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Oxidant Effects on Rat and Human Lung Proteinase Inhibitors

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Includes the Commentary of the Institute's Health Review Committee

Research Report Number 37

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Oxidant Effects on Rat and Human Lung Proteinase Inhibitors

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ABSTRACT

This project tested the hypothesis that inhaled oxidants could cause lung damage by inactivating the proteinase inhibitors that normally protect the lung from proteolysis. Rat alpha-1-proteinase inhibitor (α_1-PI)[2] was purified from blood plasma, and antibodies to this inhibitor were prepared. The activity of α_1-PI in lung lavage fluids from rats was measured by elastase inhibition, and the immunological concentration of α_1-PI was quantified in an enzyme-linked immunoassay. The ratio of the amount of active α_1-PI relative to its immunological concentration was examined as a measure of the inhibitor's functional activity. This ratio and the ratio of the immunological concentration of α_1-PI to the total protein concentration were determined in lung lavage fluids from rats exposed to air, 10 parts per million (ppm) nitrogen dioxide, and diesel emissions (3.5 mg/m^3 particulates) for 12, 18, and 24 months. Only diesel exposures resulted in a statistically significant reduction in the functional activity of α_1-PI of 30 percent ($p < 0.05$). Similar studies were performed on rats exposed to nitrogen dioxide (0.5 ppm background with peaks of 1.5 ppm) and ozone (0.06 ppm background with peaks of 0.25 ppm) for 12 and 18 months. No statistically significant effects were observed in the functional activity of α_1-PI or its immunological concentration. In other protocols, rats were acutely exposed to 0.8 ppm or 1.2 ppm ozone for two, four, or eight hours, and to 0.5 ppm or 0.8 ppm ozone in conjunction with 8 percent carbon dioxide for two or seven hours. Although these acute exposure conditions did not reduce the functional activity of α_1-PI, the immunological concentration of α_1-PI and the elastase inhibitory activity, relative to other proteins, were significantly increased in relation to the total amount of ozone inhaled.

The functional activity of α_1-PI also was measured in the bronchoalveolar lavage fluids of human subjects exposed to nitrogen dioxide (0.05 ppm with 2 ppm peaks, or to 1.5 ppm continuously) for three hours and to ozone (0.4 ppm) for two hours during exercise. These exposures did not result in significant changes in the functional activity of α_1-PI or its immunological concentration. These findings do not support the hypothesis that inhaled oxidants lead to the inactivation of α_1-PI in the lung.

The bronchial leukocyte proteinase inhibitor, which is produced in the upper airways of humans, also inhibits neutrophil elastase. No similar inhibitor was found in the rat, suggesting that rats may not be a good model for the study of chronic lung disease. The functional activity of bronchial leukocyte proteinase inhibitor was measured in nasal lavage fluids from humans exposed to 0.4 ppm ozone for two hours and was found to be decreased by 60 percent ($p < 0.002$) immediately after exposure, but unchanged 18 hours after exposure. The immunological concentration of bronchial leukocyte proteinase inhibitor was not altered by exposure in this study or in bronchoalveolar lavage fluids from individuals exposed in the protocol with 2 ppm nitrogen dioxide peaks.

Human mast cell tryptase, which is a marker for mast cell degranulation, was measured immunologically in nasal lavage and bronchoalveolar lavage fluids from subjects exposed to 0.4 ppm ozone for two hours with exercise. Tryptase was elevated significantly in the nasal fluids immediately after exposure ($p = 0.015$) and 18 hours after exposure ($p = 0.026$). In addition, tryptase was elevated in eight of the nine bronchoalveolar lavage fluid samples obtained from these subjects 18 hours after exposure. These data indicate that ozone exposure results in mast cell degranulation.

INTRODUCTION

The pathogenesis of chronic obstructive lung diseases, exemplified by emphysema, involves the proteolytic destruction of the lung's elastin-rich alveoli. This degradation of the lung by proteinases apparently results from a failure of proteinase inhibitors to control properly the proteinases of phagocytic cells such as the polymorphonuclear leukocyte. This view was brought to light when Laurell and Eriksson (1963) demonstrated a predisposition for emphysema in individuals with genetically deficient blood levels of a plasma protein called alpha-1-proteinase inhibitor, which is also known as alpha-1-antitrypsin. Alpha-1-proteinase inhibitor is the principal blood plasma inhibitor of elastases (proteolytic enzymes that hydrolyze elastin). Subsequent studies have shown that the instillation of elastase into the lungs of animals will produce emphysema-like lesions (Jan-

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[2] A list of abbreviations appears at the end of this report for your reference.

off et al. 1977; Sloan et al. 1981). Human polymorphonuclear leukocyte elastase is the major elastolytic proteinase with access to the lung, and it is generally considered responsible for the elastin degradation in emphysema.

Carp and Janoff (1978) showed that cigarette smoke condensate would inactivate α_1-PI and that antioxidants protected α_1-PI from inactivation. Subsequently, we demonstrated that oxidation of the methionine residue at the inhibitory site of α_1-PI dramatically reduced its elastase inhibitory activity (Johnson and Travis 1978, 1979). The oxidation actually decreased the association rates of α_1-PI with proteinases to the point that no complex was formed with porcine pancreatic elastase and the association rate with human leukocyte elastase was reduced 2,000-fold (Beatty et al. 1980), making the reaction physiologically insignificant.

Regarding automotive exhaust oxidants, in vitro studies have shown that ozone (O_3) (Johnson 1980) and nitrogen dioxide (NO_2) in the presence of hydrogen peroxide (Dooley and Pryor 1982) will oxidatively inactivate human α_1-PI. Since this project began, two reports have been published dealing with the in vivo effects of O_3 and NO_2 on α_1-PI. Pickrell and associates (1987) found that exposing rats to 0.5 and 1.0 ppm O_3 for 48 hours resulted in 35 percent and 80 percent reductions in the elastase inhibitory activity in serum, respectively. In a study on humans, Mohsenin and Gee (1987) found that the elastase inhibitory activity of bronchoalveolar lavage fluids from subjects exposed to 3 or 4 ppm NO_2 for three hours, with intermittent exercise, was decreased 45 percent relative to that from unexposed control subjects.

This project involved the study of rats exposed to O_3, NO_2, or diesel emissions, with air-treated animals as controls. This approach had several advantages relative to performing similar studies on humans, among which were the lack of genetic and nutritional variations and the general ability to manipulate animals, resulting in consistent sampling of the lung fluids.

The one major shortcoming of this design was that rats are not humans and, consequently, may or may not react in the same way as humans to oxidant exposure. This question was reviewed by Menzel (1984), who concluded that "ozone may thus be an ideal compound for quantitative extrapolation of toxicity from animals to humans." One of the major points made in this review was that the lesion occurred at the same anatomical site in animals and humans, which is the junction of the conducting airway and the respiratory region. This centriacinar position is the junction between mucus and surfactant and the point of minimal protection by either secretion. It is known that deficiency of blood plasma α_1-PI, a surfactant component, leads to panlobular emphysema, and that smoking results in centrilobular destruction. Human bronchial mucous secretions contain bronchial leukocyte proteinase inhibitor (BLPI), which, like α_1-PI, inhibits leukocyte elastase (Hochstrasser et al. 1972; Smith and Johnson 1985).

On the basis of these observations, we proposed that oxidants damage the lung in the centriacinar region because this is the area where proteinase inhibitors are at their lowest concentration. Consequently, oxidant inactivation of these inhibitors to favor proteolysis would most likely occur at the junction of mucus and surfactant. A test of this hypothesis required examination of the activity of lung proteinase inhibitors in rats exposed to oxidant air pollutants. Because emphysema is a chronic disease, lung lavage fluids from rats chronically exposed to air, O_3, NO_2, or diesel emissions were obtained from other workers performing the exposures for different purposes. Samples from rats exposed to air, 10 ppm NO_2, or diesel emissions for 12, 18, and 24 months were obtained from J.L. Mauderly at the Inhalation Toxicology Research Institute (ITRI), Albuquerque, NM. Additional samples were obtained from D. Costa and J. Graham at the U.S. Environmental Protection Agency (EPA) Health Effects Research Laboratory (HERL) in Research Triangle Park, NC. These collaborations greatly reduced the expense that would otherwise have been associated with a study of this type.

By studying the proteinase inhibitors of the rat, we hoped to develop a model system for the study of O_3 and NO_2 effects on these lung proteins in an in vivo situation. This was an important step because the rat has been used in numerous toxicological studies of O_3 and NO_2. In addition, this project focused on a molecular-level mechanism for the lung tissue destruction associated with the inhalation of O_3 and NO_2. Finally, an adequate understanding of the mechanism of oxidant-associated lung damage might lead to methods of prevention.

SPECIFIC AIMS

The specific aims of this project were as follows:

1. To purify rat α_1-PI from rat blood plasma.
2. To prepare antibodies to rat α_1-PI and develop an immunological assay for α_1-PI in lung fluids.
3. To determine the susceptibility of rat α_1-PI to oxidant inactivation.
4. To determine whether or not chronic exposure to O_3, NO_2, or diesel emissions results in decreased elastase inhibitory activity in lung lavage fluids.
5. To examine rats for an inhibitor corresponding to human BLPI.

6. To determine whether or not the acute exposure of rats to O_3 results in decreased α_1-PI activity in lung fluids.

During the course of these studies, the objectives were expanded to include some analyses of human bronchoalveolar and nasal lavage samples:

1. To determine the activity of α_1-PI in bronchoalveolar lavage fluids from humans exposed to O_3 or NO_2.
2. To determine the activity of BLPI in nasal lavage fluids from humans exposed to O_3.
3. To examine nasal and bronchoalveolar lavage fluids for mast cell tryptase, as a marker of mast cell degranulation.

RAT STUDIES

EXPERIMENTAL DESIGN

Chronic Exposure Studies

Chronic exposure studies were chosen because the development of emphysema is a slow, progressive process and samples were available from two chronic exposure studies that were in progress. The details of these exposure protocols will not be presented, since the exposures were performed elsewhere. A summary of the exposure protocols for rats and humans is presented in Table 1.

The first exposure protocol was conducted at ITRI by J.L. Mauderly, under an HEI-supported project entitled "Influence of Experimental Pulmonary Emphysema on Toxicological Effects from Inhaled Nitrogen Dioxide and Diesel Exhaust." The details of the exposure protocol are given in the final report for that project (Mauderly et al. 1989). Briefly, young adult male Fisher-344 (F344) rats were exposed seven hours per day, five days per week, for 12, 18, or 24 months to 10 ppm of NO_2, to diesel exhaust at 3.5 mg of soot/m^3, or to clean air as controls. Pulmonary emphysema was induced in half of the animals via intratracheal instillation of elastase six weeks prior to the beginning of exposures. Exposures continued until the Friday of the week before the animals were killed; animals were killed and lung washes were performed on Monday, Tuesday, or Wednesday. This schedule resulted in a recovery period of two to five days. Airway fluids were obtained by lavaging the right lung with two 5-mL portions of saline. The lavage fluids, after removal of cells by centrifugation, were stored at −80°C and shipped to our laboratory on dry ice. The samples arrived well frozen and were stored at −80°C until analysis.

A second chronic exposure protocol of F344 rats was performed by J. Graham and D. Costa at the EPA-HERL in Research Triangle Park, NC (Gross et al. 1989). This protocol, entitled "Chronic O_3 and NO_2 Inhalation Study," involved background O_3 levels of 0.06 ppm and nine-hour spikes to 0.25 ppm. Exposures to NO_2 used background levels of 0.5 ppm and spikes to 1.5 ppm of two hours' duration (Figure 1). At the end of the chronic exposure periods of 12 and 18 months, the rats were maintained at background levels for two days before being killed. One lung from each animal was lavaged twice with 5 mL of 0.15 M saline and centrifuged to remove cells. The lavage fluids were coded, frozen at −80°C, shipped to us on dry ice, and stored at −80°C until analysis.

Each lung lavage fluid sample was analyzed for total protein concentration, active α_1-PI concentration, and immunological concentration of α_1-PI. The ratio of active α_1-PI to its immunological (or total) concentration was analyzed to test the hypothesis that oxidant exposure inactivates lung α_1-PI. Inactivation of α_1-PI would result in a decrease in the ratio of active to immunological α_1-PI. We also examined the ratio of immunological concentration of α_1-PI to total protein concentration to test the hypothesis that oxidant exposure lowers the amount of α_1-PI in the lung. To facilitate data comparisons, the ratios of active α_1-PI to its immunological concentration were normalized to a mean of 1.0 for the air-exposed control rats for each exposure period. Likewise, the ratios of the immunological concentration of α_1-PI to the total protein concentration were normalized to a mean value of 10.0 for the air-exposed control rats for each exposure period.

Acute Exposure Studies

When we realized that the chronically exposed animals had been given two to five days to recover before being killed and lavaged, we became concerned that the recovery period may have resulted in dilution of inactive α_1-PI in the lung with active inhibitor from the blood. The amount of α_1-PI recovered in the lavage fluids of the chronically exposed rats was 25 μg, whereas the amount in the blood was estimated to be 45 mg, based on a plasma concentration of 2 mg/mL and a 45-mL blood volume. Thus, the amount of α_1-PI in the blood would be 2,000 times the amount in the lung. It is generally held that the lung concentration of α_1-PI is in diffusion-controlled equilibrium with the blood pool. Therefore, α_1-PI in the lung that may be inactivated would not accumulate, but would diffuse back into the blood and be replaced by fresh inhibitor from the blood. Although the rate of replacement is unknown, it is probably similar to the six-hour half-time for serum albumin.

Because of these problems, we became concerned that our data from chronic exposures would not answer the critical question of whether or not inhaled oxidants result in the

Table 1. Summary of Exposure Protocols

Species and Laboratory	Pollutant	Duration	Sampling	Miscellaneous Comments
Rat ITRI	10 ppm NO_2	7 hours/day, 5 days/week, 12, 18, 24 months	Bronchoalveolar lavage fluid: 2 to 5 days after exposure	Half animals healthy, half with elastase-induced emphysema
	3.5 mg diesel soot/m^3 air	Same	Same	Same
Rat EPA-HERL	0.06 ppm O_3 + 0.25-ppm spikes	20 hours/day, 12, 18 months	Bronchoalveolar lavage fluid: 2 days after exposure	
	0.5 ppm NO_2 + 1.5-ppm spikes	Same	Same	
Rat EPA-HERL	0.8 ppm or 1.2 ppm O_3	2, 4, 8 hours	Bronchoalveolar lavage fluid: 0 hours after exposure	
	0.5 ppm or 0.8 ppm O_3	2, 7 hours	Bronchoalveolar lavage fluid: 0 hours after exposure	8 percent CO_2 given intermittently
Human EPA-Clinical Research Branch	0.4 ppm O_3	2 hours	Bronchoalveolar lavage fluid: 18 hours after exposure Nasal lavage: pre-, post-, 18-hours-post-exposure	Intermittent exercise
Human University of Rochester	0.05 ppm NO_2 + 2.0-ppm peaks	3 hours	Bronchoalveolar lavage fluid: 3.5 hours after exposure	Intermittent exercise
	1.5 ppm NO_2	Same	Same	Same

inactivation of α_1-PI. Consequently, a three-month extension of this project was requested and granted so we could measure the functional activity of α_1-PI in the lungs of rats that had been killed and lavaged immediately after acute exposure to O_3. The choice of O_3 as the test oxidant was based on in vitro studies in our laboratory that had shown that O_3 completely inactivated α_1-PI after exposure to 40 moles of O_3 per mole of α_1-PI (Smith et al. 1987), and that 800 moles of NO_2 per mole of α_1-PI was required for only 50 percent inactivation (D. Brown, D. Hood, D. Johnson, unpublished observations). These results indicated that direct inactivation of α_1-PI was far more likely with O_3 than with NO_2. Furthermore, we were examining the activity of α_1-PI in lung lavage fluids of humans exposed to NO_2, which would provide data regarding NO_2 effects.

Lung lavage fluids were obtained from acutely exposed rats that had been killed and lavaged immediately after exposure. These samples were provided by D. Costa of the EPA-HERL in Research Triangle Park, NC. F344 rats (more than 90 days old) were exposed to 0.8 or 1.2 ppm O_3 for two, four, or eight hours, and to 0.5 or 0.8 ppm O_3, with intermittent 8 percent carbon dioxide (CO_2) superimposed on the O_3 exposures, for two or seven hours. The addition of 8 percent CO_2 served as an analog to exercise by increasing the respiration rate threefold. The details of this protocol have been reported by Costa and colleagues (1989).

METHODS

Inhalation Toxicology Research Institute Chronic Exposure Statistics

To avoid bias, the samples were coded before shipment from ITRI to our laboratory. This code used letters and numbers, with each letter group containing one sample of each

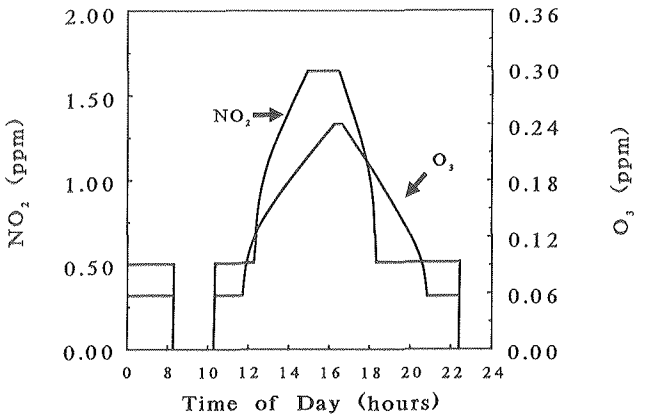

Figure 1. Chronic O_3- and NO_2-exposure protocols performed at the EPA-HERL in Research Triangle Park, NC. Rats were exposed to either of these regimens or to air for 12 or 18 months prior to being killed, and their lungs were lavaged.

type of treatment, and determinations were performed on each letter group at the same time. Such a design avoided the possibility of analyzing a certain exposure on one day and assaying the controls on another, and technique variations were spread uniformly over all samples. The code was not broken until all determinations were complete.

A three-way analysis of variance (ANOVA) was performed to compare the three treatments (air, diesel, and NO_2) over the three time periods (12, 18, and 24 months), with and without emphysema. These analyses indicated that the ratios of α_1-PI activity to its immunological concentration (active:immuno) for all treatments were consistently higher at 18 months than at the other times. Because the determinations had been made on each time period as exposures were completed, we concluded that this difference was due to variation in the assay techniques rather than representing an effect of exposure period. In addition, over the course of the study, techniques were improved and the staff became more experienced with the methods. To level out these differences, and as an aid in the graphical comparison of the data, all of the data for the different treatments were normalized to the air control (without emphysema) for each exposure period, which was given a value of 1.0. This also reflected the logical belief that air-exposed control animals were homogeneous with respect to the quantity and activity of the α_1-PI in their lungs.

Because exploratory plots of these data did not reveal periodic effects over time, and the protocol did not involve following individual animals over time, the data for the different exposure periods were pooled and treatment effects were assessed conservatively with a two-way ANOVA. Residual plots showed the need for a stabilizing transformation because the usual ANOVA assumes homogeneous variance. To avoid negative numbers, the natural logarithm of the response plus one was taken.

U.S. Environmental Protection Agency Health Effects Research Laboratory Chronic Exposure Statistics

These lavage fluid samples were coded in a fashion similar to that in the ITRI study, and the code was not broken until all the assays were finished. For the 12-month exposure period, there were 12 air-exposed control animals, 8 animals exposed to O_3, and 12 animals exposed to NO_2, as described earlier. The 18-month exposure period contained 11 air-exposed control animals, 10 animals exposed to O_3, and 11 animals exposed to NO_2. The determinations of activity, immunological concentration, and total protein were made at a later date than for the ITRI study, and this may account for the lower standard deviations observed in the data. These data were analyzed by the same methods used for the ITRI study data. Unlike in the ITRI study, no periodic effect was observed, resulting in even greater justification for normalizing the data to the air-exposed control values. In addition, the effects of exposure were analyzed after combining the 12- and 18-month exposure data.

U.S. Environmental Protection Agency Health Effects Research Laboratory Acute Exposure Statistics

These data were analyzed using a two-way ANOVA and conditional sums of squares. A single set of eight randomly selected control samples was chosen from the animals exposed to air in parallel with each O_3 exposure. These mixed control samples were used as the controls for the entire series of exposures. This was a logical choice because all exposures (to air and O_3) were for only two to eight hours. The mixed control group was internally consistent and yielded values in close agreement with the data from the group exposed to 0.8 ppm O_3 for two hours (the lowest concentration $\times$ time of treatment). The 0.8 and 1.2 ppm O_3 exposure groups each had eight animals at each time point. Data for one animal in the group exposed to 0.8 ppm for four hours was an obvious outlier and was not included in the analyses. The 0.5 and 0.8 ppm O_3 exposures with 8 percent CO_2 had six animals per group.

Determination of Total Protein

Total protein concentrations of lavage fluids were determined by the orthophthalaldehyde (OPA) method, as reported by Peterson (1983). This technique is based on the formation of a fluorescent product when OPA reacts with the amino groups of amino acids in proteins and is, therefore, very sensitive. Samples containing 100 to 2,000 ng of protein were adjusted to a final volume of 10 μL with dis-

tilled water (for example, 3 μL of sample + 7 μL of distilled water) and mixed with 100 μL of OPA reagent. After at least 15 minutes, 2.0 mL of 0.5 M sodium hydroxide was added and the fluorescence was read, relative to a reagent blank, in a Perkin-Elmer (Norwalk, CT) 650–40 fluorimeter with an excitation wavelength of 340 nm and an emission wavelength of 450 nm (10-nm slit settings). A standard curve was prepared each time using highly purified human serum albumin as the standard protein.

The protein concentrations of rat lung lavage fluids from the chronic exposure studies were determined by the OPA method. The one exception with regard to rat lung lavage protein measurement was the 12-month ITRI samples, which were assayed by the Bradford (1976) method. Protein concentration data based on this method were provided by D. Costa for the rat lung lavage samples in the acute O_3 exposure study. The protein concentrations of the nasal lavage and bronchoalveolar lavage fluid samples (from EPA-HERL, 0.4 ppm O_3) were determined in this laboratory by the OPA technique. The protein concentrations of the bronchoalveolar lavage fluid samples from the NO_2-exposed humans (University of Rochester; see Human Studies section) had been determined before they were sent to us, and those values were used. Protein concentration data are relative to a standard, such as albumin, rather than being absolute values. Thus, internal consistency is more important than absolute accuracy, but there is no reason to think that the data are not accurate.

Microtiter Plate Assay for Elastase Inhibition

Active α_1-PI concentrations were determined by elastase inhibition assays performed in 96-well, flat-bottom microtiter plates. Lung lavage fluid was added to each well and the volume was adjusted to 100 μL using 0.1 M *N*-[2-hydroxyethyl]piperazine-*N'*-ethanesulfonic acid] (HEPES), 1 mM calcium chloride, and 0.01 percent Tween-20, pH 7.5, containing 50 μg of human serum albumin per milliliter.

The porcine pancreatic elastase contained 85 percent active sites by titration, with 100 percent active human α_1-PI. Elastase was dissolved in the 0.1 M HEPES buffer to a concentration of 10.99 nmol/mL (285 μg/mL, active enzyme), based on an $E^{1\%}_{280}$ of 22.0 and a molecular weight of 25,900 (Shotton 1970). This stock solution was diluted to a concentration of 55 pmol/mL. Control and sample wells each received 50 μL (2.75 pmol of active elastase) of this porcine pancreatic elastase solution, and the mixture was allowed to incubate for 90 minutes for the enzyme-inhibitor reaction to reach equilibrium.

The remaining active elastase was assayed by adding 50 μL of the succinyl-$(Ala)_3$-*p*-nitroanilide (0.72 mg/mL in the 0.1 M HEPES buffer) to each microtiter well. After 30 minutes, 10 μL of glacial acetic acid was added to each well to stop the reaction, and the absorbance was read at 405 nm in the Titertek (Flow Laboratories, McLean, VA) microtiter plate reader, relative to wells containing only buffer and substrate. Each lavage fluid sample was assayed in triplicate at three different concentrations. Because α_1-PI and elastase react 1:1 on a molar basis, the amount of active α_1-PI was calculated from the amount of elastase inhibited relative to pure, fully active inhibitor.

Electrophoretic and Western Blot Analyses

The purity of the α_1-PI preparation and the specificity of the antisera produced by immunizing rabbits with this preparation were tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis. Purified rat α_1-PI and rat plasma proteins were denatured by boiling in 1 percent SDS with reduction, and were electrophoresed according to the method of Bury (1981). After electrophoresis, one gel was stained with Coomassie Blue and one gel was electroblotted onto nitrocellulose, as described by Towbin and coworkers (1979). The primary antibody was the IgG fraction isolated by ammonium sulfate precipitation and chromatography on DEAE-Affigel Blue (Bio-Rad, Richmond, CA), as described by the manufacturer. Detection of the primary antibody that bound to the α_1-PI was based on the reaction of affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), with final color development as described for dot-blot immunoassays.

The antibodies to rat α_1-PI were purified by ammonium sulfate precipitation (0 to 40 percent saturation) and chromatography on DEAE-Affigel Blue (Bio-Rad). The specificity of this IgG fraction for rat α_1-PI was tested by Western blot analysis. Protein standards, whole rat plasma, and pure rat α_1-PI were electrophoresed in a 7 percent SDS polyacrylamide gel to separate the various components. After electrophoresis, the proteins were electroblotted onto a sheet of nitrocellulose placed next to the SDS polyacrylamide gel. All of the proteins were transferred from the gel to the nitrocellulose, because staining the gel for protein failed to reveal any bands. In addition, the standard proteins were detected on the nitrocellulose by staining that one lane with Amido Black protein stain. The portion of the nitrocellulose with the rat plasma and pure α_1-PI samples was soaked in bovine serum albumin (BSA) for four hours to block binding sites remaining on the paper. After it was washed to remove unbound BSA, the membrane was incubated in a 1:500 dilution of the IgG fraction of rabbit anti-rat α_1-PI for four hours, followed by washing to remove any nonspecifically bound antibodies. The membrane was then soaked in a 1:2,000 dilution of peroxidase-linked goat anti-

rabbit IgG for an additional four hours. After the membrane was washed again, the peroxidase was detected by reaction with peroxide and 4-chloro-1-naphthol. This resulted in dark blue staining of the white nitrocellulose in only those places where rat α_1-PI was originally bound to the paper from the SDS polyacrylamide gel.

Enzyme-Linked Immunoassays

The antibodies to rat α_1-PI were used in enzyme-linked immunoassays to quantify this protein in lavage fluids from rats. Because quantification was so important to this project, we investigated the effects of sample treatment on protein binding to nitrocellulose and the quantification of the bound antigen. These studies (Smith et al. 1989) showed that sensitivity, specificity, and quantification were improved by pretreatment of the protein samples with SDS and boiling for two to three minutes. On the basis of these findings, samples and standards were diluted to total protein concentration of 20 µg/mL, and SDS was added to a final concentration of 10 µg/mL. Because the available amount of pure rat α_1-PI was limited to a few milligrams, the α_1-PI concentration in a pool of rat plasma was established by rocket immunoelectrophoresis to be 2.32 mg/mL, and this pool was used as a secondary standard. The responses of both the pure α_1-PI and the secondary plasma standard were the same in the dot-blot assay. After they were boiled, the cooled samples and standards were blotted on nitrocellulose in a Bio-Rad Bio-Dot 96-well apparatus with 3-mm wells. The nitrocellulose membrane was blocked with 1 percent BSA for four hours and soaked in a 1:1,000 dilution of the primary rabbit anti-rat α_1-PI antibody for eight hours. After it was washed, the membrane was incubated overnight in peroxidase-linked goat anti-rabbit IgG (1:2,000 dilution). As with the Western blot analysis, detection was accomplished by reaction of the bound peroxidase with peroxide and 4-chloro-1-naphthol, which yielded a blue insoluble product where the rat α_1-PI was bound. Because the amount of enzyme in each spot was proportional to the amount of α_1-PI bound, the intensity of the dots could be used to quantify the α_1-PI. Standards of pure α_1-PI were blotted onto each membrane, along with samples. Quantification was achieved by photographing the developed nitrocellulose membrane, followed by densitometric scanning of the photographic negative. The computer-integrated areas of these scans yielded a standard curve from which the concentrations of samples were read based on their areas. Figure 2 shows a typical standard curve.

Similar assays were established to quantify the concentrations of human α_1-PI, lung mast cell tryptase, and BLPI in nasal lavage and bronchoalveolar lavage fluids. Antiserum to tryptase was prepared as previously described

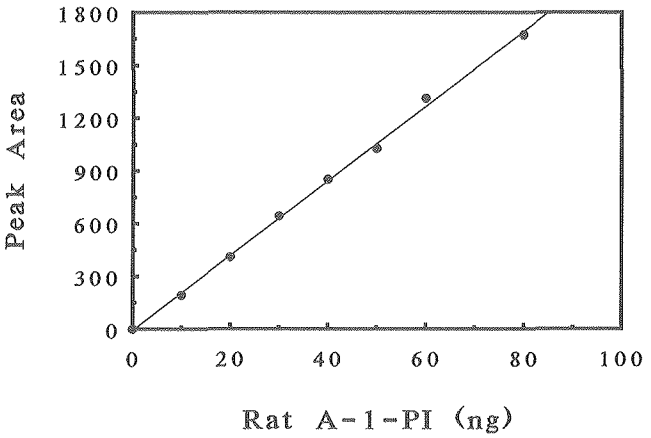

Figure 2. Standard curve for the enzyme-linked immunoassay of rat α_1-PI in lung lavage fluids. Rat α_1-PI, over the concentration range shown, was blotted onto nitrocellulose, reacted with antibodies prepared in rabbits, and then reacted with affinity-purified peroxidase-linked goat antibodies to rabbit IgG.

(Smith et al. 1984), and was affinity-purified by chromatography on a column prepared by coupling tryptase to Sepharose. Antibodies to BLPI were prepared in this laboratory, using our published methods (Smith and Johnson 1985). The immunoenzyme dot-blot technique was described earlier (Smith et al. 1989). This method involved treating the samples with less than saturating concentrations of SDS, followed by blotting to nitrocellulose. The blotted proteins were then reacted with primary antibodies and, finally, incubated with enzyme-linked secondary antibodies.

Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis, as described by Monthony and coworkers (1978), and previously employed to quantify human α_1-PI (Johnson et al. 1986b), was used to quantify the concentration of rat α_1-PI in pooled rat plasma. This pooled rat plasma subsequently served as a secondary standard in dot-blot enzyme-linked immunoassays to conserve pure α_1-PI. The use of this secondary standard also had the advantage that it, like the lung fluids, contained additional proteins. The response of the standard plasma was checked relative to pure rat α_1-PI on several occasions and was found to be equivalent.

RESULTS

Purification of Rat Alpha-1-Proteinase Inhibitor

These studies required the purification of rat α_1-PI. While this should have been a relatively easy task, the process was complicated by the use of commercially prepared rat plasma, which was hemolyzed. Exactly why the pres-

ence of hemoglobin and other erythrocyte proteins caused problems with the isolation of rat α_1-PI is unclear, but a couple of higher molecular weight proteins copurified with α_1-PI through several chromatographic steps. After struggling with this problem for some time, we contacted D. Costa at the EPA-HERL, and he provided several rat plasma samples from which we selected the ones with low hemoglobin content.

This rat plasma was fractionated by ammonium sulfate precipitation, which collected the precipitate that resulted from between 40 and 80 percent saturation with ammonium sulfate. This fraction was dialyzed and chromatographed on a Cibacron Blue Sepharose column (Ciba Geigy Corp., Ardsley, NY) to remove most of the albumin. The α_1-PI fraction, which did not bind to Cibacron Blue Sepharose, was pooled and applied to an Accell-QMA anion exchange column (Waters Associates, Milford, MA). The α_1-PI bound to this column at pH 8.8 in 50 mM Tris-HCl buffer, and was eluted with a linear sodium chloride (NaCl) gradient from zero to 0.2 M NaCl. The peak of α_1-PI was detected by inhibition assays against porcine pancreatic elastase. Trypsin inhibition assays are normally used to detect human α_1-PI, but elastase inhibition must be used when working with rat α_1-PI because rat plasma contains another trypsin inhibitor, in addition to α_1-PI, that is not present in human plasma. The partially purified rat α_1-PI was then chromatographed on a Mono Q anion exchange column (Pharmacia LKB, Gaithersburg, MD) in 10 mM Bis-Tris buffer at pH 6.0, again using a gradient of NaCl for elution. This yielded highly purified rat α_1-PI, as evidenced by a single protein staining band on SDS-PAGE (Figure 3). Titration of a constant amount of porcine elastase with increasing amounts of pure α_1-PI indicated that the inhibitor preparation was 100 percent active, a further indication of purity.

The susceptibility of rat α_1-PI to oxidants was examined by treating samples of rat plasma with O_3 under in vitro conditions (Johnson 1980). These exposures resulted in the complete loss of α_1-PI's ability to inhibit elastase, demonstrating that rat α_1-PI, like the human inhibitor, is inactivated by oxidants.

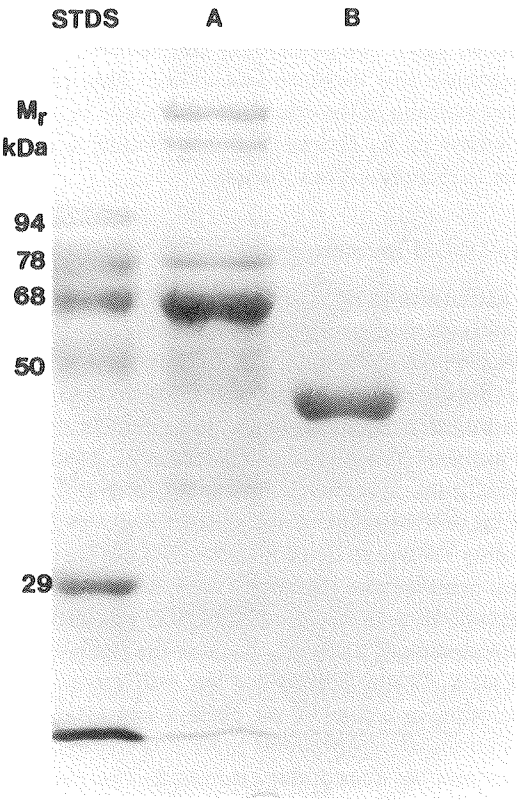

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoretic analysis of rat plasma proteins and pure rat α_1-PI. Standard proteins, rat plasma, and pure rat α_1-PI were analyzed by SDS-PAGE on a 7 percent gel with reduction. After electrophoresis, proteins were stained with Coomassie Brilliant Blue and photographed. The standards lane (STDS) contained phosphorylase A (100 kDa), transferrin (78 kDa), BSA (68 kDa), IgG (50 and 25 kDa subunits), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome *c* (12.75 kDa), and bovine pancreatic trypsin inhibitor (6.5 kDa). Lane A contained rat plasma and lane B contained pure rat α_1-PI (25 µg).

Production of Antibodies to Rat Alpha-1-Proteinase Inhibitor

The purified rat α_1-PI was mixed with Freund's complete adjuvant, and rabbits were immunized by subcutaneous injection. After boosting with rat α_1-PI protein combined with incomplete adjuvant, the rabbits' plasma contained antibodies to the rat α_1-PI that gave strong precipitin lines on Ouchterlony immunodiffusion plates.

The results of Western blot analysis of purified rat α_1-PI can be seen in Figure 4. Only one band was immunostained in the lane where the rat plasma was applied, and this band is in the same electrophoretic position as the immunostained band for the pure rat α_1-PI sample. These data show that the rabbit anti-rat α_1-PI antiserum was monospecific and reacted with only rat α_1-PI, and not with other rat plasma proteins.

Search for Bronchial Leukocyte Proteinase Inhibitor Analog in the Rat

The question of whether or not rats, like humans, contain an inhibitor analogous to the BLPI was addressed by examining rat lung lavage fluids and extracts of parotid glands for an immunologically similar proteinase, using antibodies to human BLPI (Smith and Johnson 1985). No immunologically cross-reacting proteins were found. In addition, parotid gland extracts were examined for inhibitory activity against human neutrophil elastase, but no inhibition was

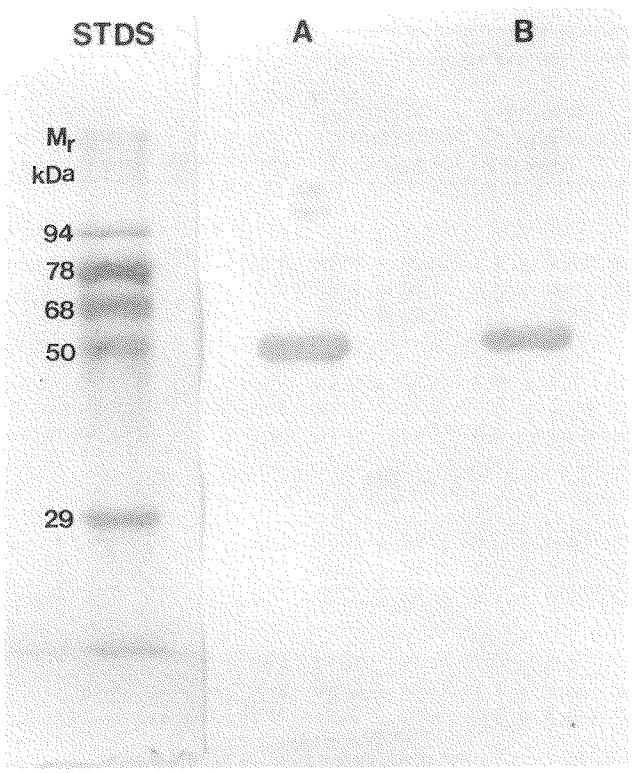

Figure 4. Western blot analysis of rat plasma proteins and pure rat α_1-PI. After electrophoresis as in Figure 3, the proteins in the polyacrylamide gels were electrophoretically transferred to nitrocellulose. The standards lane (STDS) was cut off and stained for protein with Amido Black. The portions of the nitrocellulose with lanes A and B were reacted with primary antibodies to rat α_1-PI and horseradish peroxidase-linked secondary antibodies. A dark blue, insoluble precipitate resulted from the conversion of 4-chloro-1-naphthol by peroxidase where the α_1-PI antigen was bound to the nitrocellulose. Lane A contained rat plasma and lane B contained pure rat α_1-PI.

detected. Human BLPI is produced by the serous mucus-secreting cells and is in high concentration in parotid glands (C.E. Smith and D.A. Johnson, unpublished data). Preliminary studies using complementary DNA coding for human BLPI (Stetler et al. 1986) in hybridization experiments with various mammalian DNAs failed to detect a corresponding gene in rat DNA.

Chronic Exposures to Nitrogen Dioxide or Diesel Emissions

The functional integrity of α_1-PI in the lung lavage fluids of the rats exposed to NO_2 (10 ppm) or diesel emissions (3.5 mg of soot/m^3) was assessed by examining the ratio of active α_1-PI to the immunological concentration of α_1-PI (active:immuno) in the lung fluids. There were some inconsistent variations in this active:immuno ratio over time, which apparently resulted from differences in techniques or inherent variations resulting from performing the analyses at different times, or both. The data resulting from these studies are presented in Table 2. Because each time point (12, 18, and 24 months) had a corresponding set of control samples, the data for each time point were normalized to a value of 1.0 for the air-exposed controls, and the data were pooled for the analysis of exposure effects (Figure 5). The active:immuno ratio was slightly above the control value for the NO_2-exposed animals, but this was a statistically insignificant increase. The active:immuno ratio for the diesel-exposed animals was lower than for control animals, and this effect was statistically significant ($p < 0.05$), based on Tukey's HSD test. No differences were observed in the ratios of immunological α_1-PI to total protein (immuno:protein) with either exposure (Figure 6). The presence of preexisting emphysema was without effect, but the diesel-exposed emphysematic animals, like the diesel-exposed normal rats, had significantly lowered active:immuno ratios.

Chronic Exposures to Ozone or Nitrogen Dioxide

The functional activity of α_1-PI was assessed on the basis of ratio of active α_1-PI to its immunological concentration in the lung lavage fluids of rats exposed at the EPA-HERL to O_3 (0.25 ppm peak) or NO_2 (1.5 ppm peak) for 12 or 18 months (Table 3). There were no statistically significant differences in the active:immuno ratios over the time course of exposure. As previously stated, the data were normalized to the controls for each time point, which were given values of 1.0 (Figure 7). The ratio of the immunological concentration of α_1-PI to total protein (immuno:protein) was examined for evidence of changes in the relative amount of α_1-PI in the lung, but no statistically significant differences in these ratios resulted (Figure 8).

Acute Ozone Exposures

The ratio of active α_1-PI to its immunological concentration in the lung lavage fluids of rats exposed to 0.8 or 1.2 ppm O_3 did not change significantly ($p = 0.4$) with exposure for two, four, or eight hours (Table 4 and Figure 9). A similar result was obtained with exposure of 0.5 ppm O_3 and 0.8 ppm O_3 with 8 percent CO_2 for two or seven hours (Figure 10). However, the ratios of active α_1-PI to total protein (active:protein) and immunological α_1-PI to total protein both increased significantly ($p < 0.0001$) with exposures of more than two hours. Eight hours of exposure at 1.2 ppm caused the active:protein ratio to increase to 2.5 times the control value. Twofold and fivefold increases in the active:protein ratio resulted from exposure, with 8 percent CO_2 superimposed on the O_3, for seven hours to 0.5 ppm and 0.8 ppm O_3, respectively. Similarly, eight hours of exposure at 1.2 ppm caused the ratio of immuno:protein to increase 1.7-fold. Twofold and fourfold increases resulted

Table 2. Data from Inhalation Toxicology Research Institute Chronic Exposures of Rats[a]

Treatment	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)	Active: Protein (μg/mg)	Immuno: Protein (μg/mg)	Active: Immuno (μg/μg)	Macrophages[b] (cells/mL)	Polymorphonuclear Leukocytes (cells/mL)
12 Months								
Normal								
Air (n = 8)	257 ± 19	2.0 ± 0.5	3.1 ± 0.2	7.2 ± 1.4	12.6 ± 1.1	0.66 ± 0.18	520 ± 96	110 ± 55
Diesel (n = 8)	215 ± 21	1.0 ± 0.2	2.2 ± 0.1	4.8 ± 0.8	10.6 ± 0.8	0.46 ± 0.07	272 ± 59	110 ± 31
NO_2 (n = 8)	205 ± 14	1.4 ± 0.1	2.9 ± 0.2	7.1 ± 0.5	15.2 ± 2.1	0.52 ± 0.06	387 ± 65	51 ± 16
Emphysema								
Air (n = 8)	244 ± 15	1.1 ± 0.3	2.4 ± 0.2	4.3 ± 1.1	9.9 ± 0.7	0.50 ± 0.16	542 ± 109	175 ± 40
Diesel (n = 8)	212 ± 18	0.7 ± 0.2	2.5 ± 0.3	3.3 ± 0.5	11.4 ± 0.7	0.29 ± 0.04	550 ± 77	168 ± 43
NO_2 (n = 8)	224 ± 18	1.3 ± 0.3	2.8 ± 0.2	5.4 ± 1.0	13.2 ± 0.9	0.44 ± 0.10	781 ± 145	114 ± 28
18 Months								
Normal								
Air (n = 8)	377 ± 58	1.2 ± 0.1	1.5 ± 0.3	3.7 ± 0.7	4.5 ± 0.8	0.88 ± 0.09	245 ± 38	4 ± 3
Diesel (n = 8)	551 ± 42	1.3 ± 0.1	2.4 ± 0.2	2.4 ± 0.3	4.6 ± 0.6	0.56 ± 0.06	95 ± 19	106 ± 22
NO_2 (n = 8)	407 ± 77	1.4 ± 0.2	1.9 ± 0.3	4.6 ± 1.0	6.0 ± 1.4	0.75 ± 0.05	185 ± 34	7 ± 2
Emphysema								
Air (n = 7)	518 ± 50	1.2 ± 0.1	1.7 ± 0.2	2.5 ± 0.4	3.5 ± 0.7	0.79 ± 0.12	383 ± 59	6 ± 3
Diesel (n = 5)	321 ± 37	1.0 ± 0.1	1.7 ± 0.3	3.2 ± 0.5	5.9 ± 1.7	0.64 ± 0.10	168 ± 15	104 ± 41
NO_2 (n = 8)	397 ± 71	1.3 ± 0.1	1.7 ± 0.3	3.9 ± 0.7	4.7 ± 0.7	0.82 ± 0.07	238 ± 35	8 ± 2
24 Months								
Normal								
Air (n = 8)	327 ± 32	1.1 ± 0.2	2.4 ± 0.3	3.5 ± 0.4	8.4 ± 1.9	0.49 ± 0.06	123 ± 14	1 ± 0.4
Diesel (n = 8)	463 ± 34	1.4 ± 0.2	4.3 ± 0.3	3.1 ± 0.3	9.5 ± 0.7	0.34 ± 0.04	59 ± 21	52 ± 5.7
NO_2 (n = 8)	335 ± 48	2.2 ± 0.3	2.8 ± 0.3	7.1 ± 1.2	8.9 ± 0.8	0.77 ± 0.07	104 ± 14	1 ± 1.1
Emphysema								
Air (n = 7)	327 ± 55	1.7 ± 0.5	2.5 ± 0.2	4.7 ± 0.6	8.6 ± 0.9	0.62 ± 0.13	140 ± 22	0 ± 0
Diesel (n = 8)	366 ± 32	1.7 ± 0.5	4.0 ± 0.4	4.3 ± 0.8	11.0 ± 0.7	0.40 ± 0.07	85 ± 15	28 ± 8.5
NO_2 (n = 8)	407 ± 48	2.2 ± 0.4	2.9 ± 0.3	5.1 ± 0.4	7.4 ± 0.5	0.72 ± 0.08	164 ± 30	8 ± 2.8

[a] Values are presented as means ± SEM. These values were subsequently normalized relative to the data of the normal air-exposed rats at each time point for Figures 5 and 6.

[b] Data of Mauderly and associates (1989).

Table 3. Data from Environmental Protection Agency Health Effects Research Laboratory Chronic Exposures of Rats[a]

Treatment	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)	Active: Protein (μg/mg)	Immuno: Protein (μg/mg)	Active: Immuno (μg/μg)
12 Months						
Air (n = 12)	350 ± 51	4.4 ± 0.3	9.3 ± 0.7	14.2 ± 1.3	31.4 ± 4.2	0.49 ± 0.03
O_3 (n = 9)	376 ± 66	4.4 ± 0.6	8.1 ± 0.8	12.9 ± 1.2	26.7 ± 4.3	0.55 ± 0.05
NO_2 (n = 12)	306 ± 22	4.5 ± 0.5	10.3 ± 0.7	14.7 ± 1.4	35.5 ± 2.9	0.43 ± 0.03
18 Months						
Air (n = 11)	466 ± 22	5.4 ± 0.9	11.2 ± 0.9	11.5 ± 1.7	24.5 ± 2.0	0.47 ± 0.05
O_3 (n = 10)	563 ± 142	4.5 ± 1.0	9.8 ± 1.6	10.1 ± 1.9	22.8 ± 3.5	0.44 ± 0.03
NO_2 (n = 11)	446 ± 26	5.3 ± 0.7	11.3 ± 1.4	12.0 ± 1.4	27.1 ± 4.5	0.47 ± 0.04

[a] Values are presented as means ± SEM. These values were subsequently normalized relative to the data of the air-exposed rats at each time point for Figures 7 and 8.

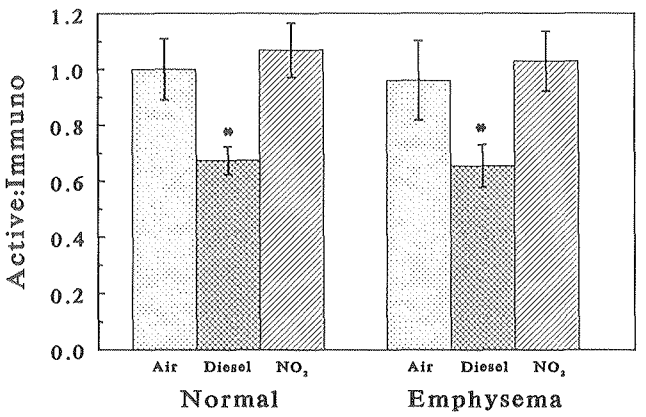

Figure 5. Effects of chronic exposure of rats to NO_2 or diesel emissions on the functional activity of α_1-PI in lung lavage fluids. Ratios are shown of active α_1-PI to its immunological concentration (active:immuno) in the lung fluids of rats exposed at the ITRI facility for 12, 18, or 24 months to air, diesel, or NO_2. Both normal rats and animals that had been made emphysematous by instilling elastase into their lungs (four weeks prior to beginning exposures) were exposed to air, 10 ppm NO_2, or diesel emissions (3.5 mg/m^3 particulates, 16 ppm carbon monoxide, 9 ppm hydrocarbons, and 1 ppm NO_2). There were eight animals per group, except for the 18-month emphysema control group (n = 7), the 18-month emphysema-plus-diesel group (n = 6), and the 24-month emphysema control group (n = 7). Exposures were for seven hours per day, five days per week. Active α_1-PI concentrations were based on elastase inhibitory activity assays using porcine elastase as the test enzyme. The immunological concentration of α_1-PI in the lavage fluids was determined by quantitative enzyme-linked immunoassays. Data were normalized to values of 1.0 for the air-exposed control groups of normal rats at each time point. Since time-dependent changes could not be detected, the data at each time point were pooled for the analysis of exposure effects. Error bars represent the SEM. An asterisk indicates a statistically significant ($p < 0.005$) difference.

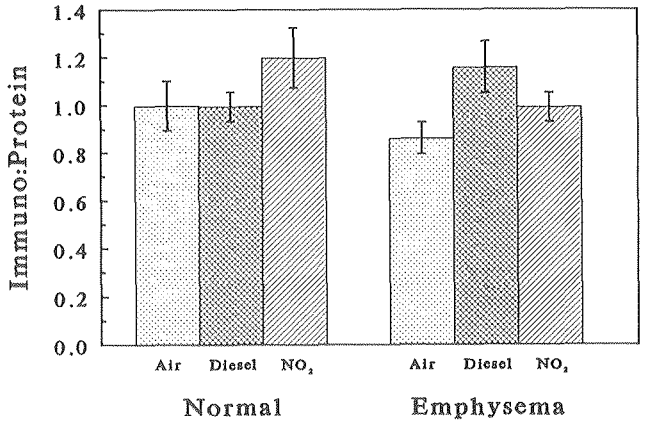

Figure 6. Effects of chronic exposure of rats to NO_2 or diesel emissions on the immunological concentration of α_1-PI in lung lavage fluids relative to total protein. Ratios of the immunological concentration of α_1-PI to total protein (immuno:protein) in lung fluids of rats exposed for 12, 18 or 24 months to air, diesel, or NO_2 (as in Figure 5) are shown. Data were normalized to values of 1.0 for the air-exposed control groups of normal rats at each time point. Since time-dependent changes could not be detected, the data were pooled for the analysis of exposure effects. Error bars represent the SEM.

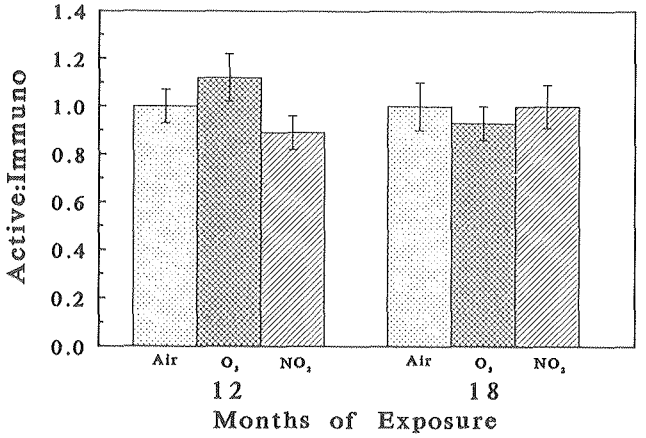

Figure 7. Effects of chronic O_3 or NO_2 exposure on the functional activity of α_1-PI in rat lung lavage fluids. Ratios of the concentration of active α_1-PI to its immunological concentration in lung lavage fluids are shown. Rats were exposed at the EPA-HERL to air, O_3 (0.25 ppm peak, 0.06 ppm background), or NO_2 (1.5 ppm, 0.5 ppm background) for 12 or 18 months. For air n = 12, for O_3 n = 9, and for NO_2 n = 12 for the 12-month exposures; and for air n = 12, for O_3 n = 10, and for NO_2 n = 11 for the 18-month exposures. Exposures were for 22 hours per day, five days per week, with O_3 peaks for one hour and NO_2 peaks for two hours. Active α_1-PI concentrations were based on elastase inhibitory activity assays using porcine elastase as the test enzyme. The immunological concentrations of α_1-PI in the lavage fluids were determined by quantitative enzyme-linked immunoassays. Average values are plotted with standard error bars, after normalizing the data to a value of 1.0 for the air-exposed normal rats without emphysema at each time point.

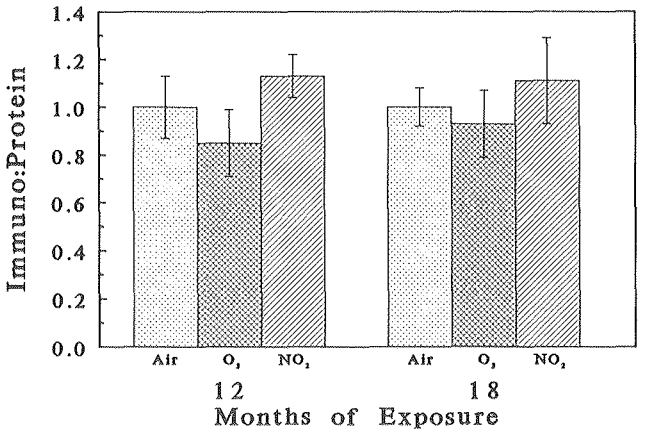

Figure 8. Effects of chronic O_3 or NO_2 exposure on the immunological concentration of α_1-PI in lung lavage fluids relative to the concentration of total protein. Ratios of the immunological concentration of α_1-PI to the concentration of total protein (immuno:protein) are shown. Exposures were performed as in Figure 7. Error bars represent the SEM.

from exposure for seven hours, with 8 percent CO_2, to 0.5 ppm and 0.8 ppm O_3, respectively. The total protein concentration also increased with the long-term and higher concentration exposures, suggestive of edema due to the influx of blood plasma proteins into the lung air space. These

Table 4. Acute Ozone Exposure Data for Rats[a]

Treatment	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)	Active: Protein (μg/mg)	Immuno: Protein (μg/mg)	Active: Immuno (μg/μg)
Air controls[b]	185 ± 23	4.4 ± 0.6	11.0 ± 1.7	24.3 ± 1.6	59.3 ± 6.4	0.44 ± 0.05
0.8 ppm O_3, 2 hours[b]	162 ± 11	5.4 ± 0.6	10.0 ± 1.1	33.0 ± 1.6	61.6 ± 5.6	0.56 ± 0.04
0.8 ppm O_3, 4 hours	183 ± 31	6.5 ± 1.7	11.2 ± 3.0	33.5 ± 2.6	56.9 ± 5.7	0.62 ± 0.06
0.8 ppm O_3, 8 hours	258 ± 41	10.4 ± 2.6	22.2 ± 6.1	37.3 ± 3.0	77.7 ± 8.7	0.50 ± 0.03
1.2 ppm O_3, 2 hours[b]	228 ± 29	7.0 ± 1.2	15.1 ± 3.1	29.5 ± 1.5	62.0 ± 7.0	0.51 ± 0.05
1.2 ppm O_3, 4 hours	240 ± 34	8.5 ± 2.0	14.5 ± 2.8	32.8 ± 2.7	57.8 ± 4.4	0.57 ± 0.03
1.2 ppm O_3, 8 hours	479 ± 35	32.1 ± 5.8	52.8 ± 10.1	63.0 ± 7.3	102.9 ± 13.2	0.62 ± 0.02
0.5 ppm O_3 + CO_2, 2 hours[c]	362 ± 39	10.3 ± 1.3	24.8 ± 2.6	28.1 ± 1.2	69.7 ± 5.3	0.41 ± 0.03
0.5 ppm O_3 + CO_2, 7 hours	950 ± 52	50.2 ± 8.1	113.4 ± 11.8	51.3 ± 5.4	118.0 ± 6.8	0.43 ± 0.03
0.8 ppm O_3 + CO_2, 2 hours[c]	559 ± 66	17.4 ± 2.6	34.1 ± 3.6	30.5 ± 1.3	62.5 ± 4.4	0.51 ± 0.06
0.8 ppm O_3 + CO_2, 7 hours	1,319 ± 21	167.9 ± 10.8	331.0 ± 18.6	126.8 ± 6.4	250.4 ± 11.8	0.51 ± 0.02

[a] Values are presented as means ± SEM.

[b] n = 8 for air control, 0.8 ppm O_3, and 1.2 ppm O_3 exposure groups.

[c] n = 6 for 0.5 ppm and 0.8 ppm O_3 + CO_2 exposure groups. The CO_2 concentration was 8 percent in all cases.

data, shown in Table 4, indicate that α_1-PI entered the lung in preference to other proteins or was locally produced by macrophages (White et al. 1981), but it is doubtful that macrophages could produce such a large amount of α_1-PI in a short time (Krivit et al. 1988). In addition, macrophages are known to produce elastase (Banda and Werb 1981) and cathepsin L, both of which have been shown to inactivate α_1-PI (Banda et al. 1980; Johnson et al. 1986a).

DISCUSSION

Rat Alpha-1-Proteinase Inhibitor Purification and Immunoassay

Rat α_1-PI was successfully purified with the use of techniques similar to those previously employed to isolate human α_1-PI. Why problems in isolation occurred when hemolyzed plasma was used as the starting material remains unexplained, but the problem was solved by selecting plasma with minimal visible hemolysis. The resulting α_1-PI was homogeneous by SDS-PAGE, as evidenced by a single, strongly stained band. The estimated molecular weight from the SDS-PAGE experiment was 45,000 which is close to the value reported by Roll and Glew (1981). The inhibitor preparation was 100 percent active, as determined by the inhibition of active-site titrated elastase. Rat α_1-PI, like human α_1-PI, was sensitive to oxidant inactivation under in vitro conditions. Thus, rat α_1-PI seems to be very similar to its human analog.

Antibodies to rat α_1-PI prepared by immunizing rabbits with the purified rat α_1-PI were monospecific. Only one component of whole rat plasma reacted with the antibodies, as evidenced by a single band on Western blot analysis. This band was also at the same position as pure rat α_1-PI in the same experiment. Thus, we are confident that the immunoassay using these antibodies was specific for rat α_1-PI in the lung lavage fluids.

Search for Rat Bronchial Leukocyte Proteinase Inhibitor

Analysis of rat lung lavage fluids and extracts of rat parotid glands failed to detect an inhibitor similar to human BLPI. Human parotid glands contain considerable BLPI

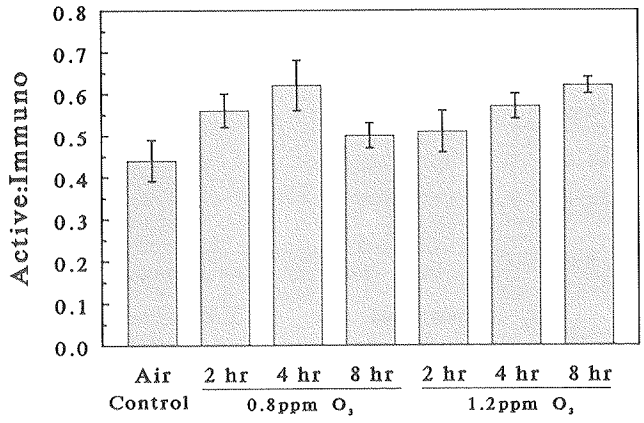

Figure 9. Effects of acute O_3 exposure on the functional activity of α_1-PI in rat lung lavage fluids. Ratios are shown of active α_1-PI to its immunological concentration (active:immuno) in the lung fluids of rats exposed to 0.8 or 1.2 ppm O_3 for two, four, or eight hours. There were eight animals in each exposure group. Error bars represent the SEM.

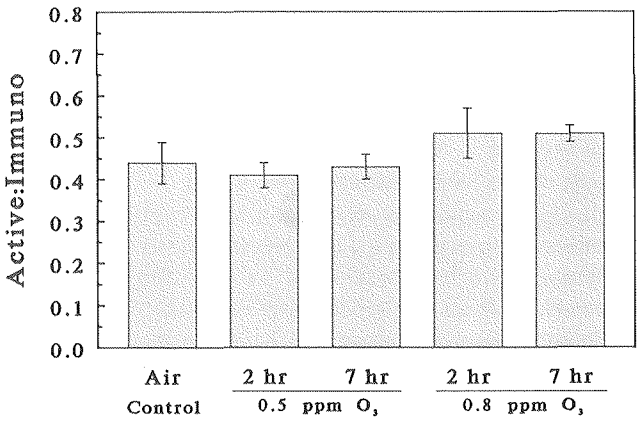

Figure 10. Effects of acute O_3 exposures with 8 percent CO_2 on the functional activity of α_1-PI in rat lung lavage fluids. Ratios are shown of the concentration of active α_1-PI to its immunological concentration (active:immuno) in the lung fluids of rats exposed to 0.5 or 0.8 ppm O_3 with 8 percent CO_2 for two or seven hours. There were six animals in each exposure group. Error bars represent the SEM.

and neutrophil elastase inhibitory activity (C. Smith and D. Johnson, unpublished data), but no inhibitory activity was found in rat parotid glands. Immunoassays using antisera to human BLPI were negative also. Although these data are not conclusive, they strongly suggest that rats do not have an analog to human BLPI.

Analyses of rat and other mammalian DNAs showed no indication of a similar gene in the rat. Thus, the rat seems to differ from humans and some other mammals in this regard. The absence of such an inhibitor may or may not be significant with regard to protecting the lung from proteolysis, but it raises some question concerning the extrapolation of data from the rat to humans.

Chronic Exposure Effects

The chronic exposure of rats to 10 ppm NO_2 (ITRI study) or 1.5 ppm NO_2 (EPA-HERL study) did not result in significant alteration in the functional activity of α_1-PI in the lungs of the exposed rats. None of the bronchoalveolar lavage fluids contained fully active α_1-PI (Tables 2 and 3), but this was to be expected due to handling and storage. Afford and colleagues (1988) have reported that the activity of α_1-PI in bronchoalveolar lavage fluid decreases with time, even when frozen. Irrespective of why these active:immuno ratios were less than 1.0, the conclusions are based on the changes in the ratio relative to air-exposed control animals. If there had been inactivation of the lung α_1-PI, the ratio of active α_1-PI to its immunological concentration would have decreased. This did not occur. The amount of α_1-PI in the lung fluids relative to the total protein concentration did not change either. Thus, under the conditions of the chronic exposure protocols employed, exposure of rats to 10 ppm NO_2 for 12, 18, or 24 months, or to 1.5 ppm NO_2 for 12 or 18 months, did not result in appreciable alterations in the integrity or relative amount of α_1-PI in the lung. Our data show that NO_2 exposure does not result in a prolonged inactivation of lung α_1-PI, as with diesel exhaust. The lung damage associated with NO_2 exposure (Menzel 1984) may result from short-term effects of NO_2 on α_1-PI in the lung, but short-term inactivation is not supported by our data on α_1-PI in bronchoalveolar lavage fluids from humans.

These results are contrary to the report of Mohsenin and Gee (1987) that exposure of humans to NO_2 (3 or 4 ppm) for three hours with intermittent exercise caused a 45 percent reduction in the functional activity of the α_1-PI found in bronchoalveolar lavage fluids. These workers performed the lavage 3.5 to 4 hours after exposure, whereas lavage was performed two to five days after the last exposure of the rats. The delay between the end of exposure and lavage of the rats may have obscured any short-term effects of exposure on the activity of α_1-PI, but without the recovery periods we would not know whether or not the inactivation observed with diesel exposure was due to acute or chronic effects.

The results obtained show that chronic exposure to diesel exhaust causes a reduction in the activity of α_1-PI that persists two to five days after exposure ceases. Similarly, the absence of an effect with O_3 or NO_2 shows that these agents do not result in a sustained inactivation of lung α_1-PI. There is very little α_1-PI on the air-space side of the lung that could be sampled by lavage. The amount of α_1-PI recovered in the lavage fluids was approximately 25 μg after washing with 10 mL of saline (Table 2). In contrast, the amount of α_1-PI in the blood is 2 mg/mL, or approximately 2,000 times the total amount on the air-space side of the lung. Because the concentration of α_1-PI inside the lung is apparently maintained by diffusion from the blood, inactive α_1-PI resulting from exposure would equilibrate over time with the large pool of active α_1-PI in the blood. Thus, by two to five days after the last exposure, most or all of the α_1-PI inside the lung during the last exposure may well have been replaced by fresh inhibitor from the blood. While the rate of replacement is not known, it is probably rapid because the lung is highly vascularized. Although chronic exposure to diesel emissions reduces α_1-PI activity, the acute effects of O_3 or NO_2 were not addressed by the chronic exposure protocols. This knowledge gap has been bridged by examining samples from rats acutely exposed to O_3 and immediately lavaged, and by examining bronchoalveolar lavage fluid samples from humans exposed to NO_2, with lavage occurring three to four hours after exposure. Results of these analyses will be discussed later.

Chronic exposure to diesel emissions (3.5 mg of soot/m^3) for 12, 18, and 24 months reduced the functional activity

(active:immuno) of α_1-PI in lung fluids by approximately 30 percent in both normal rats and rats with emphysema (Figure 5). This effect was statistically significant at the 95 percent confidence level. Why diesel emissions lowered the functional activity of α_1-PI, whereas NO_2 did not, is somewhat unclear. These animals were exposed under the same protocol as the NO_2-exposed rats, which included a recovery period of two to five days at the end of the exposures. This result is probably related to the increased inflammation in the lung of the diesel-exposed animals relative to the NO_2-exposed rats. Mauderly and colleagues (1989) found that with diesel exposure, "focal epithelial hyperplasia, squamous metaplasia, and fibrosis were prominent, and emphysematous changes were observed adjacent to some contracted fibrotic foci." These workers also found significant increases in the number of neutrophils in the lungs of diesel-exposed rats. The animals killed at 12 months had elevated numbers of neutrophils in their lavage fluids, compared to the animals exposed for 18 and 24 months. Because this work is concerned with the control of neutrophil proteases, the numbers of polymorphonuclear leukocytes in the lung lavage fluids reported by Mauderly and coworkers (1989) are included in Table 2. The rats exposed to air and NO_2 for 12 months had particularly high levels of polymorphonuclear leukocytes as compared to similarly exposed animals at 18 and 24 months. Although the numbers of polymorphonuclear leukocytes found at 18 and 24 months are in the range expected for normal rats, the rats exposed for 12 months had elevated levels, corresponding to an inflammatory state.

Interestingly, fewer macrophages were recovered in the lavage fluids from diesel-exposed animals, with the exception of the animals with emphysema exposed for 12 months. Because macrophages produce α_1-PI (White et al. 1981), one might expect the lower numbers of macrophages to result in lower amounts of α_1-PI in the lungs of these animals, but this is not the case. The total amount of α_1-PI for the normal animals exposed for 12 months was below that for the air-exposed control animals, and was greater than or equal to that for the control animals in the other exposure groups (Table 2). Thus, the lower numbers of macrophages does not correlate with lower amounts of α_1-PI, indicating that macrophages do not contribute significantly to the total α_1-PI in the lung.

There was no evidence of infection in these animals from serological tests, pathological examinations, and colony histories (J.L. Mauderly, ITRI, personal communication). The diesel-exposed rats had elevated polymorphonuclear leukocytes at all time points, but the animals exposed for 24 months had somewhat lower numbers than the rats exposed for 12 or 18 months. Thus, all exposure groups at 12 months, including control animals, appear to have been in an inflammatory state, whereas only the diesel-exposed animals contained numbers of polymorphonuclear leukocytes corresponding to inflammation at 18 and 24 months. The increased number of polymorphonuclear leukocytes would have resulted in an additional elastase burden in the lung, which would have combined with α_1-PI; this partially explains the generally lower active:immuno ratios in the 12-month exposure groups. Likewise, the elevated number of polymorphonuclear leukocytes in the lungs of diesel-exposed rats at all time points would have resulted in an additional burden of elastase and would account for the reduced active:immuno ratios in these animals relative to the air-exposed control animals. In addition, the increased number of neutrophils and other inflammatory cells would have increased the lung's burden of oxidants derived from such cells, which may have contributed to the inactivation of α_1-PI.

Neutrophil myeloperoxidase is known to inactivate α_1-PI oxidatively (Matheson et al. 1979). In addition, only serine proteases are inhibited by α_1-PI, and several metallo and cysteine proteases have been found to inactivate α_1-PI catalytically by proteolytic cleavage in the reactive site region. Two examples of such proteases are the lysosomal cysteine proteinase known as cathepsin L (Johnson et al. 1986a) and the metalloelastase of macrophage (Banda et al. 1980). Activated macrophages also contain cysteine proteases capable of degrading elastin (Chapman and Stone 1984), and we have shown that cathepsin L has considerable elastolytic activity (Mason et al. 1986). These reports indicate that macrophages contain two proteases capable of inactivating α_1-PI. Thus, increased numbers of macrophages, or their activation, or both may explain the inactivation of α_1-PI in lung fluids from diesel-exposed rats via proteolysis by nonserine proteases. Such an enzymatic process would not stop when exposure stops, but would continue for as long as the enzymes remain active in the lung. Actually, the observation that diesel emissions decrease α_1-PI activity should not be too surprising since cigarette smoking, which also results in particle deposition, is known to cause emphysema. Whether or not the higher number of polymorphonuclear leukocytes in the lungs of the diesel-exposed rats at 18 and 24 months was due to the particulate in the diesel exhaust or to other components in the diesel emissions is not clear.

Chronic exposure of rats to O_3 (0.25 ppm, EPA-HERL) for 12 or 18 months did not cause any significant change in the ratio of α_1-PI activity to its immunological concentration (active:immuno), nor was there any change in the ratio of immunological α_1-PI to total protein (immuno:protein). Therefore, chronic exposure to low levels of O_3 did not result in prolonged inactivation of lung α_1-PI. These data are consistent with the histological observations on the ani-

mals in this study, which showed only minor inflammation in the lungs (Gross et al. 1989).

Acute Exposure Effects

Although acute exposure of rats to O_3 did not result in changes in the functional activity of α_1-PI, as measured by the ratio of active α_1-PI to its immunological concentration, the elastase inhibitory activity in lung lavage fluids was increased relative to total protein (active:protein). The immunological concentration of α_1-PI relative to total protein (immuno:protein) was similarly elevated with increased exposure. This was particularly true of the longer time points and of exposures with 8 percent CO_2. Exposure to 0.8 ppm O_3 for two, four, or eight hours or to 1.2 ppm for two or four hours resulted in virtually no change in active:protein or immuno:protein ratios, but increases in both ratios were seen with 1.2 ppm O_3 for eight hours and with both 0.5 ppm and 0.8 ppm at seven hours when it was administered in conjunction with 8 percent CO_2 (Figure 11). All of the increases seen at seven and eight hours were highly significant ($p < 0.0001$).

These data indicate that the amount of α_1-PI in the lung fluids increased relative to other proteins. The longer exposures also resulted in higher concentrations of total protein per milliliter of lavage fluid, and such increases are indicative of edema. While the total protein concentration in the lung lavage fluids increased sevenfold upon exposure to 0.8 ppm O_3 with 8 percent CO_2 for seven hours, the concentrations of active and immunological α_1-PI were increased 35- and 28-fold, respectively. Although human α_1-PI is known to be an acute-phase reactant, this has not been established clearly for rat α_1-PI. Lonberg-Holm and associates (1987) reported some elevation in the plasma of rat α_1-PI in response to adjuvant-induced arthritis, but it is doubtful that the blood concentration could increase as much as fourfold in only seven hours, because such responses usually require more than 12 hours. Blood plasma from some of the exposed rats was assayed for α_1-PI activity, which would have increased in an acute-phase response, but the elastase inhibitory activity was the same in the exposed and control animals (D. Costa, EPA-HERL, personal communication). Thus, it seems that α_1-PI is preferentially entering the lung, because the active:protein and immuno:protein ratios both increased about fourfold relative to the increase in total protein with exposure to 0.8 ppm O_3 with 8 percent CO_2 for seven hours. Such increases in α_1-PI would protect the lung from neutrophil-elastase-mediated degradation.

Because 8 percent CO_2 caused the respiratory rate of the animals to increase threefold, the amount of O_3 inhaled was three times as great when exposure was combined with 8 percent CO_2 as when it was administered without CO_2. The total amount of O_3 inhaled can be calculated by multiplying the exposure concentration by the duration of exposure, or three times the length of exposure for those animals breathing three times as fast, to obtain a concentration × time value. Figure 11 was generated by plotting the concentration × time values (with units of ppm•hours) against the immuno:protein and active:protein ratios. The curve shown is a third-order polynomial fit of the data. This graph shows that the increases in the immuno:protein and active:protein ratios are dependent on exposure concentration, with a threshold of approximately 5 ppm•hours.

Pickrell and colleagues (1987) have reported that exposure of rats to 0.5 and 1.0 ppm O_3 for 48 hours decreased the elastase inhibitory capacity of serum and lung homogenates, and that exposure to 1.5 ppm O_3 for 48 hours resulted in recovery of the serum elastase inhibitory capacity and dramatic elevation of the airway fluid elastase inhibitory capacity. These studies are difficult to interpret because the exposures resulted in marked inflammation and the elastase inhibitory activity values in lung fluids were reported relative to the weight of control lungs, an obvious error due to the edema in the lungs. In addition, no immunological measurements of α_1-PI were performed, without which it is impossible to tell if α_1-PI was actually inactivated in this study. The lowered serum and increased airway fluid elastase inhibitory activities that these investigators found could have resulted from preferential movement of α_1-PI from the serum to the lung, as shown by our data.

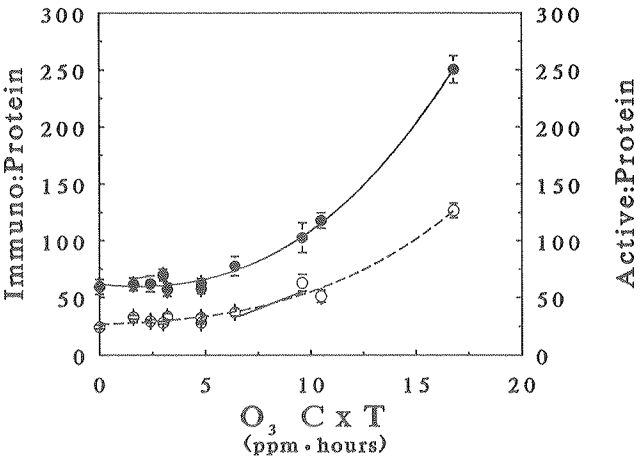

Figure 11. Effect of O_3 concentration × time (C × T) on the immunological concentration and activity of α_1-PI relative to the total protein concentration in lung lavage fluids from acutely exposed rats. The C × T factor for each exposure group was obtained by multiplying the exposure concentration by the exposure time period. For those animals that also received 8 percent CO_2, the C × T term was multiplied by 3 because the inclusion of CO_2 increased the ventilation rate threefold. Error bars represent the SEM. ● = Immuno:protein; ○ = active:protein.

HUMAN STUDIES

INTRODUCTION

During the course of this project, the opportunity arose to examine bronchoalveolar lavage fluids from humans exposed to O_3 and NO_2, as well as nasal lavage fluids from humans exposed to O_3. The NO_2 exposures were performed by M. Utell, M. Frampton, and P. Morrow at the University of Rochester, and the O_3 exposures were conducted at the EPA Clinical Research Branch at Chapel Hill, NC, in protocols under the direction of H. Koren and D. Graham. These studies were approved by the Research Committee of HEI and some additional support was provided in the third year.

Through this work, we sought to determine the effects of O_3 and NO_2 exposure on the functional activity of α_1-PI in the lungs of humans. We also examined the effects of exposure on the immunological and functional levels of BLPI in nasal lavage fluids from O_3-exposed subjects in order to gauge the importance of this inhibitor of neutrophil proteinases in the upper airways. Because many of the physiological responses observed with oxidant exposure are similar to those in allergic reactions, we wanted to know whether or not mast cell degranulation was occurring in exposed individuals. Tryptase is a trypsin-like serine protease of unknown physiological function that is found in the granules of mast cells (Schwartz et al. 1981; Smith et al. 1984), and it has been shown to serve as a specific marker of mast cell activation (Schwartz et al. 1987). We had purified tryptase from human lung tissue and had produced antibodies to it in rabbits (Smith et al. 1984). Consequently, we were one of only two laboratories in the world capable of using tryptase as a marker of mast cell degranulation, and we felt a responsibility to perform these studies.

METHODS

The procedures used in this work were essentially the same as for the studies on rats, except that commercial antiserum to human α_1-PI (Atlantic Antibodies, Scarborough, ME) was used in the immunoassays. Human bronchoalveolar and nasal lavage fluids were analyzed for BLPI (Smith and Johnson 1985) and mast cell tryptase (Smith et al. 1984) using antibodies to these proteins that were prepared in this laboratory. The immunoenzyme dot-blot technique was described earlier and has been published (Smith et al. 1989). This method involved treating the samples with less than saturating concentrations of SDS, followed by blotting to nitrocellulose. The blotted proteins were then reacted with primary antibodies and, finally, incubated with enzyme-linked secondary antibodies.

Human Exposures to Ozone

Human volunteers were exposed, at the EPA Clinical Research Branch in Chapel Hill, NC, to 0.40 ppm O_3 or air during a two-hour period with alternating 15-minute periods of rest and heavy treadmill exercise, which resulted in a minute ventilation of 35 L/minute/m^2 of body surface area and a heart rate of 160 beats/minute. The same subjects served as their own air-exposed controls, with at least six weeks between exposures. Bronchoalveolar lavage was performed approximately 18 hours after exposure. Nasal lavage washings were obtained prior to exposure, immediately after exposure, and 18 hours after exposure. The details of these protocols have been reported (Koren et al. 1990). Cell-free frozen lavage fluids were shipped to our laboratory on dry ice and stored at −80°C until they were analyzed. Both bronchoalveolar and nasal lavage fluids were assayed for total protein by the OPA method. Bronchoalveolar lavage fluids were assayed for α_1-PI activity and for the immunological concentrations of α_1-PI and human lung mast cell tryptase. Nasal washings were assayed for immunological levels of BLPI and tryptase. Elastase inhibition assays of nasal lavage fluids using human neutrophil elastase were performed to measure the activity of BLPI. Inhibition assays of nasal lavage fluids using porcine pancreatic elastase, which measured α_1-PI but not BLPI, were performed also.

Human Exposures to Nitrogen Dioxide

Exposures to nitrogen dioxide were conducted at the University of Rochester by M. Utell, M. Frampton, P. Morrow, and coworkers (Utell et al. 1991). Air and NO_2 exposures were random, blind, and separated by at least two weeks. Healthy, nonsmoking male volunteers were selected in the age range from 19 to 37 years. Bronchoalveolar lavage fluid samples from their "peaks" and "1.5-ppm" protocols were sent to us for analysis. The peaks protocol involved exposure to a base-line level of 0.05 ppm NO_2 with three 15-minute peaks to 2.0 ppm over a three-hour period. Bronchoalveolar lavage was performed 3.5 hours after exposure. Data from only 9 of the 15 subjects in the peaks protocol are presented because samples from the first 6 subjects were analyzed in the early stages of this project when the methods were not well refined, a situation that resulted in considerable scatter in the data, and in which the lack of duplicate determinations was not consistent with the quality control exercised with subsequent determinations. In spite of these problems, no effect of NO_2 exposure was seen. The 1.5-ppm protocol involved a three-hour exposure of 15 subjects to 1.5 ppm NO_2, followed by bronchoalveolar lavage 3.5 hours after exposure.

With both protocols, all subjects exercised for 10 minutes, approximately every 30 minutes, on a bicycle ergometer at

a level previously determined to result in a fourfold increase in minute ventilation. In the peaks protocol, exercise was timed to coincide with the peak NO_2-exposure level. Bronchoalveolar lavage was performed using three 50-mL aliquots of normal saline. After the fluids were filtered through gauze and centrifuged to remove debris, mucus, and cells, the fluids were frozen at −70°C until analysis. Total protein concentration was determined at the University of Rochester. Details of these protocols and attendant procedures are discussed elsewhere (Utell et al. 1991).

Statistical Analyses

All determinations on bronchoalveolar lavage fluid were performed concurrently on both the air- and NO_2-exposed samples for each subject, and the investigators performing the analyses were blinded to the exposure. Data were analyzed by comparing the data from NO_2 exposures with the air-exposed control subjects, using the paired t test. This test was chosen because the same subjects were used for controls and exposures. The 95 percent confidence level ($p < 0.05$) was taken as the significance limit.

The statistical analysis of the data resulting from the measurements on the bronchoalveolar and nasal lavage fluids from humans exposed to 0.4 ppm O_3 at the EPA Clinical Research Branch was conducted using the paired t test and the nonparametric sign test. This approach was taken because only the effect of exposure was being evaluated. Pre-exposure, post-exposure, and 18-hours-post-exposure data on the immunological concentration of BLPI relative to total protein were analyzed. Nasal lavage samples from nine air-exposed control subjects were available for analysis from the pre-, post-, and 18-hours-post-exposure groups. The pre-O_3-exposure group contained samples from 10 subjects after excluding one obvious outlier, based on Chauvenet's criterion test. Samples from nine subjects in the post-O_3-exposure group were statistically analyzed after omitting one obvious outlier; and the 18-hours-post-exposure group contained samples from 11 subjects. These data also were analyzed for the six subjects for whom complete data was available at all time points from both the air-exposed control and O_3-exposed groups. A significant difference again was evident between the immediate post-air-exposure and post-O_3-exposure samples (paired t test, $p = 0.039$; paired sign test, $p = 0.036$).

The effects of O_3 exposure on the concentration of mast cell tryptase in nasal lavage fluids were determined by ANOVA. Two outliers in the control groups were omitted from the analysis. One value in the pre-exposure group was five times the mean for that group, and one value in the 18-hours-post-exposure control group was 3.5 times the mean for its group. These data analyses showed no effect of time; each O_3 exposure time was compared to its corresponding air control time using a t test. Only the immediate post-O_3-exposure group, compared to the immediate post-air-exposure group, had significantly higher levels of tryptase ($p = 0.0008$). Complete data at all time points were available on only five subjects because some samples were not obtained or not shipped; because the same individuals served as the control group, the data from those five subjects were analyzed with a paired t test. These analyses showed that both the immediate post-O_3- and 18-hours-post-O_3-exposure groups were significantly higher than their corresponding control groups, with p values of 0.015 and 0.026, respectively.

Tryptase concentrations per milliliter of bronchoalveolar lavage fluid were obtained on nine subjects, with the same individuals having been exposed to air (controls) and to 0.4 ppm O_3 at different times. These data were analyzed using the paired t test, and the tryptase concentration in the bronchoalveolar lavage fluids with O_3 exposure was found to be significantly higher ($p = 0.01$) than in the samples with air exposure.

RESULTS

Nasal Lavage, Ozone, and Bronchial Leukocyte Proteinase Inhibitor

The activity and immunological concentration of BLPI was measured in the nasal lavage fluids from humans exposed to 0.4 ppm O_3 for two hours, with exercise, to determine if O_3 exposure would compromise the function of this extravascular inhibitor of neutrophil elastase. The ratios of neutrophil elastase inhibitory activity to the immunological concentration of BLPI for the pre-O_3- and 18-hours-post-O_3-exposure groups were not significantly ($p > 0.4$) different from the corresponding air-exposed control groups, as shown in Figure 12. The basic data from these studies are presented in Table 5, which includes data on the two subjects who were considered outliers and were omitted from the statistical analyses. However, the ratios of neutrophil elastase inhibitory activity to the immunological concentration of BLPI in the immediate post-O_3-exposure samples were significantly ($p = 0.0012$) lower than in the corresponding air-exposed samples (Figure 13).

To minimize effects due to variation in subjects, statistical analyses were performed on data from six subjects for whom complete data were available at all time points of both air and O_3 exposure. A significant difference was again evident between the immediate post-air-exposure and post-O_3-exposure subjects (paired t test, $p = 0.039$; paired sign test, $p = 0.036$). These results show that BLPI activity was depressed immediately after exposure. This could be

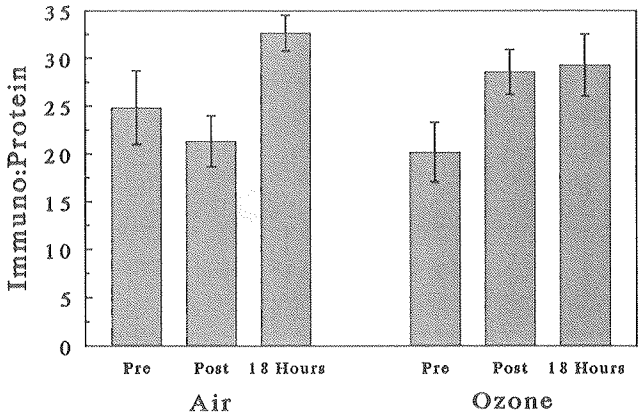

Figure 12. Immunological concentration of BLPI in nasal lavage fluids of human subjects exposed to air and O_3. Subjects were exposed to either air or 0.4 ppm O_3 for two hours. Nasal lavage was performed before exposure (pre), immediately after exposure (post), and 18 hours after exposure (18-hours). The immunological concentration of BLPI was determined by enzyme-linked immunoassay on nitrocellulose. For statistical analyses, two data points were excluded because they were obvious outliers. The *t* test was used to compare air samples to O_3 samples: pre-air (n = 9) to pre-O_3 (n = 11), post-air (n = 9) to post-O_3 (n = 10), and 18-hours-post-air (n = 9) to 18-hours-post-O_3 (n = 11). The OPA method was used to determine total protein concentrations, with BSA serving as the standard. Statistical analyses did not reveal any significant differences in the concentration of BLPI in samples from O_3-exposed subjects relative to air-exposed individuals. Error bars represent the SEM.

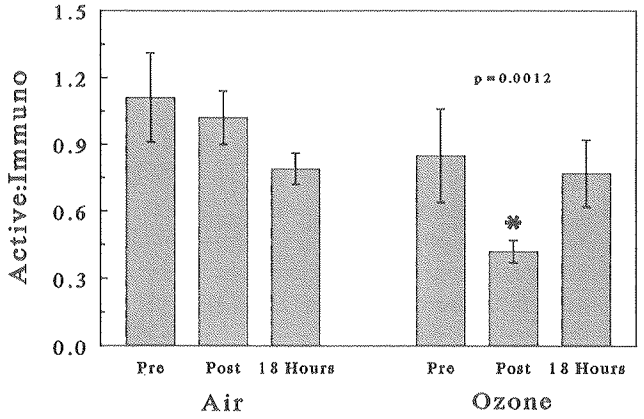

Figure 13. Effect of O_3 on the ratio of BLPI activity to its immunological concentration (active:immuno) in human nasal lavage fluids. Subjects were exposed to either air or 0.4 ppm O_3 for two hours. Nasal lavage was performed before exposure (pre), immediately after exposure (post), and 18 hours after exposure (18-hours). Bronchial leukocyte proteinase inhibitor activity was measured by inhibition of human neutrophil elastase. The immunological concentration of BLPI was determined by enzyme-linked immunoassay on nitrocellulose. For statistical analyses, two data points were excluded because they were obvious outliers. The *t* test was used to compare air samples to O_3 samples: pre-air (n = 9) to pre-O_3 (n = 11), post-air (n = 9) to post-O_3 (n = 10), and 18-hours-post-air (n = 9) to 18-hours-post-O_3 (n = 11). The pre and 18-hours-post samples were not significantly ($p > 0.4$) different, but the immediate post-O_3 samples were significantly (p = 0.0012) lower than the immediate post-air samples. Error bars represent the SEM.

due to direct or indirect oxidative damage to the BLPI protein, or the inhibitor could be bound to proteases released from neutrophils. The latter case seems more likely, because the nasal lavage contained significantly ($p < 0.003$) higher numbers of neutrophils in both the immediate post-O_3- and the 18-hours-post-O_3-exposure samples (Koren et al. 1990). Although there were no significant increases in the immunological concentrations of BLPI upon exposure, somewhat higher levels are suggested by Figure 12 for the 18-hours-post-air-, post-O_3-, and 18-hours-post-O_3-exposure groups, possibly indicating that BLPI increases in response to the lavage process.

Table 5. Bronchial Leukocyte Proteinase Inhibitor in Nasal Lavage Fluids from Humans Exposed to Ozone or Air[a]

Treatment	Total Protein (μg/mL)	Active BLPI (μg/mL)	Immuno BLPI (μg/mL)	Active: Protein (μg/mg)	Immuno: Protein (μg/mg)	Active: Immuno (μg/μg)
Air Controls						
Pre (n = 9)	436 ± 79	8.3 ± 1.3	9.9 ± 1.9	25.1 ± 6.9	24.8 ± 3.9	1.11 ± 0.20
Post (n = 9)	439 ± 71	7.7 ± 0.8	8.2 ± 0.9	19.6 ± 2.0	21.3 ± 2.7	1.02 ± 0.12
18-Hours-post (n = 9)	303 ± 33	7.0 ± 0.3	9.8 ± 1.1	25.5 ± 2.4	32.7 ± 1.9	0.79 ± 0.07
O_3						
Pre (n = 10)	602 ± 121	6.1 ± 0.8	9.5 ± 2.0	12.6 ± 1.9	20.2 ± 3.1	0.85 ± 0.21
Post (n = 9)	398 ± 47	4.5 ± 0.6	10.3 ± 2.0	11.7 ± 1.6	28.5 ± 2.4	0.42 ± 0.05
18-Hours-post (n = 11)	473 ± 68	8.6 ± 1.1	14.4 ± 2.9	19.1 ± 1.7	29.3 ± 3.2	0.77 ± .015
Outliers Omitted from Analysis of O_3-Exposed Subjects						
Pre	552	8.2	0.59	15.7	1.1	14.0
Post	314	3.2	0.06	10.2	0.2	53.7

[a] Concentration of O_3 was 0.4 ppm. Values are presented as means ± SD after omitting outliers. These data were used for the construction of Figures 12 and 13.

Nasal Lavage and Mast Cell Tryptase

The concentration of mast cell tryptase was measured in the nasal lavage fluids of human volunteers exposed to 0.4 ppm O_3 for two hours, with exercise, to see if exposure had an effect on these cells. Mast cells are prevalent in the airways and have been shown to comprise 2 percent of the alveolar cells (Fox et al. 1981). Mast cell tryptase is a granule component of human mast cells that is released with histamine (Schwartz et al. 1981) when these cells are stimulated to degranulate, and it has been shown to be a specific marker of mast cell degranulation (Schwartz et al. 1987).

Tryptase concentrations relative to the total protein concentrations were significantly increased in the nasal lavage fluids of humans obtained immediately after exposure to 0.4 ppm O_3 (p = 0.0008), when each sampling time point was compared with its corresponding air-exposed control time point (Figure 14). These data are presented in Table 6, along with the data from three subjects who were considered outliers and were excluded from the statistical analyses. However, the concentration of tryptase was not significantly increased in nasal fluids obtained prior to or 18 hours after exposure. When the data were analyzed for those five subjects from whom data were available at all time points, both the immediate post-exposure and 18-

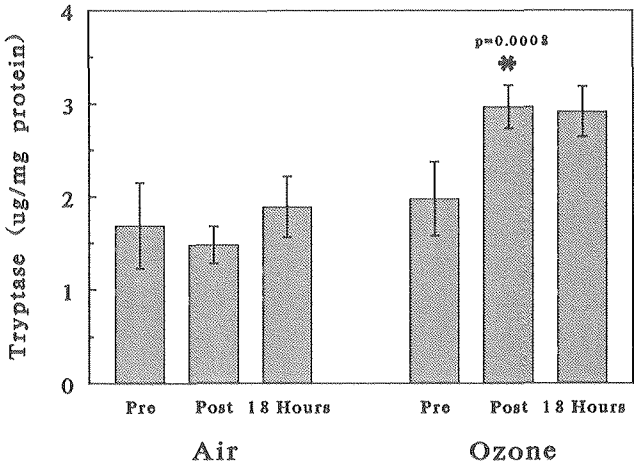

Figure 14. Effect of O_3 exposure on the concentration of mast cell tryptase in nasal lavage fluids, as determined from data from all available samples. Human subjects were exposed to 0.4 ppm O_3 for two hours, with exercise, and nasal lavage was done prior to exposure (pre), immediately after exposure (post), and 18 hours after exposure (18-hours). The immunological concentration of tryptase relative to the total protein concentration was determined. For statistical analyses, two data points were excluded because they were obvious outliers. The t test was used to compare air samples to O_3 samples: pre-air (n = 8) to pre-O_3 (n = 11), post-air (n = 9) to post-O_3 (n = 9), and 18-hours-post-air (n = 8) to 18-hours-post-O_3 (n = 11). The pre and 18-hours-post samples were not significantly different (p = 0.5 and 0.15, respectively), but the immediate post-O_3 samples were significantly (p = 0.00078) lower than the immediate post-air samples. Error bars represent the SEM.

Table 6. Mast Cell Tryptase in Nasal Lavage Fluids from Humans Exposed to Ozone or Air[a]

Treatment	Total Protein (μg/mL)	Tryptase Immuno (μg/mL)	Tryptase: Protein (μg/mg)
Air Controls			
Pre (n = 8)	465 ± 88	0.75 ± 0.16	1.83 ± 0.40
Post (n = 9)	439 ± 79	0.65 ± 0.14	1.52 ± 0.18
18-Hours-post (n = 8)	314 ± 37	0.59 ± 0.07	2.06 ± 0.28
O_3			
Pre (n = 11)	610 ± 140	1.26 ± 0.24	2.22 ± 0.35
Post (n = 9)	427 ± 53	1.22 ± 0.25	2.84 ± 0.24
18-Hours-post (n = 11)	473 ± 75	1.26 ± 0.21	2.66 ± 0.27
Outliers Omitted from Analysis of Air Controls			
Pre	206	3.91	19.0
18-Hours-post	221	2.67	12.1
Outliers Omitted from Analysis of O_3-Exposed Subjects			
O_3			
Post	314	ND[b]	ND

[a] Concentration of O_3 was 0.4 ppm. Values are presented as means ± SD after omitting outliers. These data were used for the construction of Figure 14.

[b] ND = not detectable.

Table 7. Tryptase in Nasal Lavage Fluids from Five Human Subjects Exposed to Ozone or Air[a]

	Air			Ozone		
Subject No.[b]	Pre	Post	18-Hours-Post	Pre	Post	18-Hours-Post
3	2.56	0.60	2.74	4.25	3.44	3.17
5	0.78	1.28	1.17	1.93	2.60	2.91
6	0.84	1.60	2.43	1.77	2.58	2.83
10	4.18	2.31	3.32	1.81	3.20	4.41
11	0.92	0.98	0.97	1.86	2.19	2.40
Mean	1.86	1.35	2.13	2.32	2.80	3.14
SEM	0.60	0.26	0.41	0.43	0.20	0.30

[a] Tryptase values are given in μg/mg of protein. Concentration of O_3 was 0.4 ppm.

[b] Subject numbers refer to those of Koren and associates (1990). Due to missing samples at different time points, these were the only five subjects for whom complete data were available at all sampling time points.

hours-post-exposure groups that received O_3 had higher concentrations of tryptase than their corresponding controls, with p values of 0.015 and 0.026, respectively. These data are presented in Table 7, using the subject numbers of Koren and associates (1990). The differences in the tryptase levels (O_3-exposed and air-exposed groups) are displayed in Figure 15.

Bronchoalveolar Lavage Fluids, Ozone, and Tryptase

Mast cell tryptase was measured in the bronchoalveolar lavage fluids obtained from the same subjects exposed to 0.4 ppm O_3 for two hours, as in the protocol for obtaining nasal lavage fluids. Bronchoalveolar lavage was performed 18 hours after the end of the exposure period. Koren and colleagues (1989) had shown that O_3 exposure caused increases in the protein concentration of the bronchoalveolar lavage fluids, and that this was due to leakage from the blood. Therefore, the tryptase concentration of the bronchoalveolar lavage fluids was reported as micrograms per milliliter of lavage fluid, rather than micrograms per milligrams of total protein, as for the nasal lavage samples. Protein and tryptase concentrations, in micrograms per milliliter, are presented in Table 8. Tryptase is derived from the

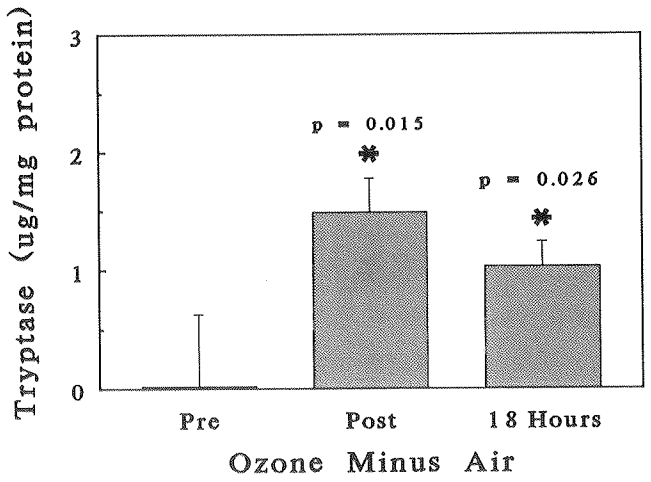

Figure 15. Effect of O_3 exposure on the concentration of mast cell tryptase in nasal lavage fluids. Five subjects were exposed to 0.4 ppm or air for two hours, with nasal lavage performed prior to exposure (pre), immediately after exposure (post), and 18 hours after exposure (18-hours). The immunological concentrations of tryptase (μg/mg protein) in the air-exposed samples were subtracted from the O_3-exposed values. The post and 18-hours-post concentrations were significantly elevated (paired t test), with p values of 0.015 and 0.026, respectively. Complete data at all time points were available on these five subjects only, which permitted this type of analysis. Error bars represent the SEM.

Table 8. Tryptase in Bronchoalveolar Lavage Fluids from Humans Exposed to Ozone or Air[a]

	Air		Ozone	
Subject No.[b]	Total Protein (μg/mL)	Tryptase (μg/mL)	Total Protein (μg/mL)	Tryptase (μg/mL)
2	206	1.53	386	3.45
3	179	1.07	413	3.88
4	212	1.90	435	3.44
5	195	1.57	355	3.22
6	206	1.10	760	10.10
7	218	0.98	491	4.66
9	186	0.70	410	0.63
10	262	3.77	419	5.15
11	154	0.12	546	5.09
Mean	202	1.42	468	4.40
SEM	20	0.32	37	0.79

[a] Bronchoalveolar lavage was performed 18 hours after exposure to air or 0.4 ppm O_3 for two hours, with intermittent exercise. The same individuals were exposed to air or O_3 in a random fashion, with a six-week period between exposures.

[b] Subject numbers refer to those of Koren and associates (1989).

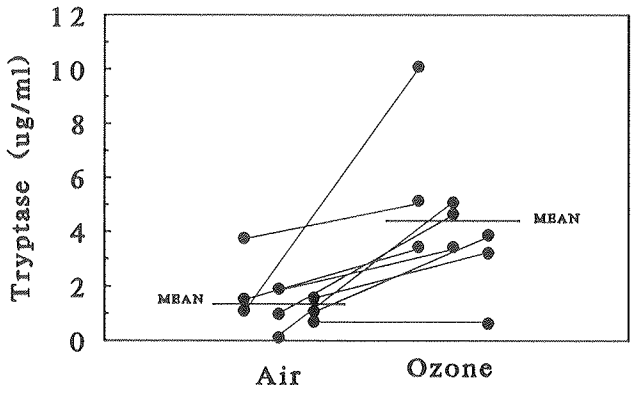

Figure 16. Effect of O_3 exposure on the concentration of mast cell tryptase in human bronchoalveolar lavage fluids. The immunological concentration of mast cell tryptase was measured in bronchoalveolar lavage fluids from nine subjects exposed to 0.4 ppm O_3 for two hours, with exercise. The same subjects served as their own air-exposed controls, and lavage was performed 18 hours after exposure. The horizontal bars mark the means for each group, which were significantly different (p = 0.01), and the data points for individual subjects are connected by a line.

mast cells of the lung, and its concentration relative to blood proteins would be an improper relationship to report because it would have obscured the effect. Both total protein (primarily from the blood) and tryptase concentrations in the bronchoalveolar lavage fluids increased, but for different reasons.

Tryptase concentrations in the bronchoalveolar lavage fluids increased upon exposure to 0.4 ppm O_3, as shown in Figure 16, and the levels in the O_3-exposed samples were significantly higher than in the air-exposed control samples. Statistical analysis by the paired t test yielded a p value of 0.01. Concentrations in the air-exposed control samples ranged from 0.12 to 3.77 μg/mL, whereas the O_3-exposed samples ranged from 0.63 to 10.1 μg/mL. Comparing individuals, all except one showed higher tryptase levels with O_3 than with air, and the one exception had low levels in both cases (air = 0.70; O_3 = 0.63).

Bronchoalveolar Lavage Fluids, Ozone, and Alpha-1-Proteinase Inhibitor

The same bronchoalveolar lavage fluid samples that had been analyzed for tryptase were assayed for α_1-PI. These individuals had been exposed to 0.4 ppm O_3 for three hours with exercise, and bronchoalveolar lavage was performed 18 hours after exposure. The concentration of active α_1-PI was based on the inhibition of porcine pancreatic elastase relative to pure α_1-PI that was determined to be 100 percent active. Immunological concentrations of α_1-PI were measured in an enzyme-linked immunoassay using commercial antibodies to human α_1-PI, with pure α_1-PI serving as the standard. The immunological concentrations of α_1-PI were too low in three of the air-exposed samples to quantify, and there was not enough sample to concentrate. Therefore, data analyses were restricted to the samples from the six individuals for whom the assays were complete.

The concentrations of total protein, active α_1-PI, and immunological α_1-PI are given in Table 9. As with the analyses of the rat lung lavage fluids, the ratio of active α_1-PI to its immunological concentration (active:immuno) and the ratio of the immunological concentration of α_1-PI to the total protein concentration (immuno:protein) were examined (Figure 17). There were no statistically significant differences in either ratio when comparing the exposures. Thus, neither the functional activity nor the relative immunological concentration of α_1-PI was altered by exposure to O_3. However, the concentrations of total protein, active α_1-PI, and immunological α_1-PI were significantly ($p < 0.005$) increased in the bronchoalveolar lavage fluids after O_3 ex-

Table 9. Alpha-1-Proteinase Inhibitor in Bronchoalveolar Lavage Fluids from Humans Exposed to Ozone or Air[a]

	Air			Ozone		
Subject No.	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)
2	206	0.74	0.73	386	2.24	3.33
3	179	0.72	1.90	413	2.24	2.55
5	195	0.85	1.31	355	1.74	2.45
7	218	0.86	0.87	491	3.56	3.53
9	186	0.81	0.89	410	2.44	2.66
10	262	0.87	1.18	419	1.91	2.35
Mean	207.7	0.81	1.15	412.3	2.35	2.81
SEM	11.2	0.02	0.16	16.9	0.24	0.18

[a] Concentration of O_3 was 0.4 ppm. Bronchoalveolar lavage was performed 18 hours after exposure.

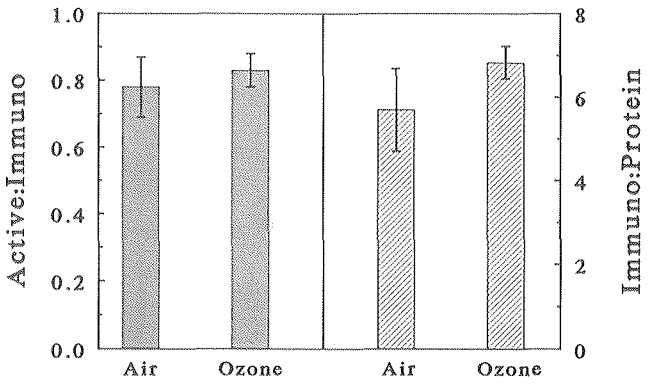

Figure 17. Effect of O_3 exposure on the functional activity and immunological concentration of α_1-PI in human bronchoalveolar lavage fluids. The ratios of active α_1-PI to its immunological concentration (active:immuno) and the immunological concentration of α_1-PI relative to the total protein concentration (immuno:protein) were determined in bronchoalveolar lavage fluids obtained 18 hours after exposure. The same subjects served as their own control group, with the exposure periods separated by six weeks. Exposures were to 0.4 ppm O_3 or air for two hours with exercise. Error bars represent the SEM.

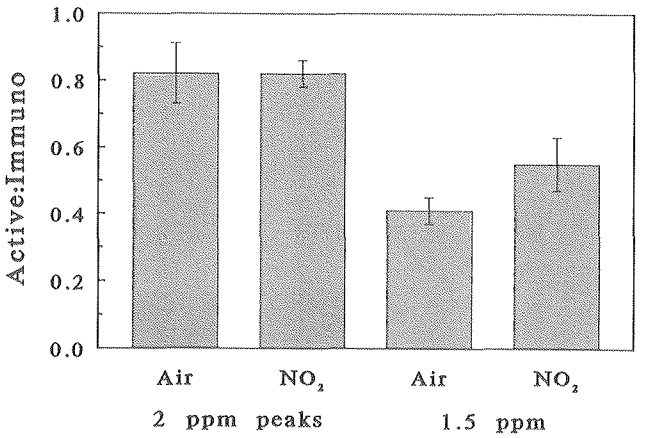

Figure 18. Effect of NO_2 exposure on the functional activity of α_1-PI in human bronchoalveolar lavage fluids. The ratio of active α_1-PI to its immunological concentration (active:immuno) was determined in bronchoalveolar lavage fluids obtained 3.5 hours after exposure. The same subjects served as their own control group, by random exposure for three hours to air or NO_2, with at least two weeks between procedures. There were 9 subjects in the 2-ppm peaks protocol and 15 subjects in the 1.5-ppm protocol. Error bars represent the SEM.

posure. These findings are consistent with those of Koren and associates (1989), who found a doubling of the total protein concentration after exposure.

Bronchoalveolar Lavage Fluids, Nitrogen Dioxide, and Alpha-1-Proteinase Inhibitor

The elastase inhibitory activity of α_1-PI and its immunological concentration were measured in bronchoalveolar lavage fluid samples from individuals exposed to NO_2 under two protocols. The results of the analyses are presented in Table 10. For the 2-ppm NO_2 peaks protocol, the ratios of active α_1-PI to its immunological concentration (active:immuno) were not significantly different for the NO_2 samples when compared to the air-exposed control samples. Neither was there a significant difference in the immunological concentration of α_1-PI relative to total protein. Similar results were obtained with the samples from the 1.5-ppm NO_2 exposure protocol. Because the principal measure of interest was the ratio of active to immunological α_1-PI, the active:immuno data from both protocols are shown in Figure 18. Although this ratio theoretically should have equaled 1.0, it averaged 0.82 for both air and NO_2 with the 2-ppm peak protocol. These ratios were lower for the 1.5-ppm protocol, with means of 0.41 for the air exposures and 0.55 for the NO_2 exposures. Why the ratios were lower with the 1.5-ppm protocol is not clear, but it probably relates to technical differences in the performance of the assays. Afford and colleagues (1988) have reported that the activity of α_1-PI in bronchoalveolar lavage fluid decreases with time, even

Table 10. Alpha-1-Proteinase Inhibitor in Bronchoalveolar Lavage Fluid Samples from Subjects Exposed to Nitrogen Dioxide[a]

Treatment	Bronchoalveolar Lavage Fluid Volume (mL)	Total Protein (μg/mL)	Active α_1-PI (μg/mL)	Immuno α_1-PI (μg/mL)
Peaks protocol (n = 9)				
Air	106.2 ± 12[a]	128.6 ± 56	0.81 ± 0.08	1.18 ± 0.63
NO_2	103.9 ± 18	126.2 ± 40	0.80 ± 0.08	1.02 ± 0.28
1.5-ppm protocol (n = 15)				
Air	113.2 ± 14	133.5 ± 46	0.42 ± 0.06	1.26 ± 0.93
NO_2	115.9 ± 8	124.8 ± 34	0.36 ± 0.05	0.89 ± 0.60

[a] Data are presented as mean ± SEM for each group. The same individuals were exposed to air or NO_2 in a random fashion, with a two-week period between exposures.

when it is frozen. Irrespective of why these active:immuno ratios are less than 1.0, the conclusion that NO_2 had no effect on the ratios relative to air-exposed control samples is clear. In the 1.5-ppm protocol, which had the lowest ratios, the mean values for the NO_2-exposed samples were slightly higher than for the air-exposed samples.

Bronchoalveolar Lavage Fluids, Nitrogen Dioxide, and Bronchial Leukocyte Proteinase Inhibitor

The immunological concentration of BLPI was measured in bronchoalveolar lavage fluids from individuals in the 2-ppm NO_2 peaks exposure protocol, but activity assays could not be performed because the concentration of α_1-PI was much greater than that of BLPI. The BLPI concentrations relative to the total protein concentrations averaged 0.273 ± 0.05 μg/mg for the air-exposed bronchoalveolar lavage fluids and 0.311 ± 0.05 μg/mg for the NO_2-exposed bronchoalveolar lavage fluids, whereas the concentrations of α_1-PI averaged 9.16 ± 0.69 μg/mg and 8.52 ± 0.78 μg/mg for the air- and NO_2-exposed bronchoalveolar lavage fluids, respectively. There were no significant differences in the concentration of BLPI in the NO_2-exposed samples compared to the air-exposed control samples. In comparison with the concentration of α_1-PI in these samples, the molar concentration of α_1-PI was approximately seven times that of BLPI.

DISCUSSION

Effects of Ozone on Nasal Lavage Bronchial Leukocyte Proteinase Inhibitor

Bronchial leukocyte proteinase inhibitor is the major inhibitor of neutrophil elastase and cathepsin G in bronchial secretions, and it apparently serves to control the activities of these two potent serine proteinases in mucous secretions (Fritz 1988). Neutrophils can leave the vasculature in response to various stimuli, reaching high concentrations in extravascular spaces such as the lung. Considerable increases in the numbers of neutrophils were observed in the nasal lavage fluids from the same 0.4-ppm O_3-exposed individuals as examined in this study (Koren et al. 1990). The rate constant for the inhibition of neutrophil elastase by BLPI is comparable to that of α_1-PI (Boudier and Bieth 1989), which suggests that BLPI is as effective as α_1-PI in controlling elastase. The number of lung epithelial cells containing BLPI increases with inflammation of the small airways (Kramps et al. 1988), and there are indications that BLPI may be more effective than α_1-PI in controlling neutrophil proteases released during phagocytosis (Axelsson et al. 1988). Thus, BLPI may play a more important role than previously thought in protecting the lung from damage by neutrophil proteinases.

Our data indicate that BLPI is the only inhibitor of neutrophil proteinases in the nasal passages, because we failed to detect α_1-PI with our sensitive immunological assay. In addition, we could not detect any inhibitory activity against porcine pancreatic elastase, which is inhibited by α_1-PI but not by BLPI (Smith and Johnson 1985). No significant changes were observed in the immunological concentrations of BLPI relative to total protein in nasal lavage fluids with O_3 exposure, indicating that BLPI is not released in response to O_3. However, the activity of BLPI against neutrophil elastase relative to its immunological concentration was significantly decreased in the nasal lavage fluids obtained immediately after O_3 exposure, compared to the same samples collected after air exposure ($p = 0.001$). These results are consistent with the finding of increased numbers of neutrophils in the nasal lavage fluids at this time point for O_3-exposed subjects (Koren et al. 1990), which may have released proteinases that would have reacted with BLPI. Why a similar decrease was not found 18 hours after exposure is not clear, because there were increased numbers of neutrophils in the lavage at this time point also. It could be that at the latter time point, the neutrophils were not releasing their enzymes. It is possible that BLPI complexed with proteases was not accurately measured because of the shielding of antigenic determinants on BLPI by protease (Kramps et al. 1984). Although we did not investigate the effects of proteases complexed with BLPI on the accuracy of the assay, we feel that treating the samples with SDS and boiling them would have sufficiently exposed protease-complexed BLPI for reaction with antibodies and quantification. Complexation of BLPI with proteases was not a concern in the bronchoalveolar lavage fluids because these samples contained active α_1-PI, which rapidly displaces BLPI from such complexes (Smith and Johnson 1985).

Effects of Ozone on Mast Cell Tryptase

The increase in tryptase concentrations found in both nasal lavage and bronchoalveolar lavage fluids indicates that O_3 exposure causes mast cell degranulation. Whether or not this results from a direct or indirect effect remains to be determined. Only the immediate post-exposure group, using all available data, had significantly ($p = 0.0008$) increased levels of tryptase, indicating that the effect is rapid. Analyses of the data from the five subjects with data at all time points showed that tryptase was significantly increased in the immediate post-exposure and 18-hours-post-exposure nasal lavage fluids, with p values of 0.015 and 0.026, respectively. These data indicate that the higher levels of tryptase are sustained for up to 18 hours. Tryptase also was elevated significantly ($p = 0.01$) in the bronchoalveolar lavage fluids obtained 18 hours after exposure, but the time at which the enzyme was released into the lung is un-

known. The measured tryptase in bronchoalveolar lavage fluid could have been released immediately upon exposure, remaining in the lung until lavage, or it may have been released at some time between exposure and the performance of the lavage. The nasal lavage data support the idea of immediate release.

These results have important implications for the effects of O_3 on the lung. The mast cell contains a host of powerful mediators. The immediate or "classic" allergic reaction results in smooth muscle contraction, vascular leakage, hypotension, mucous secretion, and pruritis, and ends within 30 to 60 minutes, but there is also a late-phase response that becomes apparent within 4 to 8 hours and lasts up to 24 hours, which involves the infiltration of eosinophils and neutrophils as well as fibrin deposition (Kaliner and Lemanske 1984). The combined action of immediate and late-phase reactants can lead to inflammation. Thus, many of the immediate and delayed physiological and biochemical responses to O_3 exposure could be attributed to mast cells. Although the release of histamine triggered by the binding of antigens to IgE on the surface of mast cells has received the most attention, several other stimuli, including heat, sunlight, cold, pressure, hypoxia, and neuropeptides, can cause mast cell degranulation (Friedman and Kaliner 1987). Thus, mast cells would appear to serve as cellular sentinels that alert the host to adverse environmental conditions. Ozone exposure was reported to enhance the asthmatic response of monkeys in a platinum-induced asthma model (Biagini et al. 1986). Bronchial hyperactivity in guinea pigs exposed to O_3 was correlated with mucosal injury and mast cell infiltration, which occurred prior to neutrophil influx (Murlas and Roum 1985). Asthmatic individuals also are thought to be at risk from the effects of air pollutants, and the mast cell is known to play a role in asthma (Friedman and Kaliner 1987).

Effects of Ozone on Alpha-1-Proteinase Inhibitor in Bronchoalveolar Lavage Fluids

Exposure to 0.4 ppm O_3 for three hours, with exercise, did not result in any decrease in the functional activity of α_1-PI in the bronchoalveolar lavage fluids of six subjects also exposed to air, nor was there any change in the immunological concentration of α_1-PI relative to the total protein concentration. A significant ($p < 0.005$) increase was seen in the concentration of total protein, and significant increases were noted in the concentrations of both active and immunological α_1-PI ($p < 0.005$). These data indicate that although O_3 did have an effect on α_1-PI (that is, α_1-PI concentrations increased along with other proteins), it appears that the O_3 exposure did not inactivate the inhibitor in the lungs of O_3-exposed subjects. Because the bronchoalveolar lavage was performed 18 hours after exposure, it is possible that inactivation occurred during exposure and that by 18 hours, any inactive α_1-PI had been replaced with active inhibitor from the blood. On the basis of the data from acute exposures in rats, it seems doubtful that any inactivation results from O_3 exposure. These results are consistent with the general absence of emphysema in O_3-exposed animals.

Effects of Nitrogen Dioxide on Alpha-1-Proteinase Inhibitor and Bronchial Leukocyte Proteinase Inhibitor in Bronchoalveolar Lavage Fluids

In two separate NO_2 exposure protocols with 9 and 15 subjects who served as their own controls, there was no effect of exposure on the functional activity of α_1-PI. Differences in the actual active:immuno ratios obtained for the two protocols are probably due to differences in sample handling or minor variations in the assay techniques. Presumably, any differences in assays or sample handling were balanced by treating the air- and NO_2-exposed samples identically. Considerable variability in the activity of α_1-PI in bronchoalveolar lavage fluids relative to its immunological concentration has been reported by different investigators (Stone et al. 1983; Abboud et al. 1985; Bridges et al. 1985; Lellouch et al. 1985), with mean values ranging from 39 to 125 percent. These differences are probably due to inherent differences among subjects and to errors in the assays. Because the functional activity of α_1-PI is based on the ratio of its inhibitory activity to its immunological concentration, errors in the measurement of either factor could be magnified or obscured depending on the direction and magnitude of the error resulting from each measurement. In the current study, individual variability was controlled for by having the same individuals serve as the control group. Thus, the lowered active:immuno ratios found in the 1.5-ppm NO_2 protocol study may result from an underestimation of elastase inhibitory activity, an overestimation of the immunological concentration of α_1-PI, or a combination of these two errors.

In the only published paper dealing with the effects of NO_2 exposure on α_1-PI activity in bronchoalveolar lavage fluid, Mohsenin and Gee (1987) examined α_1-PI activity in bronchoalveolar lavage fluid samples from subjects exposed to 3 or 4 ppm NO_2 for three hours, with exercise, as a percentage of its theoretical activity. Bronchoalveolar lavage was performed 3.5 to 4 hours after exposure. These workers found that α_1-PI functional activity was decreased 45 percent in the bronchoalveolar lavage fluids from exposed subjects relative to a separate, air-exposed control group. The apparent inactivation of α_1-PI found by Mohse-

nin and Gee (1987) may be due to inherent differences in the subject and control groups, which were composed of different individuals. This problem was avoided in our studies by using the same subjects for the control exposures with air. In their paper, Mohsenin and Gee (1987) stated that the exposures were performed in random order, but did not mention whether samples from the exposure and control groups were assayed at the same time or separately. Differences in the performance of the assays also could account for the effect they observed. Such problems were avoided in our study by simultaneously performing the assays on both the exposure and control groups. In addition, Afford and associates (1988) found that α_1-PI in bronchoalveolar lavage fluid lost activity even when frozen at −70°C. Thus, differences in the time of storage prior to assay could account for the results of Mohsenin and Gee (1987). Mohsenin and Gee also concentrated their bronchoalveolar lavage fluids by positive pressure on ultrafiltration membranes, which has been shown to result in variable recovery of α_1-PI protein and activity (Afford et al. 1985). We used methods that did not require concentration of the bronchoalveolar lavage fluids from the NO_2 exposure protocols, but the decreased α_1-PI activities of the 1.5-ppm protocols cannot be explained. The failure of 1.5 or 2 ppm NO_2 to inactivate α_1-PI is consistent with the findings of Dooley and Pryor (1982), who showed that bubbling solutions of pure α_1-PI with NO_2 did not inactivate α_1-PI unless hydrogen peroxide was included in the solution. From the ventilation rates reported by Mohsenin and Gee (1987), we calculate that their subjects were exposed to 23.5 g of NO_2 in the 4-ppm exposure and 15.8 g of NO_2 in the 3-ppm exposure. The total amounts of NO_2 received by the subjects in our study were 5.05 g of NO_2 in the 2-ppm protocol and 8.21 g of NO_2 in the 1.5-ppm protocol. Thus, the inactivation of α_1-PI reported by Mohsenin and Gee (1987) could be real, but our data show that under more environmentally relevant conditions, NO_2 does not alter the activity of α_1-PI in the lung.

Exposure to NO_2 in the 2-ppm peaks protocol did not alter the immunological concentration of BLPI in bronchoalveolar lavage fluid, suggesting that NO_2 exposure does not change the amount of this inhibitor that is secreted by lung cells. Although we found the molar ratio of α_1-PI:BLPI to be about 7, Stockley and associates (1984) reported just the opposite relationship, with a molar ratio of BLPI:α_1-PI close to 5. However, in another study, Morrison and colleagues (1986) found the molar ratio of α_1-PI:BLPI to be 4.8, in closer agreement with our results. These varied findings are not easily explained, but the differences may be due to differences in bronchoalveolar lavage techniques or in the specificity of the antibodies used in the two assays. The subjects studied by Stockley and coworkers (1984) all had chronic bronchitis, and Kramps and colleagues (1988) have reported increased numbers of BLPI-producing cells in the lungs of diseased patients. Therefore, the concentration of BLPI in the lung may vary with the health of the individual and with possible unknown genetic factors. In addition, BLPI that had formed complexes with proteinases may not have been fully detected in our assays, but this is unlikely because neutrophil elastase is rapidly transferred from BLPI to α_1-PI (Gauthier et al. 1982) and there was active α_1-PI in the bronchoalveolar lavage fluid. Further investigations on the concentration of BLPI in bronchoalveolar lavage fluid are needed to clarify the issue of whether or not α_1-PI or BLPI is the principal elastase inhibitor in the lung.

IMPLICATIONS OF THE FINDINGS

Neither O_3 nor NO_2 exposure of rats resulted in measurable reductions in the functional activity of α_1-PI in lung lavage fluids, indicating that these oxidants do not damage α_1-PI. These findings were confirmed by studies on humans exposed to O_3 and NO_2. Thus, it would seem that these oxidants do not inactivate α_1-PI in vivo and the lung should be adequately protected from neutrophil elastase. Diesel exposure of rats did result in decreased functional levels of α_1-PI in the lung, suggesting that this pollutant may contribute to chronic obstructive lung disease. Although the reduction in α_1-PI activity was not of sufficient magnitude to create a situation comparable to the human ZZ homozygous deficiency state, certain areas of the lung may have been rendered susceptible to proteolysis. Thus, there is the possibility that chronic exposure to diesel emissions contributes to chronic lung diseases, such as emphysema. Additional studies are needed to confirm these findings in rats and in humans before considering the regulation of diesel emissions.

Exposure of human subjects to 0.4 ppm O_3 for two hours, with exercise, resulted in the release of mast cell tryptase in the nasal passages and the lungs, indicative of mast cell degranulation. These studies must be repeated at lower concentrations of O_3 to determine the lowest concentration capable of producing a significant response. The possibility that some individuals, such as those with asthma, may be more susceptible to O_3-induced mast cell degranulation also needs to be examined, because such individuals may represent a susceptible population. In addition, tryptase could serve as a marker of exposure to other pollutants as well as ozone, and the specificity of this response needs to be defined. Although the regulatory significance of the present findings are unclear, they point out the potential usefulness of this marker and the involvement of mast cells in the response to O_3 exposure, which may explain previously recognized physiological responses. A better under-

standing of the effects of oxidants on particular cells may lead to better treatment methods for individuals adversely affected by exposure and may result in methods of prevention.

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ABOUT THE AUTHORS

David A. Johnson received his Ph.D. in chemistry from Memphis State University in 1973, where his dissertation work dealt with the proteinases of *Escherichia coli*. Two summers during the years in graduate school were spent in the Antigen Preparation Laboratory of the National Center for Disease Control in Atlanta, GA. Three years of postdoctoral study were spent in the laboratory of James Travis at the University of Georgia, Athens, GA, followed by two years of research as an Assistant Biochemist. During this time, work on the structure of human alpha-1-proteinase inhibitor resulted in determining the inhibitory site of α_1-PI, which contains an oxidant-sensitive methionine residue. In 1978, he moved to East Tennessee State University to participate in the establishment of a new college of medicine. A three-month period of research on mast cell tryptase was conducted at Strangeways Research Laboratory, Cambridge, England, in 1985. He received the University's Foundation

Research Award in 1987, which is given each year to the most outstanding researcher at ETSU. In 1990, he was promoted to the rank of Professor and continues his work on the effects of ozone and nitrogen dioxide on lung proteins, in addition to studies on the structure and function of mast cell tryptase.

R. Steve Winters received an M.S. degree in chemistry in 1979 and an M.B.A. in 1989 from East Tennessee State University. He was a Senior Chemist for International Telephone and Telegraph, Gray, TN, from 1977 until 1983. He is experienced in organic chemistry, spectroscopy, and biochemical techniques. He worked on this project as a Research Associate and is presently pursuing a Ph.D. in this same area of research at East Tennessee State University in David Johnson's laboratory.

Kwan R. Lee received his Ph.D. in statistics from Southern Methodist University in 1981. He was an Associate Professor of Mathematics at East Tennessee State University from 1987 to 1989, and is now at the Tennessee Eastman Co., Kingsport, TN. He is experienced with general linear models, variance component estimation, and repeated measures with the problem of missing values, and has consulted extensively for the chemical industry.

Craig E. Smith received his M.S. in Biology from East Tennessee State University in 1985 and is a candidate for the Ph.D. in Biomedical Sciences. His experience includes enzymology and molecular biology. He is employed as a Senior Research Scientist with Promega Corp., Madison, WI.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Johnson DA, Frampton MW, Winters RS, Morrow PE, Utell MJ. 1990. Inhalation of nitrogen dioxide fails to reduce the activity of human lung alpha-1-proteinase inhibitor. Am Rev Respir Dis 142:758–762.

ABBREVIATIONS

α_1-PI	alpha-1-proteinase inhibitor
active α_1-PI	α_1-PI capable of inhibiting porcine elastase
active:immuno	ratio of the concentration of active α_1-PI to the concentration of α_1-PI measured immunologically
active:protein	ratio of the concentration of active α_1-PI to the concentration of total protein
ANOVA	analysis of variance
BLPI	bronchial leukocyte proteinase inhibitor
BSA	bovine serum albumin
CO_2	carbon dioxide
EPA	U.S. Environmental Protection Agency
EPA-HERL	EPA-Health Effects Research Laboratory
F344	Fisher-344
HEPES	N-[2-hydroxyethyl]piperazine-N'–[2-ethane-sulfonic acid]
immuno α_1-PI	the immunologically measured concentration of α_1-PI, assumed to be equivalent to the total α_1-PI concentration, including both active and inactive forms of the inhibitor protein
immuno:protein	ratio of the immunologically measured concentration of α_1-PI to the total protein concentration
ITRI	Inhalation Toxicology Research Institute
NAAQS	National Ambient Air Quality Standard
NaCl	sodium chloride
NO_2	nitrogen dioxide
O_3	ozone
OPA	orthophthalaldehyde
ppm	parts per million
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

A Request for Applications (RFA 84–3), which solicited proposals for "Mechanisms of Oxidant Toxicity," was issued by the Health Effects Institute (HEI) in the summer of 1984. In response to the RFA, D.A. Johnson from the Quillen-Dishner College of Medicine, East Tennessee State University, submitted a proposal entitled, "Oxidant Effects on Rat Lung Proteinase Inhibitors." The three-year, eight-month project began in August 1985, and total expenditures were $250,377. The Investigators' Report was received at the HEI in May 1989, and was accepted by the Health Review Committee in November 1989. During the review of the Investigators' Report, the 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 Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of the HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA)[1] sets standards for oxidants (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. 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)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of certain oxidants (and other pollutants) and provide the EPA with limited discretion to modify those requirements. The Clean Air Act is currently undergoing revision, which may or may not affect regulatory policies.

In addition, Section 109 of the Clean Air Act provides for the establishment of National Ambient Air Quality Standards (NAAQS) to protect the public health. The current standards include those for ozone and nitrogen dioxide. The determination of the appropriate standards for emissions of oxidants and their precursors depends, in part, on an assessment of the risks to health that they present. Information on critical cellular and molecular targets of oxidant injury can contribute to the understanding of both acute and chronic effects caused by oxidants and, therefore, to informed regulatory decision making.

[1] A list of abbreviations appears at the end of the Investigators' Report for your reference.

SCIENTIFIC BACKGROUND

Oxidants and their precursors are derived from mobile, stationary, and natural sources. Although nitrogen dioxide is produced from the combustion of fossil fuels and decomposition of vegetation, it is primarily derived from secondary atmospheric reactions. The NAAQS for nitrogen dioxide is 0.053 parts per million (ppm) averaged annually. Ozone is not directly emitted from motor vehicles but is formed as a result of complex photochemical reactions among oxides of nitrogen and volatile organic compounds. The NAAQS for ozone is 0.12 ppm averaged over one hour, not to be exceeded more than once a year. The summertime peak hourly ambient levels range from 0.07 ppm in rural sections of the country to as high as 0.35 ppm in some urban areas (U.S. Environmental Protection Agency 1986b). Ozone is one of the oxidants of primary concern because of the inability to maintain air quality within the NAAQS for an estimated 130 million persons who reside in nonattainment areas (U.S. Office of Technology Assessment 1988).

In acute animal experiments, the inhalation of high concentrations of oxidants results in pulmonary edema, inflammation, and hyperplasia of epithelial and interstitial cells (reviewed by U.S. Environmental Protection Agency 1982, 1986a, 1988; Morrow 1984). The concentration of oxidant needed to produce injury, the site of damage in the respiratory tract, and the target cells affected differ between ozone and nitrogen dioxide, due to differences in physicochemical and oxidizing properties. Recently, efforts have focused on determining the contribution of oxidants to the development of chronic lung diseases, such as emphysema, fibrosis, and chronic bronchitis. However, because the early stages of these diseases are not well understood, it has been difficult to relate pollutant exposure to such delayed chronic effects.

Emphysema is the major cause of severe airway obstructive lung disease (reviewed by Snider 1989). Cigarette smoking is considered the major cause of this disease; the role of environmental air pollutants is unclear. Emphysema is characterized by the permanent enlargement of the alveolar airspaces, destruction of alveolar walls, and absence of fibrosis (Snider et al. 1985). The biological basis for emphysema resides in alterations in the connective tissue matrix

of the lung parenchyma. The major components of lung connective tissue are collagen, elastin, glycosaminoglycans, and glycoproteins (Turino 1985). The pathogenesis of emphysema is not completely understood, but it is believed that the disease results from the breakdown of connective tissue proteins.

Several hypotheses have been proposed to describe the mechanisms responsible for tissue destruction (reviewed by Janoff 1985; Niewoehner 1988). Most theories focus on an imbalance between proteolytic enzymes and their inhibitors. This imbalance can result from an excess of proteolytic enzymes, from a decrease in the total or functional amount of inhibitors, or from both. There are multiple mechanisms by which this imbalance can occur. In addition, nonproteolytic damage to connective tissue components may contribute to tissue destruction. It is unlikely that only one mechanism contributes to the pathogenesis of emphysema, but rather, an interdependent network of events is probably involved.

Proteolytic enzymes differ in their structure and activity, depending on their cell source. In the lung, the elastase synthesized by neutrophils has been implicated as the primary enzyme responsible for the destruction of connective tissue proteins (in particular, elastin and types III and IV collagen) (Janoff et al. 1977; Senior et al. 1977). Stored in the lysosomal granules of neutrophils, elastase is released into tissues during phagocytosis. During inflammation, when large numbers of actively phagocytosing neutrophils are present, potentially large amounts of the enzyme can be discharged. Neutrophils also synthesize and secrete lesser amounts of an acid proteinase, proteinase 3, which is maximally active under acidic conditions (Kao et al. 1988). Although the role of alveolar macrophages is less clear and may be more complex, these cells are also sources of elastolytic enzymes (reviewed by Niewoehner 1988; Sibille and Reynolds 1990). In addition, both neutrophils and macrophages produce collagenases and other proteolytic enzymes that are capable of remodeling the connective tissue matrix (reviewed by Sibille and Reynolds 1990).

Elastolytic activity is regulated by various proteinase inhibitors. Within the alveolar region, alpha-1-proteinase inhibitor appears to be the most significant inhibitor of neutrophil elastase (Gadek et al. 1981). This glycoprotein circulates in the blood but, because of its small size, it freely diffuses between the blood and the alveolar air spaces. Alpha-1-proteinase inhibitor is also synthesized in small amounts by alveolar macrophages (White et al. 1981). In the bronchiolar region, epithelial cells produce and secrete a local inhibitor, bronchial leukocyte proteinase inhibitor, also known as secretory leukoproteinase inhibitor (Hochstrasser et al. 1981; De Water et al. 1986). In the centriacinar region of the lung, where emphysematous lesions occur, the concentrations of the two inhibitors are at their lowest, thus providing minimal protection. It is not known if the low levels of proteinase inhibitors contribute to tissue injury in this region of the lung.

By serving as a pseudo-substrate, proteinase inhibitors block the elastolytic activity of elastase (Travis and Salvesen 1983). Chemical interactions between the enzyme and, in the case of alpha-1-proteinase inhibitor, a specific methionine residue in the inhibitor molecule, result in a stable enzyme-inhibitor complex, thus rendering the enzyme unavailable for proteolytic attack. If the inhibitor molecule is chemically altered so that its binding affinity to elastase is decreased, localized destruction of pulmonary connective tissue can occur. One such way to decrease the binding affinity of alpha-1-proteinase inhibitor is to oxidize the active site methionine residue (Johnson and Travis 1979).

Several sources of oxidizing agents that can inactivate proteinase inhibitors have been identified. Cigarette smoke, which contains both oxidant gases and free oxygen radicals, inactivates alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor when tested in vitro (reviewed by Janoff 1985). Ozone (Johnson 1980) and nitrogen dioxide in the presence of hydrogen peroxide (Dooley and Pryor 1982) have been shown to oxidize alpha-1-proteinase inhibitor in vitro; both oxidants also inactivate bronchial leukocyte proteinase inhibitor in vitro (Johnson 1987). In addition to exogenous sources of oxidants, inflammatory cells, in particular neutrophils, can generate free oxygen radicals when stimulated (reviewed by Weiss 1989). The subsequent formation of chlorinated oxidants can inactivate various proteinase inhibitors.

Support for the proteinase-antiproteinase imbalance hypothesis for the etiology of emphysema comes from several lines of evidence (reviewed by Janoff 1985; Snider 1989). Early-onset familial emphysema occurs in individuals with an inheritable deficiency of alpha-1-proteinase inhibitor. These individuals have, on average, only 16 percent of the normal serum levels of the inhibitor. Elastase activity is present in the bronchoalveolar lavage fluid from affected persons, but is undetectable upon replacement therapy with alpha-1-proteinase inhibitor. In animal studies, intratracheal instillation of elastase produces lung lesions similar in appearance to human emphysema.

Not all aspects of emphysema, however, can be explained simply by an imbalance between elastase and alpha-1-proteinase inhibitor (reviewed by Hoidal and Niewoehner 1983; Janoff 1985; Snider 1989). For example, cigarette smoking is considered a major risk factor in the pathogenesis of the disease, but it has not been possible to consistently demon-

strate functionally inactive alpha-1-proteinase inhibitor in the lungs of smokers. An excess of elastolytic activity in the bronchoalveolar lavage fluid from smokers has been reported; however, the majority of the activity is associated with the macrophage-derived enzyme, which is not inhibited by alpha-1-proteinase inhibitor. Also, although in vitro experiments have shown that cigarette smoke inactivates alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor through chemical oxidation, data from in vivo studies have been unable, thus far, to confirm the physiologic significance of oxidative damage. Finally, individuals who are heterozygous for alpha-1-proteinase inhibitor deficiency and have half of the circulating inhibitor are not at greater risk of developing emphysema.

In addition to attack by proteinases, other mechanisms have been proposed as contributors to the genesis of emphysema. Recent findings from in vitro studies suggest that oxidants can alter connective tissue components directly either by degrading connective tissue molecules or by increasing the susceptibility of the molecules to degradation by proteolytic enzymes (reviewed by Riley and Kerr 1985). These in vitro observations need to be confirmed, and their biological significance assessed in vivo. Repair processes also influence the effect of injury to pulmonary connective tissue. If the resynthesis of damaged connective tissue components is impaired, the resulting lesion is either worsened (Kuhn and Starcher 1980) or its characteristics are changed (Niewoehner and Hoidal 1982).

Although oxidant injury to components of the connective tissue, either directly or indirectly, has been demonstrated in experimental systems, how these forms of injury relate to human disease is not known. In animals, nitrogen dioxide exposure causes airspace enlargement with some alveolar septal fibrosis. Rats exposed to 30 ppm of nitrogen dioxide continuously for three to six weeks developed mild emphysema (Freeman et al. 1968). Although this level of nitrogen dioxide is orders of magnitude greater than ambient levels, this model has been adopted as a way of studying the morphologic and connective tissue biochemical changes during the early stages of the emphysematous lesions. During initial inflammation, large numbers of elastase-rich neutrophils are recruited from the blood to the alveolar airspace (Glasgow et al. 1987); increased numbers of alveolar macrophages have also been reported (Kleinerman et al. 1982; Glasgow et al. 1987). It is not known if the emphysema results from direct oxidative damage to proteinase inhibitors or from the presence of inflammatory cells, or both.

Data from early animal studies implied that exposure to ozone induced emphysematic lesions (Stokinger et al. 1957). However, the ozone used in these experiments was generated from air, which also produces oxides of nitrogen, a confounding pollutant. Studies that use ozone generated from pure oxygen have not shown emphysematous abnormalities (reviewed by Last 1988). There is little information from chronic, low-level studies and more is just now becoming available (Gross et al. 1989).

In addition to ozone and nitrogen dioxide, diesel engine exhaust is another pollutant derived from mobile sources that is of concern. Although most of the attention has been focused on carcinogenic effects, emphysematous lesions have been reported after chronic exposure of animals to high concentrations (Karagianes et al. 1981; Heinrich et al. 1986). Diesel engine exhaust is composed of numerous gases, including nitrogen dioxide, and particulate matter. The particulate matter elicits an inflammatory cell response. Diesel engine exhaust, as a causative agent of emphysema, has not been thoroughly investigated. The effect of the exhaust on proteinase inhibitors has also not been evaluated.

In summary, although the etiology of emphysema is not completely understood, an imbalance between proteolytic enzymes and their inhibitors probably contributes, in part, to the pathogenesis of the disease. However, for different individuals and for different insulting agents, the mechanisms that create such an imbalance may be different. Elastase, as well as other elastolytic and proteolytic enzymes, may be present in excess due to influxes of neutrophils and macrophages. Reduced amounts or oxidized forms of inhibitors could decrease lung defenses against elastolytic enzymes. Oxidizing agents may be inhaled or may be derived from activated neutrophils. Thus, neutrophils are not only a source of elastase, but they affect the proteolytic burden by generating oxidizing agents, which in turn decrease inhibitor functional activity.

With respect to oxidants derived from automotive emissions and their transformation products, ozone and nitrogen dioxide inactivate alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor, the two major proteinase inhibitors of the respiratory tract, when the inhibitors are exposed in vitro. Recent studies suggest that alpha-1-proteinase inhibitor may be susceptible to inactivation when exposed in vivo to ozone or to high concentrations of nitrogen dioxide. Pickrell and coworkers (1987) reported a decrease in elastase inhibitory activity in the serum and lung homogenates from rats exposed for 48 hours to 0.5 or 1.0 ppm ozone. Mohsenin and Gee (1987) observed a decrease in alpha-1-proteinase inhibitor functional activity in the bronchoalveolar lavage fluid from humans exposed for three hours to 3 or 4 ppm nitrogen dioxide with intermittent exercise. These studies need confirmation before the role of in vivo oxidant inactivation can be assessed.

JUSTIFICATION FOR THE STUDY

The HEI sought proposals (RFA 84–3) that would improve our understanding of the relationship between oxidant injury and lung disease. One area of particular interest to the Institute was the measurement of alterations in molecules or cells that may be affected by oxidant exposure. D.A. Johnson proposed to study the effects of chronic inhalation of automotive pollutants on the proteolytic enzyme inhibitors of rat lungs. Because most oxidant-toxicology studies had been conducted in rats, and the available assays were for human proteinase inhibitors, Johnson first proposed to develop assays for rat inhibitor molecules. He then proposed to assay serum or bronchoalveolar lavage samples from rats chronically exposed to either ozone, nitrogen dioxide, or diesel engine emissions for elastase inhibitory activity. Inhalation studies were to be conducted by J. Graham and D. Costa from the EPA Health Effects Research Laboratory at Research Triangle Park, NC, and J.L. Mauderly from the Inhalation Toxicology Research Institute, Albuquerque, NM.[2]

In addition to being responsive to the HEI's RFA, a unique feature of Johnson's proposal was the opportunity to maximize information from ongoing studies. Chronic inhalation studies represent a major investment, and Johnson would be able to obtain relevant samples from these ongoing studies without incurring additional exposure and animal costs. Furthermore, Johnson's biochemical expertise in the area of antiproteinases was a valuable addition to these toxicology studies.

During the course of the study, Johnson and his colleagues realized that a two- to five-day recovery period had occurred between the last day of exposure and the time of lavage. They were concerned that during this period, inactivated inhibitor in the lung could have been replaced by active inhibitor from the blood. They requested, and received from the HEI Research Committee, a three-month extension of the study to measure inhibitor activity in lung lavage fluid from rats acutely exposed to ozone; these experiments were done in collaboration with Costa from the EPA Health Effects Research Laboratory, Research Triangle Park, NC.[3]

Johnson and his colleagues also had the opportunity to analyze bronchoalveolar and nasal lavage material from humans acutely exposed to oxidants. The exposures of human subjects to nitrogen dioxide and the sampling of the bronchoalveolar and nasal lavage fluid were done by M. Utell, M. Frampton, and P. Morrow from the University of Rochester, NY. H. Koren and D. Graham from the EPA Clinical Research Branch, Chapel Hill, NC, performed the human ozone exposures.[4] In the subjects exposed to ozone, the investigators proposed to assay for mast cell tryptase, a trypsin-like serine proteinase, in addition to assaying for alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor. The biological significance of tryptase is unknown, but it is released during mast cell degranulation and thus, serves as a marker for mast cell activation (Schwartz et al. 1987). Although assaying for this enzyme was not directly related to the investigators' hypothesis, information about this marker could provide insight into other physiological and biochemical responses to ozone exposure. The HEI Research Committee approved these preliminary studies on human samples on the basis that they represented a complementary component to Johnson's overall project.

OBJECTIVES AND STUDY DESIGN

The primary objective of this project was to test the hypothesis that oxidant exposure decreases elastase inhibitory activity in the respiratory tract. The specific aims of the study were:

1. To purify rat alpha-1-proteinase inhibitor from rat plasma and to develop an immunologic assay;
2. To determine the susceptibility of rat alpha-1-proteinase inhibitor to ozone inactivation;
3. To determine if rats contain an inhibitor analogous to human bronchial leukocyte proteinase inhibitor;
4. To determine the effect of chronic exposure of rats to ozone, nitrogen dioxide, or diesel engine exhaust on alpha-1-proteinase inhibitor activity in lung lavage fluids;
5. To determine the effect of acute exposure of rats to ozone on alpha-1-proteinase inhibitor in lung lavage fluids;
6. To determine the effect of acute exposure of humans to ozone or nitrogen dioxide on alpha-1-proteinase inhibitor in lung lavage fluids;
7. To determine the effect of acute exposure of humans to ozone on bronchial leukocyte proteinase inhibitor in nasal lavage fluids; and
8. To determine the effect of acute exposure of humans to ozone on mast cell tryptase in nasal and lung lavage fluids.

Standard chromatography procedures were used to purify rat alpha-1-proteinase inhibitor. Polyclonal antibodies

[2] Additional data on pulmonary function, histopathology, and biochemical analyses from these chronic inhalation studies are available (Gross et al. 1989; Mauderly et al. 1989).

[3] Details of the exposure protocols as well as additional data are available in Costa and colleagues (1989).

[4] Details of the exposure protocols as well as additional data are available in Utell and associates (1991).

were raised in rabbits against the purified inhibitor (Specific Aim 1). Rat plasma was exposed to ozone in vitro, and the susceptibility of the rat inhibitor to oxidative inactivation was tested (Specific Aim 2).

Rat lung lavage fluid and parotid gland extracts were assayed for an inhibitor analogous to human bronchial leukocyte proteinase inhibitor (Specific Aim 3). Antibodies to human bronchial leukocyte proteinase inhibitor, cDNA for human bronchial leukocyte proteinase inhibitor, and the inhibition of human neutrophil elastase were used to test for the presence of the inhibitor.

For the chronic exposure studies (Specific Aim 4), Fischer-344 rats were exposed to 10 ppm nitrogen dioxide or 3.5 mg diesel soot/m^3 for seven hours per day, five days per week for 12, 18, or 24 months. These exposures were conducted at the Inhalation Toxicology Research Institute. Six weeks before the chronic exposures began, one half of these animals received intratracheal instillations of elastase to induce pulmonary emphysema. In a separate experiment carried out by the EPA Health Effects Research Laboratory, Fischer-344 rats were exposed to 0.06 ppm ozone with 0.25 ppm spikes or to 0.5 ppm nitrogen dioxide with 1.5 ppm spikes for 20 hours per day for 12 or 18 months.

For the acute exposure studies (Specific Aim 5), Fischer-344 rats were exposed to 0.8 ppm or 1.2 ppm ozone for two, four, or eight hours. In a separate experiment, rats were exposed to 0.5 ppm or 0.8 ppm ozone for two or seven hours; in this experiment, 8 percent carbon dioxide was given intermittently to increase ventilation rate and simulate exercise. Both experiments were carried out at the EPA Health Effects Research Laboratory.

In all the rat studies, lung lavage fluid was collected and assayed. In the chronic studies, the fluid was sampled two to five days after the exposure was terminated; in the acute studies, lung lavage was collected immediately after the exposure. In both types of studies, lavage fluids were assayed for total protein, alpha-1-proteinase inhibitor activity, and immunological (or total) amounts of alpha-1-proteinase inhibitor. Alpha-1-proteinase inhibitor activity was determined by measuring the inhibition of porcine pancreatic elastase in vitro. The inhibitor was quantified by an enzyme-linked immunoassay; this technique measures the total amount of the inhibitor present in both its active and inactive forms. Johnson and his colleagues called the value obtained from the immunoassay the immunological concentration of the inhibitor. They defined the functional activity of the inhibitor as the ratio of the active:immunological concentrations.

In the human studies, volunteers were exposed to 0.4 ppm ozone for two hours, with intermittent exercise, at the EPA Health Effects Research Laboratory (Specific Aims 6, 7, and 8). Bronchoalveolar lavage fluid was collected 18 hours after the exposure ended and nasal lavage fluid was sampled before, immediately after, and 18 hours after exposure. In a separate study carried out at the University of Rochester, humans were exposed to 0.05 ppm nitrogen dioxide with 2.0 ppm peaks or 1.5 ppm nitrogen dioxide continuously for three hours (Specific Aim 6). For both nitrogen dioxide protocols, exercise was intermittent during the exposure, and bronchoalveolar lavage fluid was collected 3.5 hours after exposure was terminated.

In humans exposed to ozone, the bronchoalveolar lavage fluid was assayed for total protein, alpha-1-proteinase inhibitor activity, immunological concentration of alpha-1-proteinase inhibitor, and mast cell tryptase. Nasal washings were assayed for total protein, immunological levels of bronchial leukocyte proteinase inhibitor, and mast cell tryptase. In those individuals exposed to nitrogen dioxide, the bronchoalveolar lavage fluid was assayed for total protein, alpha-1-proteinase inhibitor activity, and immunological concentrations of alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The objectives of the original proposal were attained. The additional observations from the collaborative studies in humans added to the importance and significance of this research project.

ASSESSMENT OF METHODS AND STUDY DESIGN

This project, which was well designed and competently executed, succeeded in developing a new immunologic assay for alpha-1-proteinase inhibitor in rats. A positive feature of the study was that the investigators took advantage of ongoing exposure studies in other laboratories. Although collaborations of this nature can be efficient mechanisms for utilizing various approaches and expertise to pursue a research problem, extra attention must be paid to the coordination of exposure protocols and the needs of any subsequent assays. For example, in the chronic studies, the recovery period of two to five days presumably allowed the replacement of inactivated lung alpha-1-proteinase inhibitor by serum inhibitor.

The use of bronchoalveolar lavage to sample the contents of lung surfaces is a common approach. However, inactivation of alpha-1-proteinase inhibitor may be highly localized. The washing out of lobes or whole lungs has the potential to dilute the concentration of any locally oxidized

molecule, thus making the detection of inactivated inhibitor difficult.

STATISTICAL METHODS

The method used to assay for alpha-1-proteinase inhibitor was altered during the course of the studies to make use of the best available techniques. Thus, normalization of the data to the values from the air controls for each exposure period was necessary. The changes in assay techniques and the impact of laboratory drift could have been determined if standard pools of lavage fluid had been analyzed over the course of the study.

The need to normalize is further justified by the threefold to fourfold difference observed in the base levels of active alpha-1-proteinase inhibitor between the animals from the Inhalation Toxicology Research Institute and those from the EPA Health Effects Research Laboratory. The reason the rats from the EPA laboratory had inhibitor activity levels ranging from 4.4 to 5.4 μg/ml and immunologic levels ranging from 9.3 to 11.2 μg/ml, while the animals from the Institute had activity levels of 1.1 to 2.0 μg/ml and immunologic levels of 1.5 to 3.1 μg/ml, is unexplained.

Normalization of the response in the treated animals by expressing the response as a percentage of the control response is one way to standardize, but it may not always be the most desirable. This approach is reasonable when the effect of the treatment is to multiply the level seen in the control. The same effect can be achieved by working with the differences between the logarithms of concentrations. However, the effect of treatment might be to add a constant increase to the response of the controls. The choice can be of some consequence in that the size, or even the presence or absence, of an interaction often depends on the measurement scale. In addition, the sensitivity of the analyses can be enhanced by making the appropriate choice.

In the acute exposure studies, equivalent numbers of control animals were exposed to air at the same time as each ozone exposure group. However, a total of eight animals from all the air-exposed groups were randomly selected and used as a single control group. Although reducing the number of lavage fluids from control animals decreased the number of analyses required and did not seem to affect the mean values in control animals, a large amount of potential data in the control animals was not obtained. Furthermore, it is not clear from the Investigators' Report if the control animals were assayed simultaneously with, or at different times than, the oxidant-exposed animals.

In the human studies, the paired *t* test that was used was appropriate because it maximized the use of the available data. The need for an additional nonparametric test, however, was not demonstrated. If a nonparametric test were needed, the signed rank test, because it is the nonparametric analogue to the paired *t* test, rather than the paired sign test, would have been more appropriate.

RESULTS AND INTERPRETATION

Rat alpha-1-proteinase inhibitor was successfully purified and was shown to be sensitive to oxidative inactivation by ozone in vitro. Knowing that the rat inhibitor was sensitive to oxidative damage and having the antigen, it was then reasonable to analyze for rat proteinase inhibitor activity in animals exposed in vivo. It was, however, not possible to identify an inhibitor in the rat that corresponds to the human bronchial leukocyte proteinase inhibitor.

In the rat studies, only chronic exposure to diesel soot caused a statistically significant reduction, by 30 percent, in the functional activity of alpha-1-proteinase inhibitor. Although this reduction was observed also in the emphysematous animals exposed to diesel soot, preexisting emphysema had no independent effect. With all other exposures, both chronic and acute, no consistent effects on either functional activity or immunologic levels of the inhibitor were detected. In the acute exposure studies, the ratio of active alpha-1-proteinase inhibitor to total protein was increased in the ozone-exposed animals. The reason for this interesting finding is unknown. It could be that the total protein measurements underestimated the amount of protein, especially the larger molecular weight proteins, present after ozone exposure. It might have been preferable to determine alpha-1-proteinase inhibitor concentrations relative to albumin, since the molecular weights of the two proteins are similar. Although the investigators argue against secretion of the inhibitor by macrophages, it was not possible in this study to differentiate if the molecule was being actively secreted or was preferentially entering the lung relative to other serum proteins.

In humans exposed to ozone, samples of nasal washings showed reduced bronchial leukocyte proteinase inhibitor activity and increased mast cell tryptase. Samples from bronchoalveolar lavage fluid revealed increased tryptase, but no changes in alpha-1-proteinase inhibitor. No effects on the activity and concentrations of alpha-1-proteinase inhibitor or on the concentrations of bronchial leukocyte proteinase inhibitor were detected after exposure of subjects to nitrogen dioxide. Although interesting, the results from these human studies are preliminary. Because of small sample size, additional studies are needed to confirm these findings.

In the chronic studies, a two- to five-day recovery period after pollutant exposure occurred before alpha-1-proteinase

inhibitor activity was analyzed. If the effects of exposure temporarily decreased alpha-1-proteinase inhibitor activity, then the delay potentially provided sufficient time for the replacement of functional inhibitor. Although this design did not permit the identification of a short-term effect of these pollutants on the proteinase inhibitor, a prolonged effect was apparent after exposure to diesel engine exhaust. Furthermore, in subsequent acute studies, both in rats and in humans, exposure to ozone did not inactivate alpha-1-proteinase inhibitor in lung lavage fluid obtained immediately after exposure.

The reason for the difference in effect on the inhibition of alpha-1-proteinase inhibitor between exposure to the oxidants and diesel engine exhaust is not known. The presence of large numbers of neutrophils in the diesel-exposed animals could have added to the elastase burden and contributed endogenous sources of oxidants. However, after 12 months of exposure, even the air control animals had increased numbers of inflammatory cells; the role of excess neutrophils is, therefore, difficult to interpret.

A possible confounder of the interpretation of the effects of oxidants on immunological activity is that the oxidant could have altered the antigenic site of alpha-1-proteinase inhibitor. If this were to have occurred, then the measurements of immunologic levels of alpha-1-proteinase inhibitor would have underestimated the total amounts of the inhibitor after oxidant exposure. Furthermore, the use of the active:immunological ratio as an indicator of inhibitor functional activity assumes that the denominator is unaffected by oxidants. Information on this possibility appears to be unavailable.

REMAINING UNCERTAINTIES AND IMPLICATIONS FOR FUTURE RESEARCH

The findings on the effects of diesel engine emissions on alpha-1-proteinase inhibitor have important implications for potential effects of chronic inhalation of diesel engine exhaust and should be clarified. Additional research on the mechanisms, as well as the components, of the exhaust that caused the inactivation should be pursued. The presence of inflammatory cells could alter significantly the proteinase-antiproteinase balance in a localized fashion. Also, it is not known if short-term exposure to diesel engine exhaust in rats has an effect on inhibitor activity. Finally, the implications for the development of chronic lung disease should be explored.

Preliminary findings in the human studies suggest several areas for further research. First, whether or not ozone inactivates bronchial leukocyte proteinase inhibitor in bronchial washings obtained from the main bronchus, where bronchial leukocyte proteinase inhibitor, not alpha-1-proteinase inhibitor, is the primary inhibitor, remains to be determined.

Second, the finding of increased levels of mast cell tryptase in nasal and bronchoalveolar lavage fluids after ozone exposure has significant implications for the effects of ozone on bronchial reactivity and asthma (Friedman and Kaliner 1987). The possibility that tryptase in upper airway secretions could serve as a marker of exposure or of sensitive individuals deserves further investigation.

Third, although exposure to ozone or nitrogen dioxide did not have any effect on alpha-1-proteinase inhibitor in this study, others have demonstrated an effect (Mohsenin and Gee 1987; Pickrell et al. 1987). Thus, it cannot be ruled out that ozone or nitrogen dioxide does inactivate the inhibitor in vivo. Differences between this study and the other two studies, which were discussed by the investigators in their report, could be clarified.

An assumption underlying this investigation is that the mechanism by which oxidants enhance the destructive action of proteolytic enzymes on lung tissue is through the inactivation of antiproteinases. An alternate hypothesis, which could account for enhanced proteolysis of lung tissue, is that oxidants induce alterations of connective tissue macromolecules or other crucial matrix components. Altered substrates may then become more susceptible to the action of proteinases. Research is needed on the role of oxidants in substrate-proteinase interactions.

CONCLUSIONS

The results from this project were essentially not supportive of the hypothesis that in vivo exposure to nitrogen dioxide or ozone inactivates alpha-1-proteinase inhibitor in bronchoalveolar lavage fluid. In addition, it appears that preexisting emphysema does not alter the susceptibility of chronically exposed animals to inhibitor inactivation. It cannot be ruled out, however, that alpha-1-proteinase inhibitor was inactivated in localized areas of the lung, but because of dilution by bronchoalveolar lavage, it was not detected. Furthermore, sampling of the fluid from alveolar surfaces may not have reflected accurately the proportions of proteinases and antiproteinases in the interstitium, where damage occurs.

Three findings from this project are of interest. First, in rats exposed chronically to high levels of diesel soot, a prolonged depression of alpha-1-proteinase inhibitor functional activity was seen, suggesting the importance of mixed exposures. The continued presence of inflammatory

cells several days after the exposures were terminated may have contributed to this finding. Second, in a preliminary study of humans acutely exposed to low levels of ozone, bronchial leukocyte proteinase inhibitor in nasal washings was reduced immediately after exposure. Third, in humans acutely exposed to ozone, mast cell tryptase was elevated in nasal and bronchoalveolar lavage fluids. The significance of these findings to the development of irreversible or reversible chronic obstructive pulmonary disease remains to be determined.

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LIST OF HEI PUBLICATIONS

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Title	Publication Date
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research	September 1985
Automotive Methanol Vapors and Human Health: An Evaluation of Existing Scientific Information and Issues for Future Research	May 1987
Gasoline Vapor Exposure and Human Cancer: Evaluation of Existing Scientific Information and Recommendations for Future Research (Supplement)	January 1988

Research Reports

Report No.	Title	Principal Investigator	Publication Date
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