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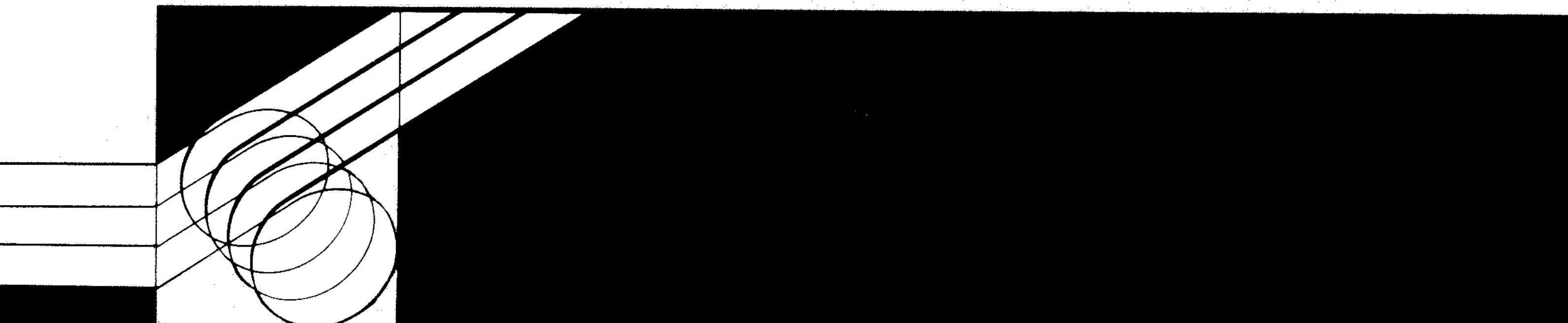

Effects of Ozone and Nitrogen Dioxide On Human Lung Proteinase Inhibitors

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Includes the Report of the Institute's Health Review Committee

The Health Effects Institute (HEI) is a non-profit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions.

Funded equally by the U.S. Environmental Protection Agency (EPA) and 26 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process, to assure both credibility and high scientific standards.

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ABBREVIATIONS

α_1-Achy	alpha-1-antichymotrypsin
α_1-PI	alpha-1-proteinase inhibitor, alpha-1-antitrypsin
BLPI	bronchial leukocyte proteinase inhibitor
Cat G	neutrophil cathepsin G, a chymotrypsin-like enzyme
DMSO	dimethyl sulfoxide
HEPES	[4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]
His	histidine
HNE	human neutrophil elastase
MeO-Suc	methoxysuccinyl
Met	methionine
NCS	N-chlorosuccinimide
NPGB	p-nitrophenylguanidine benzoate
pNA	p-nitroanilide
SOD	superoxide dismutase
Suc	Succinyl
Trp	tryptophan
Tyr	tyrosine

PREFACE

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

The Health Effects Institute (HEI) is an independent non-profit corporation which, according to its charter, is "organized and operated...specifically to conduct or support the conduct of, and to evaluate, research and testing relating to, the health effects of emissions from motor vehicles".

It is organized in the following ways to pursue this purpose:

INDEPENDENCE IN GOVERNANCE

HEI is governed by a four-member Board of Directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University; Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University; Donald Kennedy, President of Stanford University; and Charles Powers, President, Clean Sites, Incorporated. Professor Cox chairs the Board. These individuals, who select their own successors, were chosen initially, after consultation with industry and other individuals, by then Environmental Protection Agency Administrator Douglas M. Costle.

TWO-SECTOR FINANCIAL SUPPORT

The Institute receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-six leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget, in shares proportionate to the number of vehicles or engines that they sell.

RESEARCH PLANNING AND PROJECT EVALUATION

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is devised by the Health Research Committee, a multi-disciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate HEI committee, the Health Review Committee. Members are expert scientists representing a broad range of experience in environmental health sciences. The Health Review Committee has no role either in the review of applications or in the selection of projects and investigators for funding. This Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each funded proposal is assigned in advance of completion to a member of the Health Review Committee, who acts as "primary reviewer". When the draft report is received, the primary reviewer directs a peer review by technical experts and, when appropriate, by a biostatistician. After the investigator has had a chance to comment on the technical evaluations, the primary reviewer drafts a review. This document is sent to the investigator for comment. It is subsequently examined by the full Health Review Committee and revised as necessary. The investigator's final report, as well as the Health Review Committee's report, are then made available to the sponsors and to the public after evaluation by the HEI Board of Directors.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing and nature of HEI report releases are tailored to ensure that the Health Review Committee's report does not interfere with the journal publication process. The report of the Health Review Committee will be as thorough as necessary to evaluate any individual report.

INTRODUCTION

A Request for Applications (RFA 82-2), which solicited proposals on the "Cellular and Biochemical Markers Related to Non-Neoplastic Chronic Lung Disease", was issued by the HEI in the summer of 1982. Dr. David A. Johnson of the Quillen-Dishner College of Medicine, East Tennessee State University, submitted a proposal entitled, "Effects of O_3, NO_2, and SO_2 on Human Lung Proteinase Inhibitors." The HEI approved the three-year project and authorized a total expenditure of $177,184.00. The project began in May, 1983, and the final report was accepted by the Health Review Committee in January, 1987. The Health Review Committee's report, which follows the investigator's report, is intended to place the investigator's final report in perspective as an aid to the sponsors of the HEI and to the public.

THE CLEAN AIR ACT

The Environmental Protection Agency (EPA) sets standards for motor vehicle emissions of oxides of nitrogen (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 judgement cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3) and 202(b)(1) impose specific requirements for reductions in motor vehicle emissions of oxides of nitrogen (and other pollutants), and provide EPA with limited discretion to modify those requirements.

The determination of the appropriate standards for emissions of oxides of nitrogen depends, in part, on an assessment of the risks to health they present. Research on the effects of nitrogen dioxide and ozone on chronic lung disease can contribute to such risk assessment and, therefore, to informed regulatory decision-making.

In addition, Section 109 of the Clean Air Act provides for the establishment of national ambient air quality standards to protect the public health. The current standards include those for ozone and nitrogen dioxide. Research on the effects of those substances on chronic lung disease can contribute to the assessment of the appropriateness of the standards.

BACKGROUND

A major concern for a society that is heavily dependent on automobiles is whether air pollutants derived from automotive sources cause or exacerbate chronic lung disease. Obstacles encountered when trying to determine the health risks associated with air pollution exposure include the insidious onset of chronic diseases and the absence of indicators of early lesions. Thus, understanding the pathogenesis of specific lung diseases would aid in risk assessment.

Emphysema is among the most prevalent chronic lung diseases in the United States. Cigarette smoking is considered the major environmental cause of this disease; the role of other environmental air pollutants is unclear. Emphysema is characterized by the destruction of lung tissue, in particular, the alveolar walls and terminal bronchioles. The pathogenesis of emphysema is not completely understood, but it is believed that the disease results from the breakdown of connective tissue proteins by proteolytic enzymes. In the normal lung, the activity of such proteases is regulated by inhibitor molecules called antiproteinase or proteinase inhibitors. Emphysema is thought to arise when the balance between proteinase and antiproteinase activity is lost; the theory that describes this imbalance has been termed the proteinase-antiproteinase hypothesis (reviewed by Janoff, 1985).

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 macromolecules (in particular, elastin, and types III and IV collagen). 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. 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. This glycoprotein circulates in the blood, but due to its small size, freely diffuses between the blood and the alveolar air spaces. In the bronchiolar region, epithelial cells produce and secrete a local inhibitor, bronchial leukocyte proteinase inhibitor. The relative protective roles of alpha-1-proteinase and bronchial leukocyte proteinase inhibitors have not been determined.

By serving as a psuedo-substrate, proteinase inhibitors block the elastolytic activity of elastase. 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 as to decrease its binding affinity to elastase, localized destruction of pulmonary connective tissue can occur. One such way to inactivate alpha-1-proteinase inhibitor is by oxidation of the active site methionine residue (Johnson and Travis, 1979).

Support for the proteinase-antiproteinase hypothesis comes from several lines of evidence. Early onset, familial emphysema occurs in individuals with inheritable deficiency of alpha-1-proteinase inhibitor. In the bronchoalveolar lavage fluid from affected persons, elastase is present but is undetectable upon replacement therapy of alpha-1-proteinase inhibitor administered intravenously. In addition, emphysema can be induced experimentally in animals with pulmonary instillation of elastase. Details of the hypothesis, however, require clarification and confirmation before the theory can be used to determine the influence of environmental agents on the genesis of emphysema. For example, cigarette smoking is considered a major risk factor in the pathogenesis of the disease. 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 physiological significance of oxidative damage.

Because exposure to oxidant air pollutants, such as ozone and nitrogen dioxide, causes inflammation in the lung, it has been suggested that they may play a role in the pathogenesis of emphysema. Experimental studies in animals exposed to nitrogen dioxide reveal morphological, physiological, and biochemical changes indicative of small airway destruction (reviewed by Wright, 1987). Many of the nitrogen dioxide

studies used high concentrations of the oxidant, but several long-term experiments did use levels less than 1 ppm. Data from early animal studies implied that exposure to ozone induces emphysematic lesions. However, the ozone used in these experiments was generated by irradiating air, which is known to also produce oxides of nitrogen, a confounding pollutant. Studies that employ ozone generated by irradiation of pure oxygen have not shown emphysematous abnormalities, although data from chronic, low- dose ozone studies are not available.

One approach in the assessment of the potential for atmospheric oxidants to induce emphysema would be the evaluation of proteinase inhibitor activity. Experimental studies, although limited, suggest that oxidant air pollutants affect proteinase inhibitor function. *In vitro* studies have demonstrated that both ozone (Johnson, 1980) and nitrogen dioxide in the presence of hydrogen peroxide (Dooley and Pryor, 1982) inactivate alpha-1-proteinase inhibitor; similar studies have not been performed using bronchial leukocyte proteinase inhibitor. The effects of oxidant exposure *in vivo*, however, have not been evaluated.

REFERENCES

Dooley M, Pryor W, 1982. Free radical pathology: inactivation of human alpha-1-proteinase inhibitor by products from the reaction of NO_2 with H_2O_2 and the etiology of emphysema. Biochem Biophys Res Comm; 106:981-7.

Janoff A, 1985. Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. Am Rev Respir Dis; 132:417-33.

Johnson DA, 1980. Ozone inactivation of human alpha-1-proteinase inhibitor. Am Rev Respir Dis; 121:1031-8.

Johnson DA, Travis J, 1979. The oxidative inactivation of human alpha-1-proteinase inhibitor: further evidence for methionine at the reactive center. J Biol Chem; 254: 4022-6.

Wright JL, 1987. Investigation of pulmonary emphysema and small airways disease: possible relation to vehicle emissions or their components. In: Watson AY, Bates RR, Kennedy D (eds). Air Pollution, The Automobile, and Public Health: Research Opportunities for Quantifying Risk. Washington, D.C.: National Academy of Sciences Press (in press).

INVESTIGATOR'S REPORT

by David A. Johnson, Ph.D.

Effects of Ozone and Nitrogen Dioxide on Human Lung Proteinase Inhibitors

ABSTRACT

Based on available knowledge, this study shows that alpha-1-proteinase inhibitor (α_1-PI) plays an important role in protecting lung elastin from elastolytic proteinases, particularly human neutrophil elastase (HNE). Studies previous to this one showed that α_1-PI was very susceptible to inactivation by oxidants. We sought to use this oxidant sensitivity as an *in vivo* marker for ozone (O_3) and nitrogen dioxide (NO_2) exposure. The mechanism of α_1-PI inactivation by O_3 and NO_2 was examined to provide insight concerning the pathogenesis of oxidant-mediated lung damage. Attention also was focused on the bronchial leukocyte proteinase inhibitor (BLPI), which inhibits HNE in the bronchial secretions.

Careful examination of blood plasma samples from individuals exposed to 0.5 ppm O_3 for four hours on two consecutive days failed to detect any inactivation of α_1-PI. This result showed that blood α_1-PI was not a satisfactory marker for O_3 exposure, but, more importantly, demonstrated that inhaling O_3 for short periods does not grossly inactivate this important protein.

Studies on BLPI showed that it is a significant inhibitor of HNE and probably plays a more important role in protecting the lung than previously thought. BLPI, like α_1-PI, was found to be inactivated by oxidants, including O_3 and NO_2.

The mechanism of O_3 inactivation of leukocyte proteinase inhibitors was studied using α_1-PI, α-1-antichymotrypsin (α_1-Achy), BLPI, and Eglin C. While all these inhibitors differed in structure, the concentrations of O_3 required for inactivation were essentially the same, except for α_1-Achy, which only lost half of its inhibitory activity. It would seem from these results that O_3 can damage proteins via the oxidation of any of the following: tryptophan (Trp), methionine (Met), tyrosine (Tyr), or histidine (His) residues. Interestingly, Eglin C, which does not have oxidizable amino acids in its inhibitory active site, was inactivated by the same amount of O_3 as BLPI. BLPI was easily inactivated by a methionine-specific oxidant, suggesting an important role for methionine in this inhibitor.

In vitro exposure of α_1-PI and BLPI to 800 moles of NO_2 per mole of inhibitor resulted in 35% and 50% losses of HNE inhibitory activity, respectively. Tryptophan was destroyed by NO_2 and studies are in progress to examine effects on other amino acids.

INTRODUCTION

There is a clear correlation between the pathogenesis of emphysema and the proteolysis of the lung's alveoli, whose structural protein is elastin. This view results from nature's own experiment, in which individuals with genetically deficient blood levels of α-1-proteinase inhibitor (α_1-PI; also known as α-1-antitrypsin) are predisposed to the development of emphysema (Laurell and Eriksson, 1963). This principal blood plasma inhibitor of elastases (proteolytic enzymes that digest elastin) can enter the air space because of its size (52,000 MW).

Other studies showed that the instillation of elastase into the lungs of animals produces emphysema-like lesions (Janoff et al., 1977; Sloan et al., 1981). Human neutrophil elastase (HNE, also known as polymorphonuclear leukocyte elastase), the major elastase with access to the lung, generally is thought to cause the elastin degradation in emphysema.

Our *in vitro* studies (Johnson and Travis, 1979) showed that oxidation of two methionine residues in α_1-PI to methionine sulfoxide abolishes its porcine pancreatic elastase (PPE) inhibitory activity, but not its trypsin or chymotrypsin inhibitory activity. The oxidation alters the association rate of α_1-PI with each protease to the point that no complex is formed with porcine pancreatic elastase (PPE) and the association rate with human neutrophil elastase (HNE) is reduced 2,000-fold (Beatty et al., 1980).

Cigarette smoke also was shown to oxidatively inactivate α_1-PI (Carp and Janoff, 1978). Beatty et al. (1982) devised a method for measuring α_1-PI inactivation in the serum of smokers. Since oxidized α_1-PI has a normal trypsin inhibitory capacity (TIC) but lacks elastase (PPE) inhibitory capacity (EIC), the study found that the TIC/EIC ratio is a measure of α_1-PI oxidation *in vivo* and that 23% of the circulating α_1-PI of smokers was oxidized or unable to inhibit PPE. This technique is simpler and easier than examining lung lavage fluids (Gadek et al., 1979).

The possible involvement of airborne pollutants in the pathological events leading to emphysema was recognized when it was found that oxidants would inactivate α_1-PI by converting a critical methionine residue in the inhibitory site to methionine sulfoxide (Johnson and Travis, 1979). Subsequent *in vitro* studies showed that O_3 (Johnson, 1980) and nitrogen dioxide with hydrogen peroxide (Dooley and Pryor, 1982) also inactivate α_1-PI.

The major elastase inhibitor protecting mucous membranes of the upper airways from HNE is a locally-produced protein known as the bronchial leukocyte proteinase inhibitor (BLPI) (Hochstrasser et al., 1971; Ohlsson et al., 1977). BLPI, unlike α_1-PI, is a low molecular weight (11 kDa), acid stable protein that also inhibits cathepsin G (Cat G), the chymotrypsin-like protease of leukocyte. The study of pollutant effects on this protein is important because it inhibits the two major neutral

serine proteases of the PMN leukocytes and is positioned in the upper airways, which would result in its exposure to inhaled agents before alveolar α_1-PI. Thus, BLPI appears to function in the control of extravascular neutrophil proteases, since it also is found in human seminal plasma, cervical mucus, tears, and nasal secretions (Schiessler et al., 1976; Casslen et al., 1981). Gauthier et al. (1982) suggested that BLPI, due to its small size, may be a better inhibitor of elastin-bound elastase than α_1-PI, and probably plays a major role in protecting the lung. These findings suggest that α_1-PI protects the alveoli from HNE, while BLPI inhibits HNE and Cat G in the bronchioles. There is only one published report dealing with the partial inactivation (approximately 50%) of BLPI by cigarette smoke (Garp and Janoff, 1980) and no data exists on the possible inactivation of BLPI by other airborne pollutants.

SPECIFIC AIMS

The primary goal of this project was to study the effects of O_3 and NO_2 on α_1-PI and BLPI. The possible oxidative inactivation of α_1-PI was examined as a potential marker of inhaled oxidant effects in the lung. Oxidative inactivation of α_1-PI and BLPI would provide insight concerning the mechanism by which oxidants damage the lung. Additionally, α_1-PI and BLPI served as model proteins to study the effects of O_3 and NO_2 on protein structure and function.

Project Objectives:

1. The effect of O_3 on the ability of α_1-PI to inhibit elastase was studied by examining serum samples from humans exposed to O_3.
2. The effects of O_3 on α_1-PI, α_1-Achy, BLPI, and Eglin C were studied to provide comparative data of O_3 effects on proteinase inhibitors with similar functions but different structures.
3. The reaction kinetics of BLPI with HNE and Cat G were determined so BLPI's role relative to α_1-PI could be understood.
4. The effects of inhaled NO_2 on α_1-PI are being studied in bronchoalveolar lavage fluids from humans.

METHODS

MATERIALS

Porcine pancreatic elastase (chromatographically purified) (types III & IV), Coomassie Brilliant Blue G-250, electrophoresis film, Tris base, superoxide dismutase (SOD), HEPES, mannitol, porcine pancreatic trypsin, NCS, MeO-Suc-$(Ala)_3$-pNA, MeO-Suc-$(Ala)_2$-Pro-Val-pNA, Suc-$(Ala)_2$-Pro-Phe-pNA and Bz-Arg-pNA, N-benzoyl-L-Tyr-pNA, p-nitrophenylguanidine benzoate (NPGB) and Tricine base were purchased from Sigma Chemical Company, St. Louis, MO. Bovine trypsin, chymotrypsin, hen ovalbumin, soyabean trypsin inhibitor, horse heart myoglobin, horse heart cytochrome c, and 5,5'-dithiobis-(2-nitrobenzoic acid) also were products of Sigma. Goat antiserum to human α_1-PI was obtained from Atlantic Antibodies, Scarborough, ME. Sea Kem (ME) agarose was manufactured by the Marine Colloids Division of FMC Corp., Rockland, ME. Nitrogen dioxide (NO_2) was purchased from MG Industries (North Branch, N.J.) in a cylinder certified to contain 260 ppm NO_2 in nitrogen (N_2). Vitamin C (L-ascorbic acid) was the product of Fisher Scientific (Pittsburgh, PA). Sephadex G-25 was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). BLPI was purified as previously described (Smith and Johnson, 1985). The plasma proteinase inhibitors α_1-PI and α_1-Achy were prepared with slight modifications by the procedures of Travis and Johnson (1981) and Travis and Morii (1981), respectively. The human neutrophil proteinases HNE and Cat G were purified by the method of Martodam et al. (1979). Eglin C was supplied by Dr. Hans-Peter Schnebli of Ciba-Geigy (Basel, Switzerland) and Trolox C(R) (6-hydroxy-2,5,7,8-tetramethyl-chroman -2-carboxylic acid; a water soluble vitamin E analog) was a gift of Hoffman-LaRoche (Nutley, N.J.). All other chemicals were of reagent grade quality.

HUMAN OZONE EXPOSURE PROTOCOL

This was a collaborative effort with Drs. Fredrick Henderson and Delores Graham, EPA Health Effects Research Laboratory, Chapel Hill, N.C. The exposure protocol was approved by the Institutional Review Board of the University of North Carolina.

Two groups of male human volunteers (non-smokers) with one receiving 0.5 ppm O_3 and the other air (controls) were exposed for four hours on two consecutive days in exposure chambers at the Environmental Protection Agency's Human Exposure Facility in Chapel Hill, N.C. Blood was drawn by venipuncture prior to the exposures, which started at 12:00 noon (Pre 1 & Pre 2), and following exposures, at 4:00 p.m. (Post 1 & Post 2). A final sample of blood was drawn at 7:00 a.m. on the day after the second exposure (Pre 3), to look for a delayed effect. The blood was mixed with citrate anticoagulant and centrifuged to obtain the plasma fractions. The plasma was stored frozen until analyzed. Thawed plasma samples were diluted 1:100 with 0.1 M Tris-HCl pH 8.0 for subsequent assays.

Total protein was determined by the method of Bradford (1976), using human serum albumin as the standard. The protein assays gave values in the 25 μg range for samples diluted 1:100, with the values being read from standard curves with correlation coefficients of 0.99. These determinations were to the nearest 0.2 μg for an estimated error of 1%.

Rocket immunoelectrophoresis was performed as described by Monthony et al. (1978), using a Tris-Tricine buffer system. The antiserum to α_1-PI (0.1 ml of a 1:5 dilution in electrophoresis buffer) was mixed with 15 ml of melted 1% agarose

in electrophoresis buffer cooled to 60°C and poured onto a 10 X 10 cm sheet of plastic electrophoresis film. Samples of 5 μl were placed in 3 mm wells followed by electrophoresis on a horizontal apparatus at 400 volts constant voltage for 150 min. at 4°C. Soluble proteins were removed by soaking the plate overnight in 4 liters of 0.15 M NaCl. The gel then was pressed and dried with hot air, prior to staining with Coomassie Brilliant Blue G-250 to enhance peak measurement. Each plate contained four standard amounts of highly purified α_1-PI, five samples from an O_3-exposed individual, and five samples from an air-exposed subject. The sensitivity of the rocket immunoelectrophoresis technique allowed the quantitation of as little as 54 ng of α_1-PI (1 pmole). Normal human plasma contains 135 mg/dl (25 nmoles/mil) of α_1-PI and 5 μl of each sample was analyzed after diluting 100-fold with buffer. The four standards in each plate ranged from 54 ng (1 pmole) to 135 ng (2.6 pmoles), and gave linear plots with correlation coefficients of 0.99 or better. The difference in height between the lowest standard and the highest was 18 mm, with each peak being measured to the nearest 0.2 mm. Thus, a 0.2 mm error in measurement would result in an error equivalent to 0.9 ng (0.017 pmoles). All samples gave peaks higher than the lowest standard. The maximum error would have been 0.9 ng in 54, or 1.7%, and this method should have allowed detection of $\pm$ 2% in the serum levels of immunological α_1-PI.

Inhibition assays were performed in 0.1 M Tris-HCl, 0.1 M NaCl, and 1 mM $CaCl_2$, pH 8.0, in a total volume of 1.0 ml. The enzyme and the inhibitor were pre-incubated in assay buffer for 30 minutes prior to the addition of 50 μl of 20 mM substrate in dimethylsulfoxide (DMSO), to measure the amount of remaining enzyme activity. Porcine pancreatic elastase (PPE) was assayed with Suc-$(Ala)_3$-p-nitroanilide. Since representative enzyme assays gave linear increases in A_{410} with time when followed on a recorder, assay reactions normally were stopped by acidification, after 15 minutes. All samples for each subject were assayed on the same day and samples from O_3-exposed subjects were paired with air-exposed controls for the assay procedures only. In this way, variations in the assay conditions and experimental error were minimized. Assay reactions normally were stopped by acidification with 50 μl of glacial acetic acid, after 15 minutes. Elastase inhibition assays for α_1-PI activity were sensitive to 3 pmoles of inhibitor, which was sufficient for 20% inhibition of the 14.7 pmoles of PPE in each assay. These data were derived from titration plots of percent PPE activity versus volume in microliters of 1:100 diluted plasma, with correlation coefficients of 0.99. The amount of diluted plasma required for total inhibition of the PPE was determined from the X-intercepts of these plots and intersected at or near 50 μl. A 2 μl error resulting from this process would be equivalent to an estimated measurement error of 4%.

The primary standard for inhibition assays was porcine trypsin, which was found to be 85% active by titration with p-nitrophenyl guanidobenzoate (NPGB) (Chase and Shaw, 1967). When pure α_1-PI was titrated against the porcine trypsin the α_1-PI was found to be 100% active. Highly purified α_1-PI was used as the standard to check PPE inhibition assays. The amount of active α_1-PI in each plasma sample was determined by titrating a constant amount of porcine pancreatic elastase (PPE) with increasing amounts of 1:100 diluted plasma. Linear plots of percent PPE activity remaining versus amount of plasma preincubated with the elastase were obtained. The elastase inhibitory activity (EIA) or moles of PPE inhibited by each plasma sample were derived from the intercept of these plots.

STATISTICAL ANALYSIS OF DATA

The data were used to calculate the ratio of total α_1-PI/total protein and the ratio of EIA/total α_1-PI (moles of PPE inhibited per mole of α_1-PI). An increase in the total α_1-PI relative to total protein would indicate increased synthesis or release of α_1-PI, an acute phase protein. Similarly, a decrease in the amount of EIA relative to total α_1-PI would be a measure of α_1-PI inactivation.

Statistical analyses were accomplished manually or with the aid of the Statistical Analysis System Computer software package (SAS version 82.3). A fixed-effects (Model I) two-way repeated measures analysis of covariance was conducted, GROUP by TIME (with TIME being the repeated factor), covaried on the PRE measures (Freund and Littell, 1981). This analysis indicated no differences between the groups on either α_1-PI/total protein or EIA/total α_1-PI for $POST_1$ when difference in PRE_1 were controlled or for $POST_2$ when differences in PRE_2 were controlled. Changes over time were statistically significant, however, when followed up with Newman-Keuls multiple comparisons (Winer, 1971). Although 28 subjects were studied, yielding a total of 133 samples, complete data were available for only 21 subjects because blood plasma samples were not obtained at some of the pre- or post-exposure time points. This resulted in omitting four subjects from the O_3-exposed group and three subjects from the air-exposed control group. Those subjects with incomplete data were omitted for statistical comparison.

GENERAL ASSAY CONDITIONS

Inhibitory activities for all experiments were measured by assaying residual enzyme activity after incubating enzyme and inhibitor. Enzyme and inhibitor incubation times were based on either known or determined half-times of association ($t_{1/2\ on}$) and the enzyme concentration in the reaction (Bieth, 1980). HNE activity was assayed with either Suc-$(Ala)_2$-Pro-Val-pNA (Castillo et al., 1979) or Suc-$(Ala)_3$-pNA (Bieth et al., 1974). The latter substrate was used to assay porcine pancreatic elastase also. Chymotrypsin activity was assayed with either Bz-Tyr-OEt (Kang and Fuchs, 1973), or Bz-Tyr-pNA (Bundy, 1963). Suc-$(Ala)_2$-Pro-Phe-pNA and Bz-Tyr-OEt were used to assay Cat G activity. The trypsins were assayed by the method of Erlanger et al. (1961), using Bz-Arg-pNA as the substrate. One BLPI inhibitory unit was defined

as the amount of inhibitor that decreased the rate at which chymotrypsin catalysed the hydrolysis of Bz-Tyr-OEt by 1.0 A_{256} absorbance unit per minute. Assays with p-nitroanilide substrates were monitored by measuring the absorbance increase at 410 nm. Representative stopped assays were checked by continuous monitoring for linearity. All assays were linear and there was no indication of substrate-induced complex dissociation.

INHIBITORY ACTIVITY ASSAYS OF OZONE-TREATED INHIBITORS

Assays to detect inhibitory activity were based on the decrease in enzymatic activity, which resulted from the pre-incubation of proteinase with an inhibitor. Unless noted, pre-incubations were for 1,000 times the normal half-time of association, based on published association rates (Bieth et al., 1980; Smith and Johnson, 1985; Baici and Seemuller, 1984) and the concentration of proteinase used in the reaction. Generally, inhibitors (treated and non-treated) were pre-incubated with proteinases in a total volume of 0.98 ml of buffer and reactions were started by addition of 20 μl of substrate (20 mM in DMSO). Product production was monitored directly proportional to the increase in absorbance at 410 nm. HNE was assayed using either MeO-Suc-$(Ala)_2$-Pro-Val-pNA (Castillo et al., 1979) or MeO-Suc-$(Ala)_3$-pNA (Bieth et al., 1974). MeO-Suc-$(Ala)_2$-Pro-Phe-pNA was used to assay Cat G activity (Martodam et al., 1979). Porcine pancreatic trypsin was assayed by the method of Erlanger et al. (1976), using Bz-Arg-pNA in 0.1 M Tris-HCl, 0.05 M NaCl, pH 8.0. HNE and Cat G were assayed in 0.1 M HEPES pH 7.5. The buffer for HNE also contained 1 M NaCl, while the buffer for Cat G was 0.5 M in NaCl.

DETERMINATION OF MOLAR STOICHIOMETRIES

The protein concentrations used were based on published extinction coefficients ($E^{1\%}_{280}$) and molecular weight values, as follows: HNE, 9.85 and 30,000 (Baugh and Travis, 1976); Cat G, 6.64 and 23,500 (Travis et al., 1978); porcine trypsin, 13.5 and 23,400 (Travis and Liener, 1965); α_1-PI, 5.3 and 52,000 (Travis and Johnson, 1981); BLPI, 7.8 and 11,200 (Smith and Johnson, 1985); α_1-Achy, 6.2 and 68,000 (Travis and Morii, 1981) bovine trypsin, 14.3 and 23,800 (Cunningham, 1954); bovine chymotrypsin, 20.5 and 25,600 (Wilcox, 1970); porcine pancreatic elastase, 22.0 and 25,900 (Shotton, 1970). Molarities were corrected for active site concentrations, as described below. The concentrations of Eglin C solutions were based on the weight dissolved and a molecular weight of Eglin C of 8,133. The active site concentration of each protein was based on the chemical titration of porcine trypsin with NPGB, by the method of Chase and Shaw (1967: 85% active), which subsequently was used to titrate a stock solution of α_1-PI (100% active by this procedure). The α_1-PI, which forms a 1:1 complex with Cat G and HNE was used as a secondary standard to determine the activity of these enzymes (95% active). The molar binding ratios for HNE and Cat G with BLPI, Eglin C and α_1-Achy (Cat G only) were determined by their titration with either HNE or Cat G. The proteinase concentrations used were high enough to allow for short incubation times, and they gave linear titrations plots.

OZONE EXPOSURE OF PURIFIED INHIBITOR PROTEIN SOLUTIONS

Proteinase inhibitors were exposed to O_3, as previously described (1980). Briefly, O_3 diluted with nitrogen was pumped through 2 ml of sample solution at a flow rate of 5 ml/min. Inhibitor protein solutions were buffered with 25 mM Na_2HPO_4, 0.1 M NaCl, pH 7.4, as recommended by Pryor (1985). Ozone delivery into the solutions was quantitated by iodometric determination, as described by Mudd et al. (1969). The O_3 delivery rate was 25 to 30 nmoles/min and was measured before and after each series of exposures. All of the O_3 apparently reacted with the protein because no O_3 was detected in a tube of potassium iodide absorbing solution downstream from the inhibitor solutions.

To compensate for denaturation due to bubbling, control samples were bubbled with air for the maximum time period used for O_3-exposed samples. However, air bubbling did not reduce the activity of the inhibitors. Treated proteinase inhibitors were assayed for inhibitory activity and compared to controls, which were taken as having 100% activity.

Examination of free radical scavengers for protection of α_1-PI and BLPI was accomplished by O_3 treatment in the presence of one of the following: 10 μg/ml superoxide dismutase (SOD), 50 mM mannitol, 10 μg/ml ascorbic acid, or 10 μg/ml Trolox C (Bisby et al., 1984). These concentrations approximated normal physiological ranges.

IN VITRO NITROGEN DIOXIDE TREATMENT OF ALPHA-1-PROTEINASE INHIBITOR AND BLPI

A cylinder of NO_2 in N_2, certified to contain 260 ppm NO_2, was obtained from MG Industries. A gas sampling bag was filled with the NO_2/N_2 mixture and withdrawn by peristaltic pump into protein or arsenite absorbing solutions. Figure 1 is a schematic of the NO_2 exposure set-up, which was used in a chemical fume hood.

Teflon or Tygon tubing was used to carry the gas from the bag through the samples. The volume of protein or absorbing solution was 2.5 ml. The amount of NO_2 delivered per minute was determined with arsenite absorbing solution in tube A, before, during, and after a series of exposures.

The amount of NO_2 was determined by the sodium arsenite method, according to the published EPA procedure (EPA, 1981). Briefly, after bubbling gas through the arsenite absorbing solution for five minutes, the gas was reacted with sulfanilamide and the concentration of NO_2 was read from a standard curve prepared with $NaNO_2$. The concentration of NO_2 in the gas bag was determined to be 120 ppm or 2.57 nmoles/ml.

The arsenite absorbing solution used for the quantitation of NO_2 delivery was stated to be 82% efficient. In contrast, the Saltzman sulfanilic acid absorbing solution used by Dooley and Pryor is reported to be only 35% efficient (EPA,

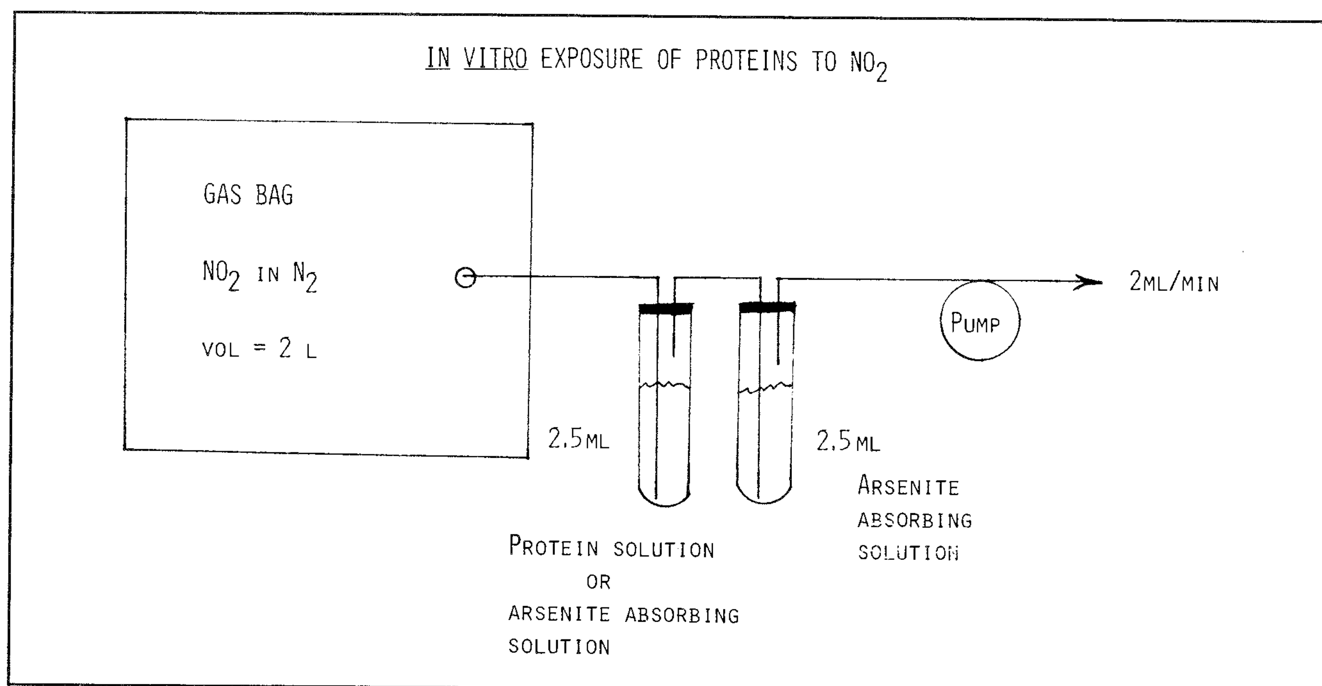


Figure 1. Set-up for the NO_2 exposure of proteins: The NO_2 delivery of this system was 3.13 nmoles NO_2 per ml.

1981). If our data are corrected for the 18% NO_2 loss due to inefficient absorption, the NO_2 concentration being bubbled through the protein solution would be 146 ppm or 3.13 nmoles/ml. Some NO_2 is lost by reaction with the gas handling bag and tubing, if the cylinder truly contained 260 ppm. However, we are confident of our results, which are based on repeated determinations.

OXIDATION WITH N-CHLOROSUCCINIMIDE (NCS)

BLPI (300 μg) was treated with NCS by the method of Schechter et al. (1975), which is specific for the oxidation of methionine to the sulfoxide. After a 20 minute incubation with NCS, samples were passed over a Sephadex G – 25 column to remove NCS, with a 90% recovery of protein.

Oxidized and native BLPI samples were assayed for inhibitory activity against HNE and Cat G by incubating equimolar mixtures of proteinase and inhibitor for not less than five times the normal half-time of association. Percent inhibitory activity for each sample was calculated based upon control samples that had undergone the same treatment. As with O_3 treatments, the controls were taken as 100% active.

AMINO ACID ANALYSIS OF BLPI

Samples were hydrolysed *in vacuo* for 12, 24, 48, and 72 hours with 6 M HCl at 110°C, followed by duplicate analyses. The values for serine and threonine were obtained by extrapolation to zero time. The 72 hour values were taken for valine and isoleucine, and the 24 and 48 hour data were averaged for the values of the other amino acids. The sample for tryptophan analysis was subjected to alkaline hydrolysis (Hugli and Moore, 1972), and the sample for amino sugar content was hydrolysed for 5 hours with 4 M HCl at 100°C. Samples were analysed on a Beckman 119 CL amino acid analyser. A newly developed chromatographic method was used in the analysis of tryptophan and glucosamine (Johnson, 1983). The $A_{280}^{1\%}$ was calculated from the recovery of amino acids relative to the A_{280} of the sample. The protein was examined for free sulfhydryl groups by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972).

AMINO ACID ANALYSIS OF OXIDIZED INHIBITORS

The destruction of tryptophan was monitored by a decrease in its 340 nm fluorescence emission peak (Guilbault and Froelich, 1973), using an excitation wavelength of 280 nm. Oxidation of methionine (to methionine sulfoxide) and other amino acids was quantitated by the method of Schechter et al. (1975), as previously reported for α_1-PI (Johnson and Travis, 1979).

ASSOCIATION RATE DETERMINATIONS FOR BLPI

The association rates (k_{on}) for BLPI with HNE, cathepsin G, bovine trypsin, and chymotrypsin were determined by the method of Vincent and Lazdunski (1972), under second order rate conditions. Equimolar mixtures of enzyme and inhibitor, based on the above titrations, were incubated for increasing time periods in a total volume of 980 μl. Remaining enzyme activity was measured as described earlier by monitoring the change in A_{410} after the addition of substrate.

These experiments were performed in 0.1 M HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)], pH 7.5 at 25°C. The reaction buffer for HNE was 1 M in NaCl, and

the buffers for cathepsin G, chymotrypsin, and bovine trypsin were 0.05 M in NaCl. In addition, the buffer for chymotrypsin reactions contained 8% dimethyl sulfoxide to maintain Bz-Tyr-pNA solubility, and 10 mM $CaCl_2$. The final enzyme concentrations used in each determination were as follows: 0.033 μM for HNE, 0.55 μM for cathepsin G, 1.04 μM for trypsin, and 2.14 μM for chymotrypsin. Substrate concentrations ranged from 0.3 to 0.8 mM.

DISSOCIATION RATE DETERMINATIONS FOR BLPI

Dissociation rates (k_{off}) were determined by adding α_1-PI to complexes of BLPI and enzyme, and measuring the amount of enzyme transferred from the BLPI-proteinase complex to α_1-PI in a time course reaction (Vincent and Lazdunski, 1972; Gauthier et al., 1982). Initially, equimolar mixtures of BLPI and enzyme, based on the aforementioned titrations, were incubated until zero enzyme activity was recorded (7 X $t_{1/2,\ on}$ for association). The dissociation of the BLPI-proteinase complex was measured by monitoring the α_1-PI inhibitory activity of 50 μl aliquots taken from the reaction mixture (500 μl) and added to 930 μl of 0.1 M Hepes, 1 M NaCl, pH 7.5, which contained an equal amount of porcine pancreatic elastase. Following a 15 minute incubation, the residual elastase activity, which equalled the amount of BLPI-proteinase complex, was measured after the addition of 20 μl of 20 mM Suc-(Ala)$_3$-pNA in dimethyl sulfoxide. The enzyme concentrations used in the initial incubations were: 2.15 μM for HNE, 5.15 μM for cathepsin G, 10.4 μM for trypsin, and 10.1 μM for chymotrypsin. The ratio of k_{off}/k_{on} was used to calculate the equilibrium dissociation constant (K_i) for the interaction of each proteinase with BLPI.

RESULTS

IN VIVO EFFECTS OF OZONE ON HUMAN PLASMA ALPHA-1-PROTEINASE INHIBITOR

The ratio of total α_1-PI/total protein was calculated from the amount of total α_1-PI determined by rocket immunoelectrophoresis and the total protein assays, as described in Methods. These ratios for each time point are displayed as a bar graph in Figure 2. The ratio of total α_1-PI/total protein increased with increasing exposure to either O_3 or air. These changes were significant ($p < 0.05$) for each time point. However, there were no significant differences in these data for the O_3-exposed subjects relative to the air-exposed subjects.

The elastase inhibitory activity (EIA) of plasma α_1-PI was measured by inhibition assays against a known concentration of active PPE. The EIA of each sample was derived from the intercept of plots of percent PPE activity versus the amount of 1:100 diluted plasma pre-incubated with PPE. By this method, three to four data points essentially were averaged for the EIA of each sample. In preliminary experiments the use of only one amount of plasma yielded widely scattered data. The reason for this is shown in Figure 3. As more plasma was added, the activity of PPE decreased to a minimum and then slowly increased. Therefore, even multiple assays performed in the non-linear area could result in erroneous data. For example, approximately 60% inhibition was obtained upon the addition of either 30 or 300 μl of plasma (Figure 3), but only the 30 μl value is indicative of the amount of α_1-PI in the sample. All titration plots were linear and the mean calculated concentration of active α_1-PI in the undiluted

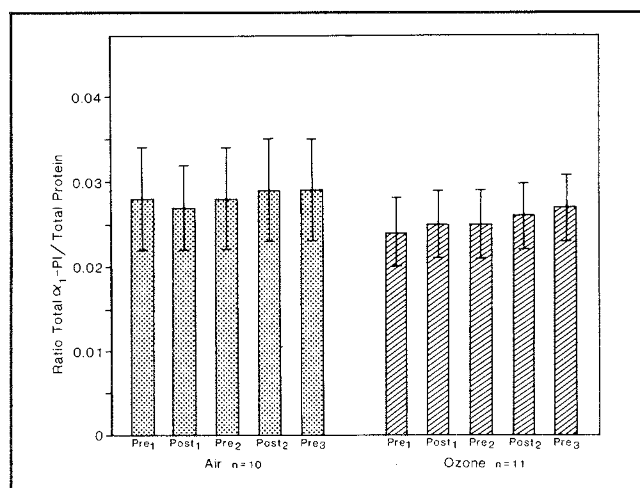


Figure 2. Ratio of total α_1-PI/total protein in plasma samples of ozone and air exposed humans: Blood was drawn before (Pre 1) and after (Post 1) exposure to 0.5 ppm ozone or air for 4 hrs, and this procedure was repeated the following day (Pre 2 and Post 2). Additionally, blood was drawn on day 3 (Pre 3), 15 hrs after exposure on day 2. The bars represent the mean of the ratios and the error bars equal one standard deviation from the mean.

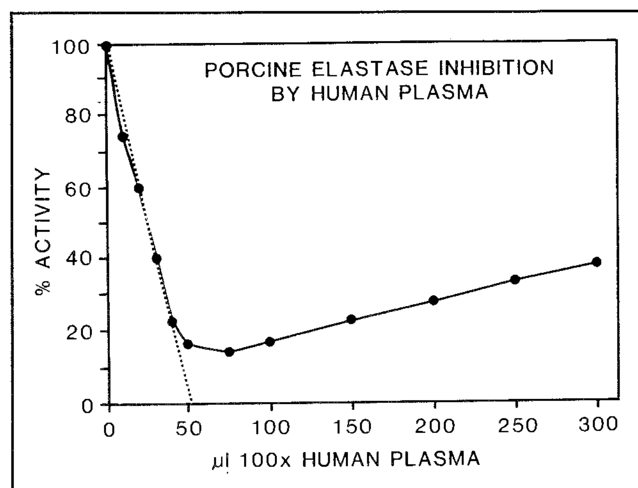


Figure 3. Titration plot of porcine elastase activity vs. increasing amounts of human plasma: PPE (14.7 pmoles, 0.38 μg) was preincubated 30 min with 10-300 μl of 100x plasma (1:100 dilution in 0.1 M Tris-HCl, pH 8.0). Remaining active elastase was assayed using the substrate, Suc-(Ala)$_3$-pNA.

samples was 148 mg/dl (range = 116-184, n = 133), which is close to the mean value reported by Laurell's group (1963) of 135 mg/dl.

The functional activity of α_1-PI was evaluated by dividing the amount of elastase inhibitory activity (EIA) by total α_1-PI (rocket immunoelectrophoresis). The results of these determinations are shown in Figure 4, in which the ratios of EIA to total α_1-PI are presented. Theoretically, these ratios should not have exceeded 1.0. Why they ranged as high as 1.7 is unclear.

Although the relative activity of α_1-PI decreased with time in a statistically significant ($p < 0.05$) fashion, there was no notable difference in this effect between groups. Additionally, the mean ratios of EIA to total α_1-PI were consistent with fully active α_1-PI. These data show that inactive α_1-PI cannot be detected in the blood plasma of subjects breathing 0.5 ppm of O_3 for 4 hours. No effect was observed even after two consecutive exposures. Additionally, the possibility of a delayed effect was examined by assaying plasma obtained the morning after the second exposure, but again no effect was seen.

ALPHA-1-PROTEINASE INHIBITOR AND ALPHA-1-ANTICHYMOTRYPSIN EFFECTS OF IN VITRO EXPOSURE TO OZONE

Ozone inactivated α_1-PI in a dose-dependent fashion (Figure 5), with total inactivation occurring at 50 moles of O_3 per mole of inhibitor. To test whether or not the observed inactivation was due to a reduction in enzyme-inhibitor association rates, pre-incubation times prior to assay of residual proteinase activity were increased to 1,000 times the half-time for association of native α_1-PI and HNE, as reported by Beatty et al. (1980). No change in inhibitory activity was noted, indicating that the inactivation involved more than the oxidation of the inhibitory site P_1 methionine residue. Amino acid analysis and spectrofluorometric data showed extensive oxidation of tryptophan (Trp), histidine (His), and tyrosine (Tyr) in addition to methionine (Met) residues (Table 1).

The *in vitro* exposure of α_1-Achy to ozone also led to a loss of inhibitory activity, but in contrast to α_1-PI, only 50% of α_1-Achy's Cat G inhibitory activity was lost, even at 250 moles of ozone per mole of α_1-Achy (Figure 5). Maximum inactivation occurred at 50 moles of ozone per mole of α_1-Achy, which was the same ozone:inhibitor ratio resulting in total inactivation of α_1-PI. Because of this similarity, amino acid analyses were performed at the 50 mole/mole point (Table 1). Although α_1-Achy contains more Met and Trp, the oxidation of these residues was 25% lower than for α_1-PI. The percentage of His residues attacked was approximately the same as for α_1-PI. Relative to α_1-PI, 30% more of the Tyr residues in α_1-Achy were oxidized.

BLPI AND EGLIN C, EFFECTS OF IN VITRO EXPOSURE TO OZONE

Both the Cat G and HNE inhibitory activities of BLPI and Eglin C decreased with exposure to O_3 (Figure 6). BLPI

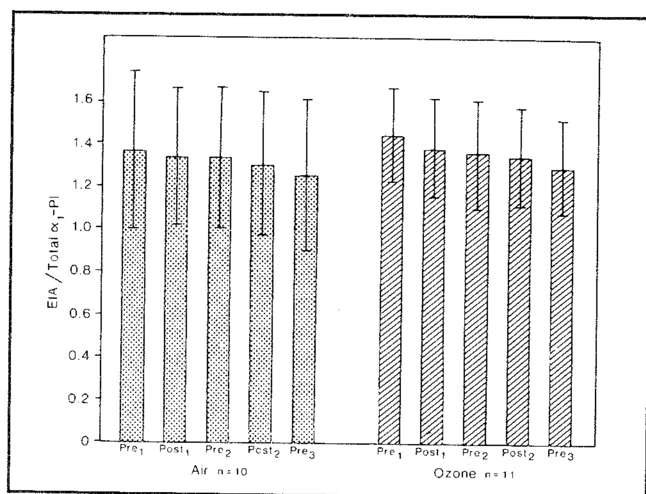


Figure 4. Ratio of EIA/total α_1-PI in plasma samples of ozone and air exposed humans: Blood was drawn before (Pre 1) and after (Post 1) exposure to air or 0.5 ppm ozone for 4 hrs. This procedure was repeated the following day to obtain corresponding samples (Pre 2 and Post 2). On the 3rd day, 15 hrs after exposure on day 2, an additional sample was obtained (Pre 3), but subjects were not exposed. The means of the ratios for each time point are represented by the bar graph and the error bars equal one standard deviation.

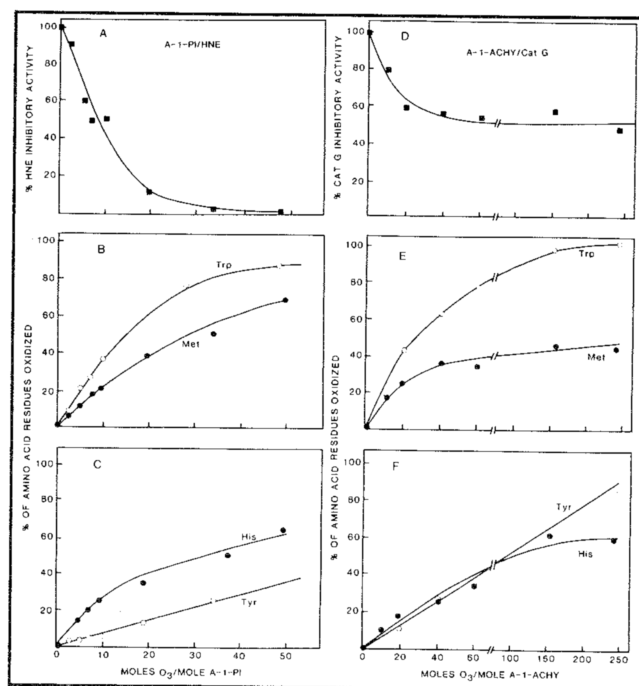


Figure 5. Reaction of O_3 with α_1-PI and α_1-Achy: Ozone was bubbled through a 2.5 ml solution containing 10 nmoles of α_1-PI and a 1.75 ml solution containing 4.1 nmoles of α_1-Achy. Both inhibitor solutions were buffered with 25 mM Na_2HPO_4, 0.1 M NaCl, pH 7.4. (A) α_1-PI inhibitory activity vs. HNE, (B) α_1-PI, Trp o-o, and Met •-• oxidation, (C) α_1-PI, Tyr o-o, and His •-• oxidation, (D) α_1-Achy inhibitory activity vs. Cat G, (E) α_1-Achy, Trp o-o, and Met •-• oxidation, (F) α_1-Achy, Tyr o-o, and His •-• oxidation.

Table 1. Number of Amino Acid Residues Oxidized at Initial and Maximum Exposure Levels

Inhibitor		Moles O_3 per mole	% Inhib. Act vs. HNE	% Inhib. Act vs. Cat G	Met	Trp	His	Tyr	Total Residues Oxidized	% of Total Residues Oxidized
α_1-PI	Initial	10	43	ND[a]	2.0 (9)	[b] 0.7 (2)	3.4 (13)	0.4 (6)	6.5	22
	Maximum	50	2	ND	6.2	1.7	8.1	2.0	18.0	60
α_1-Achy	Initial	10	ND	77	1.9 (12)	1.0 (8)	0.6 (8)	0.5 (9)	4.0	12
	Maximum	50	ND	52	4.3	2.8	2.7	2.9	12.7	38
BLPI	Initial	10	68	61	2.6 (3)	0.4 (1)	NA	0.5 (2)	3.5	58
	Maximum	40	21	13	3.0	0.8		1.3	4.4	73
Eglin C	Initial	10	75	68	NA[c]	NA	0.6 (3)	3.2 (6)	3.8	42
	Maximum	40	19	8			2.4	5.9	8.3	92

[a] ND - Not determined
[b] Total number of residues is given in parenthesis
[c] NA - Non-applicable

inactivation leveled off at 40 moles of O_3 per mole BLPI, while the inactivation of Eglin C leveled off at 64 moles of O_3 per mole. These points of maximum inactivation were chosen for amino acid analyses. Met, Trp, and Tyr were extensively oxidized (Table 1) in the BLPI sample and, almost all of the Tyr and His residues in Eglin C were destroyed.

The inhibitory site amino acid sequence of BLPI has not been reported, but mild oxidation with Chloramine-T and NCS was shown to cause partial inactivation, suggesting that oxidizable residues are important to the inhibition process (Carp and Janoff, 1980). The possible involvement of Met in the inhibitory site was examined by selective oxidation with NCS. As shown in Figure 7, treating BLPI with increasing concentrations of NCS per mole of Met resulted in the progressive loss of Cat G and HNE inhibitory activities. Cat G inhibitory activity was more susceptible to inactivation by Met oxidation than HNE inhibitory activity. Two moles of NCS per mole of methionine resulted in an 80% loss of Cat G inhibitory activity, but only a 40% loss of HNE inhibitory activity. Therefore, Met is more important to Cat G than to HNE inhibition. Amino acid analysis at 2.5 moles NCS/mole methionine showed that all three methionine residues had been ozidized to methionine sulfoxide.

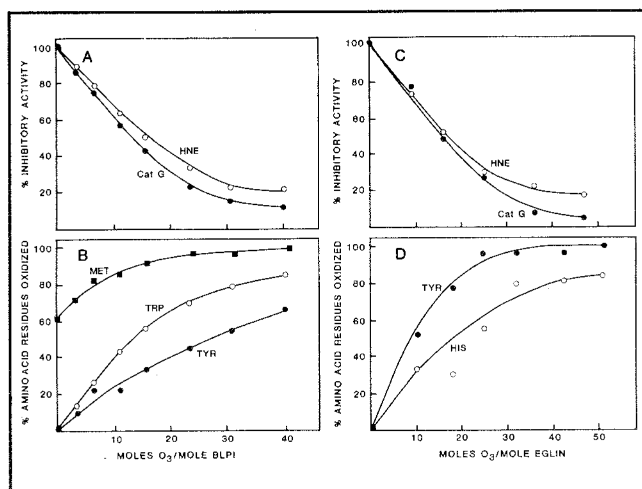


Figure 6. Reaction of ozone with BLPI and Eglin C: Ozone was bubbled through 2.5 ml solutions containing 20 nmoles of BLPI and 10 nmoles of Eglin C in 25 mM Na_2HPO_4, 0.1 M NaCl, pH 7.4. (A) BLPI inhibitory activity vs. HNE o-o and Cat G •-•, (B) BLPI, Met ■-■, Trp o-o, and Tyr •-• oxidation, (C) Eglin C inhibitory activity vs. HNE o-o and Cat G •-•, (D) Eglin C, Tyr •-•, and His o-o oxidation.

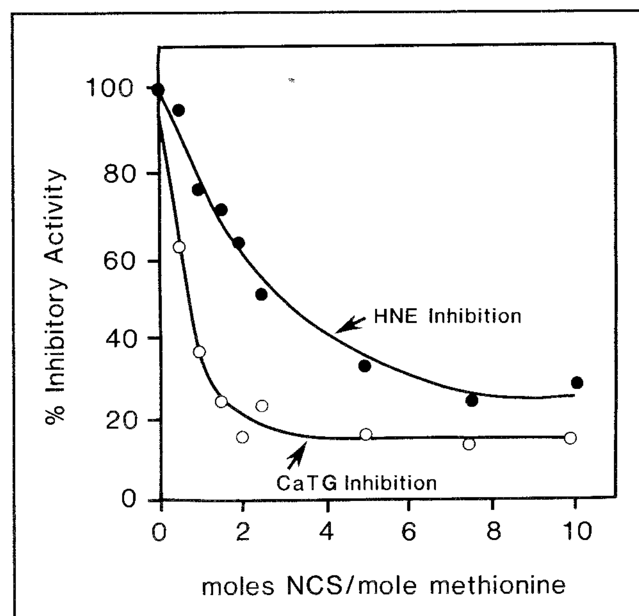


Figure 7. Effect of methionine oxidation on the inhibitory activities of BLPI: BLPI (300 μg) was incubated with increasing mole/mole ratios of NCS/methionine for 20 min. NCS was removed by chromatography on Sephadex G-25 (Methods), followed by assay of HNE (•-•) and Cat G (o-o) inhibitory activities.

ANTI-OXIDANT EFFECTS ON OZONE INACTIVATION OF ALPHA-1-PROTEINASE INHIBITOR AND BLPI

Mannitol, vitamin C, Trolox C, and SOD were examined for their ability to protect α_1-PI and BLPI from O_3 inactivation. The anti-oxidants were added to solutions of either α_1-PI or BLPI prior to O_3 exposure. Mannitol and vitamin C, which are free radical scavengers, were somewhat protective of both inhibitors. These results (Table 2) suggest some oxidation via hydroxyl radicals, which may have been derived from O_3 attack on tyrosine residues (Pryor et al., 1984). Trolox C, which has been shown to repair radicals resulting from the oxidation of Trp, Tyr, Met, and His (Bisby et al., 1984), was the best protectant of α_1-PI, but only 1% of the BLPI inhibitory activity was protected. Superoxide dismutase failed to provide any protection, showing that superoxide is not involved in the inactivation process. The sum of these results indicate that O_3 inactivates α_1-PI and BLPI primarily via direct attack on the susceptible amino acids.

PURIFICATION AND CHARACTERIZATION OF HUMAN BLPI

Sputum was collected daily from patients with chronic bronchitis and was stored at -15°C until it was used. The thawed sputum was made 0.5 M in NaCl and 20mM in HCl in order to dissociate BLPI-proteinase complexes. Trichloroacetic acid was added to a final concentration of 3% (w/v), which denatured humoral inhibitors and several other proteins. The viscosity of this mixture was reduced by stirring in a blender for 15 seconds at low speed prior to centrifugation at 30,000 g for 20 minutes. The supernatant fraction was made 0.05 M in Tris base, adjusted to pH 8.0 with 10 M NaOH, and added to chymotrypsin-Sepharose (25 ml of gel/l of sputum) which had been prepared by coupling the enzyme to CNBr-activated Sepharose at pH 6.5 (Marsh et al., 1974). After stirring it for 1 hour at room temperature, the BLPI-chymotrypsin-Sepharose was collected on a glass fritted Buchner funnel and washed with 0.05 M Tris-HCl, 0.5 M NaCl, pH 8.0. The gel was poured into a chromatography column and washed with the pH 8.0 buffer until the A_{280} was 0. The BLPI was eluted, in a trailing peak (Figure 8), with 0.025 M HCl, 0.5 M NaCl, pH 1.75. Active fractions were pooled and concentrated on an Amicon UM-2 (2,000 MW cut-off) membrane. The yield from six isolations averaged 5 mg/l of sputum. Chromatography of the preparation on Sephadex G-50 superfine yielded a single symmetrical peak, corresponding to a molecular weight of 12,900 (Figure 9). This column was monitored at 225 nm in order to detect other components such as peptides, but none were found. The preparation was also homogeneous by electrophoresis (Figure 10) at pH 6.8 in a high resolution system for cationic proteins (Thomas and Hodes, 1981). Titration of chymotrypsin (0.12 μM) and Cat G (0.26 μM) with BLPI using Bz-Tyr-OEt as substrate yielded linear plots intersecting at 1.0 mole of BLPI per mole of proteinase, indicative of 1:1 binding.

Amino acid analysis of this preparation (Table 3) was in very close agreement with that of Ohlsson et al. (1977) for BLPI and Schiessler et al. (1978). Examination of the protein for free thiol groups with Ellman's reagent after denaturation in sodium dodecyl sulfate (Habeeb, 1972) indicated that all 14 of the cysteine residues were disulfide linked. The combined cystine and proline content equals 25%, and these residues probably make a significant contribution to the stability of this protein.

Table 2. Antioxidant Protection of α_1-PI and BLPI

	Percent Protection of HNE Inhibitory Activity*	
Antioxidant	**α_1-PI**	**BLPI**
Mannitol (50 mM)	28	14
Vitamin C (10 μg/ml)	12	12
Trolox C (10 μg/ml)	35	1
SOD (10 μg/ml)	0	0

*Relative to maximally inactivated inhibitor; A-1-PI (50 moles O_3/mole; 0% HNE inhibitor activity) BLPI (40 moles O_3/mole; 13% HNE inhibitor activity).

Table 3. Amino acid analysis of human BLPI

	Analysis (residues/molecule)			
	Present Work		Data of Ohlsson et al.	Data of Schlessler et al.
	Found	Integer	(1977)	(1978)
Asp	8.51	9	9	9
Thr	3.90	4	4	4
Ser	5.88	6	6	6
Glu	6.69	7	5	8
Pro	13.23	13	11	12
Gly	9.13	9	9	9
Ala	3.03	3	4	3
Cys	14.14	14	14	12
Val	5.02	5	7	5
Met	3.26	3	3	3
Ile	0.76	1	1	2
Leu	4.99	5	5	6
Tyr	1.77	2	2	2
Phe	1.96	2	2	2
His	0	0	0	0
Lys	14.20	14	13	15
Arg	4.44	4	4	5
Trp	0.62	1	0	0
Glucosamine	0.44	1	0	0
Total		103	99	103
Calculated M_r		11243	10332	10970

The major difference between present data and those of others is that one residue/mol each of tryptophan and glucosamine was found, while others found none or did not analyze for these residues. These are difficult residues to quantify, and they may have been missed by previous investigators. From amino acid analysis data, a minimum M_r of 11,243 was calculated, which was close to the value obtained by gel filtration. An $A^{1\%}_{280}$ of 7.8 was determined from amino acid analysis data, corresponding to a molar extinction coefficient of 8,770 $M^{-1} cm^{-1}$ for M_r = 11,200. These values, which have not been reported previously, are consistent with the presence of one tryptophan and two or three tyrosine residues, in good agreement with the amino acid analysis given in Table 3. The $A^{1\%}_{280}$ and M_r of 11,243 were used to calculate the inhibitor's molar concentration in subsequent studies.

Whereas other methods for BLPI purification have employed ion exchange and gel filtration chromatography

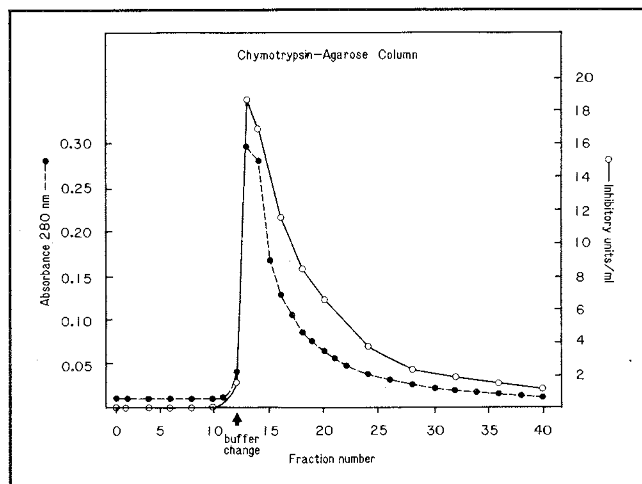


Figure 8. Chymotrypsin-Sepharose affinity chromatography: The trichloroacetic acid supernatant fraction from 1 liter of sputum was added to 25 ml of chymotrypsin-Sepharose at pH 8.0. The affinity gel was washed with 2,000 ml of 0.05 M Tris-HCl, 0.5 M NaCl, pH 8.0, and poured into a column. The column (1.5 cm X 14 cm) was washed with the pH 8.0 buffer until the A_{280} was 0, and BLPI was eluted with 0.025 M HCl, 0.5 M NaCl, pH 1.75. Fractions (2.8 ml each) were collected every 3 min at room temperature.

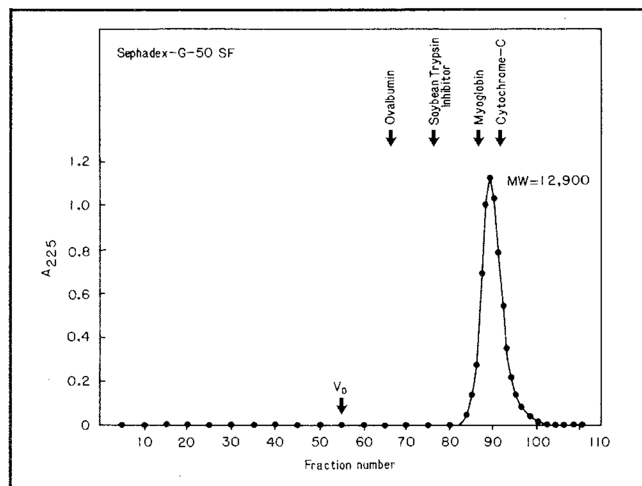


Figure 9. Gel filtration chromatography: BLPI (1.16 mg) was chromatographed on a Sephadex G-50 (superfine grade) column (1.6 cm X 85 cm) in 0.05 M Tris-HCl, 0.1 M NaCl, pH 8.0, at room temperature. Fractions (1.0 ml) were collected at 5 min intervals and their A_{225} was measured. The elution positions of the standard proteins used to calibrate the column are indicated. The correlation coefficient for the log M_r vs. $K_{av.}$ plot of standards was 0.99; the M_r of BLPI was calculated to be 12,900.

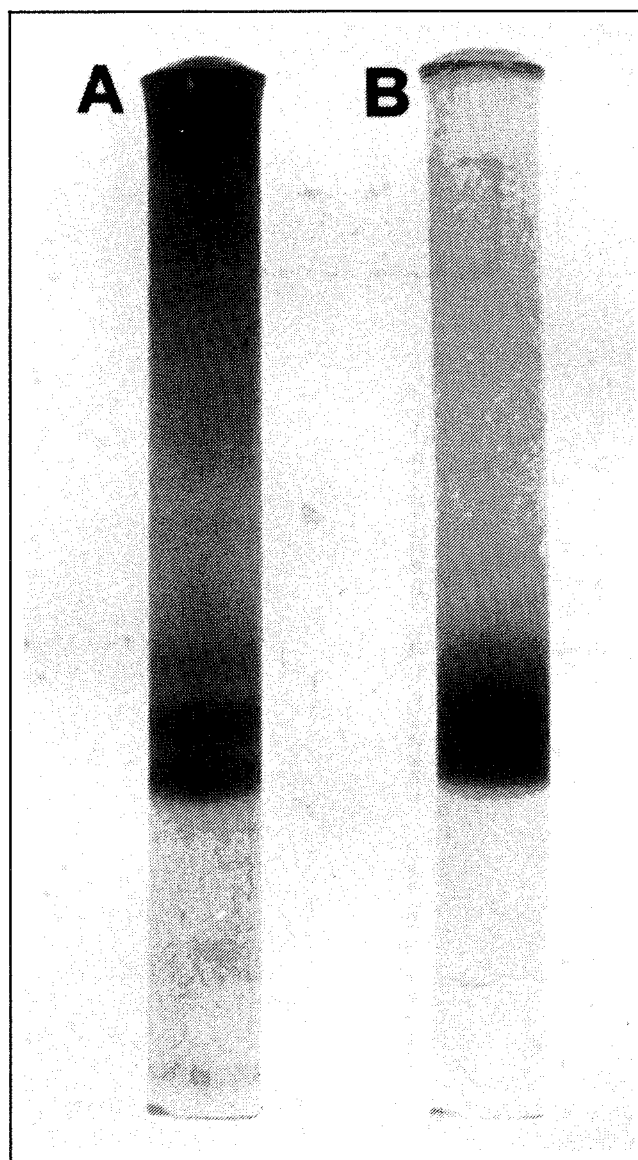


Figure 10. Polyacrylamide-gel electrophoresis: The trichloroacetic acid supernatant fraction (0.5 ml) (a) and purified BLPI (40 μg) (b) were electrophoresed in 10% polyacrylamide gels. The pH of the 3% stacking gel was 8.0 and the separating gel had a pH of 6.8. The gels were buffered with MoPS (4-morpholine propane sulfonic acid) only while the reservoir contained MoPS/histidine at pH 6.8. The anode was at the top and cathode at the bottom. After electrophoresis for 4 hrs at 23 mA/gel, the gels were stained with Coomassie Brilliant Blue R-250 containing formaldehyde and acetic acid as fixatives.

Table 4. Comparison of Kinetic Data for Various Proteinases with BLPI. For an explanation of experimental details see the text. Complex stability is equivalent to the half-time of dissociation, as explained in the text. Calculations of delay time and complex stability are based on the molar concentrations of BLPI in sputum as determined by Kramps et al. (1984).

Enzyme	k_{on} ($M^{-1} \cdot rs^{-1}$)	k_{off} (s^{-1})	K_i (M)	Delay time (s)	Complex stability
HLE	1.06×10^5	1.99×10^{-5}	1.87×10^{-10}	8.04	9.67
Cathepsin G	2.48×10^4	1.04×10^{-4}	4.18×10^{-9}	34.35	1.85
Trypsin	2.24×10^4	1.85×10^{-4}	8.28×10^{-9}	38.03	1.04
Chymotrypsin	1.16×10^3	3.05×10^{-5}	2.63×10^{-8}	734.30	6.31

in addition to affinity chromatography on trypsin-bound agarose, the present method, using chymotrypsin-Sepharose, yielded a homogeneous preparation in a single chromatographic step. As subsequent data show, BLPI- chymotrypsin complexes are more stable than trypsin complexes and may explain why chymotrypsin proved to be a superior affinity ligand. Both electrophoresis and gel filtration data demonstrate the homogeneity of the preparation. The purity of our preparation is consistent with that of Ohlsson et al. (1977), as evidenced by amino acid analysis and immunological cross reactivity (K. Ohlsson, personal communication). Schiessler et al. (1978) found that BLPI inactivated HNE, cathepsin G, bovine trypsin, and bovine chymotrypsin, but failed to inhibit porcine elastase or porcine trypsin. The inhibition spectrum of our preparation is in total agreement with this report. Thus, there seems to be little doubt that this inhibitor is the major leukocyte proteinase inhibitor of sputum described by Hochstrasser et al. (1972), and Ohlsson et al. (1977).

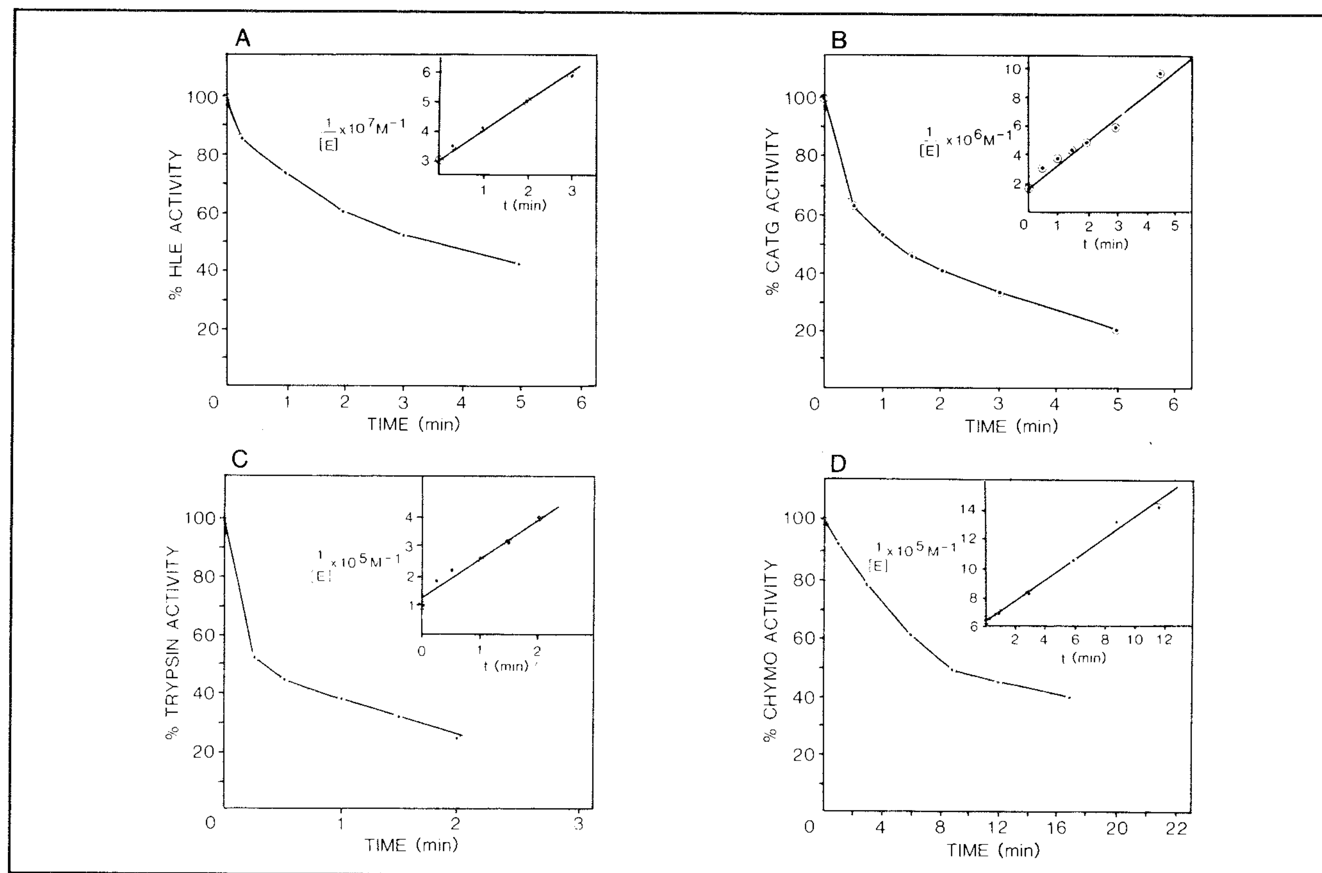
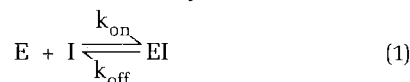


Figure 11. Association rates for BLPI with various proteinases: Equimolar mixtures of enzyme and inhibitor were incubated for increasing time intervals, and the percentage of enzyme activity remaining plotted vs. time. A second order replot of these data (inset), in accordance with eq. (3) (see the text), gives a slope equal to k_{on}, and the y-intercept equals $[E_o]$. The enzymes studied were: (A), HLE; (B), cathepsin G; (C), trypsin; and (D), chymotrypsin.

INHIBITION KINETICS OF BLPI WITH HNE AND CAT G

The rates of association for BLPI with HNE, Cat G, bovine trypsin, and chymotrypsin were determined according to Vincent and Lazdunski (1972), as described in the Experimental section. In its simplest form, the interaction of enzyme and inhibitor, for reversible inhibition, may be written as



$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \qquad (1)$$

where k_{on} is the rate of association and k_{off} is the rate of dissociation for EI complexes. The association rates of BLPI with the aforementioned proteinases were determined under second order rate conditions where the rate was dependent on the concentration of both enzyme and inhibitor (Fig. 11). The rate of complex formation is given by the equation (Bieth, 1980):

$$\frac{d\,(E)}{dt} = k_{on}\,(E)\,(I) - k_{off}\,(EI) \qquad (2)$$

Since no complex dissociation was observed over the time course of each assay, the last term in equation (2) may be excluded. Under these conditions, k_{on} may be analysed by equation 3:

$$\frac{1}{[E]} = \frac{1}{[E_o]} + k_{on}(\text{time}) \qquad (3)$$

where $[E_o]$ is the initial (or total) enzyme concentration, [E] is unassociated (or free) enzyme, and t is time. Plots of 1/[E] versus time (Figure 11 insets) were linear and intercepted the 1/[E] axis at or near $1/[E_o]$, as expected. The slopes of these lines equalled the respective association rates (k_{on}). The dissociation rates of BLPI-proteinase complexes were determined by adding an equimolar amount of α_1-PI to previously formed (1:1) BLPI-proteinase complexes. Alpha-$_1$-PI forms irreversible complexes with dissociated proteinase and serves as an enzyme 'trap'. Samples of this mixture were assayed to determine the amount of free and enzyme-complexed α_1-PI. This assay is based on the observation that α_1-PI inhibits porcine pancreatic elastase on a mole/mole basis, whereas BLPI does not inhibit this enzyme. Plots of ln BLPI-proteinase complex (%) versus time (Figure 12) were linear with slopes equal to the respective dissociation rates (k_{off}). The association and dissociation rates are listed in Table 4 for comparison. Equilibrium dissociation constants (K_i) were calculated from the ratio of k_{off}/k_{on} (Table 4).

PRELIMINARY

ELISA FOR ALPHA-1-PROTEINASE

An enzyme-linked immunoassay (ELISA) was set up to quantitate α_1-PI in dilute solutions. In this competitive ELISA, 96-well microtiter plates are coated with pure α_1-PI (0.1 μg/ml) in 0.05 M carbonate buffer pH 9.6, at 4°C, 12 hours. All other binding sites are blocked with 1 mg/ml bovine serum albumin (BSA) in the same buffer (1 hour at room temperature). Unbound protein is removed by washing it with phosphate buffered saline (PBS) pH 7.0 containing 0.01%

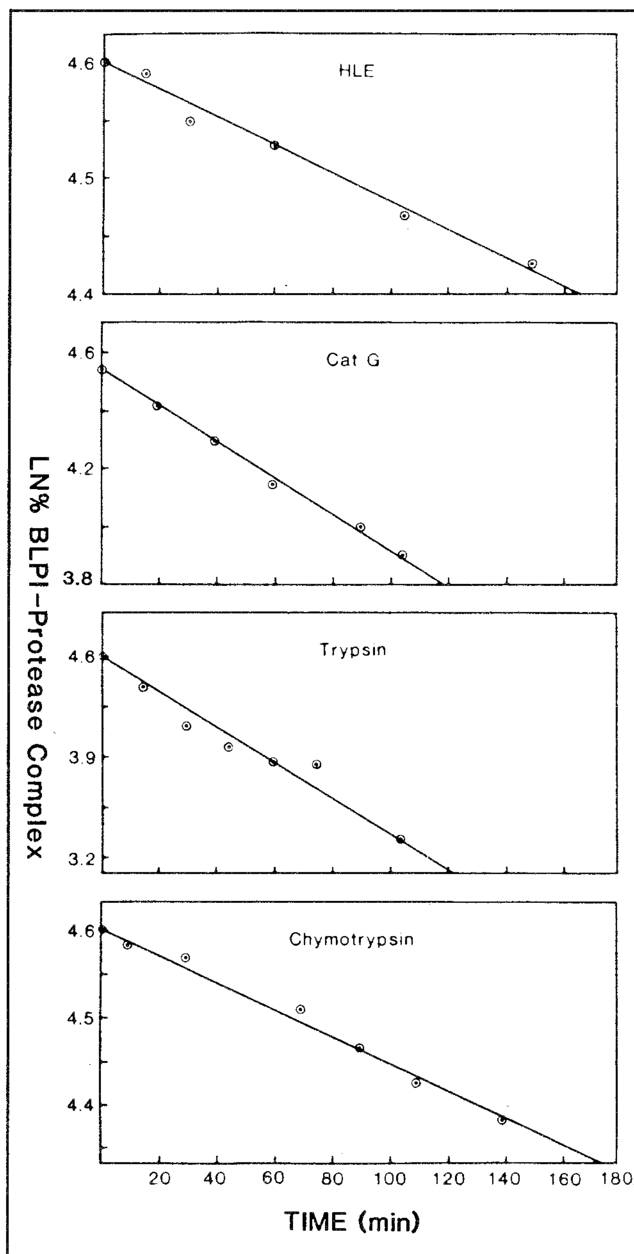


Figure 12. Dissociation rates for BLPI with various proteinases: Equimolar mixtures of BLPI and proteinase were incubated until zero proteinase activity was recorded, at which time an equal molar amount of α_1-PI was added. By measuring the inhibitory activity of α_1-PI against porcine pancreatic elastase in 50 μl aliquots of the reaction mixture, the percentage of BLPI-proteinase complex remaining over a period of time could be determined. Plots of ln [BLPI-proteinase complex (%)] vs. time were linear, with slopes equal to the respective dissociation rates (k_{off}). Enzymes: HLE; cathepsin G; trypsin; chymotrypsin.

Tween-20. Standards of various concentrations of α_1-PI (4,000 ng/well to 10 ng/well) or bronchoalveolar lavage fluid (BALF) are mixed with rabbit antisera to α_1-PI (1:2,000 dilution in PBS) and added to the wells. Plates are incubated for 3 hours at 30°C, during which time the unbound α_1-PI competes with bound α_1-PI for the antibody. The more α_1-PI in the standard or BALF, the less anti-α_1-PI is bound to the plate. After washing as above, alkaline phosphatase linked to goat anti-rabbit IgG (1:1,000 dilution) is added, which results in alkaline phosphatase being bound to the rabbit anti-α_1-PI. The plate is washed again and 200 μl of 0.1 M glycine buffer pH 10.4 containing 1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 0.25 mg/ml p-nitrophenylphosphate, (alkaline phosphatase substrate) is added to each well. Bound enzyme cleaves the substrate over 30 minutes to release yellow p-nitrophenol. The reaction is stopped by adding 50 μl of 3 M NaOH. The 405 nm absorbance of each well is read on a Titer-Tech plate reader. The samples containing the most α_1-PI in the competition step produce less color, since less anti-α_1-PI and, subsequently, less alkaline phosphatase, is bound to the plate.

This technique is being used to quantitate α_1-PI in bronchoalveolar lavage fluids from individuals exposed to NO_2. We are presently comparing this method with the rocket immunoelectrophoresis technique on the same samples.

IN VIVO EFFECTS OF NITROGEN DIOXIDE ON ALPHA-1-PROTEINASE INHIBITOR AND BLPI

These studies are being performed in collaboration with Dr. Mark Utell (University of Rochester). We have examined the bronchoalveolar lavage fluids (BALF) from volunteers exposed to 0.6 ppm NO_2 for 3 hours, followed by lung lavage. Control samples were obtained from the same subject after air exposure two weeks prior to or two weeks after the initial exposure. Because BALF is very dilute, these samples were concentrated by pressure dialysis on an Amicon YM-30 membrane. However, this procedure resulted in variable recoveries of protein. Rocket immunoelectrophoresis of these samples also gave varied results and attempts to measure elastase inhibitory activity were unsuccessful. Afford et al. (1985) recently showed that positive-pressure dialysis results in variable protein recoveries and considerable losses of elastase inhibitory activity, which explains some of the problems. Subsequently, 18 more BALF samples from 9 individuals exposed to peak NO_2 concentrations of 2 ppm were received. Protein assays were performed with unconcentrated samples. For elastase inhibition assays, the samples were concentrated 10-fold by lyophilization. The α_1-PI and BLPI concentrations of the unconcentrated samples were determined by ELISA and immunoblot assays.

IN VITRO EFFECTS OF NITROGEN DIOXIDE ON ALPHA-1-PROTEINASE INHIBITOR AND BLPI

Nitrogen dioxide exposure of α_1-PI resulted in a 35% loss of HNE inhibitory activity, which was linear from 200 moles NO_2/mole of α_1-PI to 800 moles NO_2/mole α_1-PI. Additional NO_2 exposure did not result in a further decrease in inhibitory activity. It should be noted that NO_2 did not affect PPE inhibitory activity as severely as HNE inhibitory activity; the opposite is the case with methionine-specific NCS oxidation. BLPI's HNE inhibitory activity was reduced to 50% of normal by NO_2, after which it too did not decrease with additional exposure. At present, it is known that tryptophan residues are destroyed. Effects of NO_2 on other amino acid residues are being studied, to better understand the mechanism of inactivation (Figure 13).

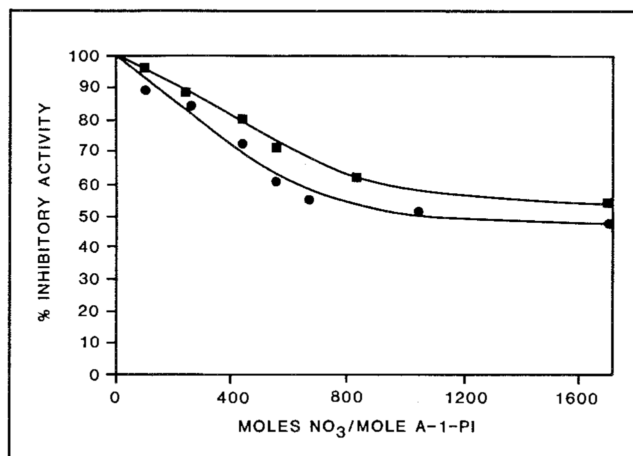


Figure 13. Effect of NO_2 on the elastase inhibitory activity of human α_1-PI and BLPI: α_1-PI (□—□) and BLPI (o—o), after exposure to the indicated amounts of NO_2, were incubated with human neutrophil elastase, and assayed for remaining elastase activity. Results are expressed as % inhibitory activity relative to unexposed control samples.

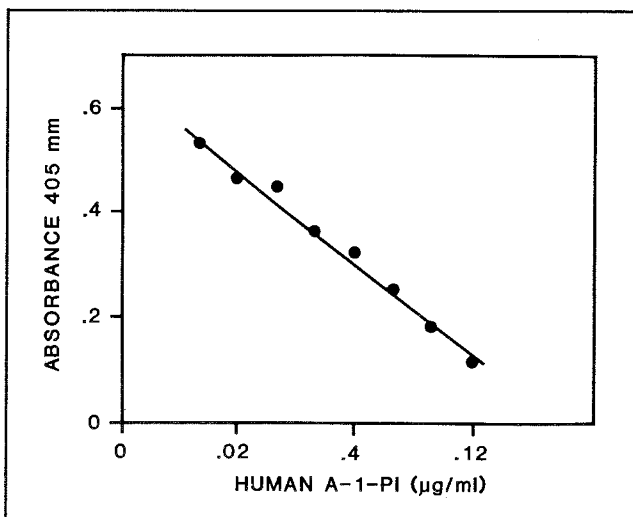


Figure 14. Competitive ELISA for α_1-PI: Dilution curve (3X) obtained with competitive ELISA for α_1-PI. Absorbence is plotted as a function of concentration (given vs. μg/ml α_1-PI). Concentration ranges from 12 μg/ml to 0.016 μg/ml. Correlation coef = 0.993.

DISCUSSION

IN VIVO EFFECTS OF OZONE ON BLOOD PLASMA ALPHA-1-PROTEINASE INHIBITOR

No difference was found in the ratios of total α_1-PI/total protein between O_3-exposed and control subjects. The statistically significant increase in this ratio with time may have been due to the trauma of venipuncture and/or exposure process, resulting in higher plasma concentrations of α_1-PI.

EIA/total α_1-PI ratios were used to measure the functional activity of α_1-PI in the plasma samples. The lack of any significant difference in this ratio between the two groups, or with time for the exposed group, showed that acute exposure to O_3 did not oxidatively inactivate plasma α_1-PI. The importance of constructing titration plots for the determination of EIA values was demonstrated by Figure 3, in which high concentrations of plasma did not inhibit PPE in a concentration dependent fashion. This effect probably resulted from the binding of PPE by alpha-2-macroglobulin, which would allow the bound enzyme to hydrolyze the synthetic substrate (Barrett, 1981). Why the mean EIA/total α_1-PI ratios exceeded the theoretical maximum of 1.0 is not entirely clear, but most likely, it results from an underestimation of total α_1-PI by rocket immunoelectrophoresis. Since there is no statistically significant difference in this ratio between the two groups, it does not affect the comparison of these ratios.

When the present study was begun, the published literature stated that inactive α_1-PI could be detected in the blood of cigarette smokers when compared to non-smokers (Beatty et al., 1981; Chowdury et al., 1982; Janoff et al., 1979). Since that time, two reports appeared that did not find a significant difference in the blood plasma activity of α_1-PI in smokers relative to non-smokers (Boudier et al., 1983; Cox and Billingsley, 1984). It seems that the present results also fail to support the hypothesis that inhaled oxidants directly inactivate α_1-PI, but plasma α_1-PI activity may not reflect the situation in the lung. The amount of lung α_1-PI exposed to inhaled oxidants is not easily estimated, but it probably represents a small percentage of the total circulating inhibitor. In addition, the rate of α_1-PI diffusion to and from the air space side of the lung is unknown, and this uncertainty complicates the understanding of this system. However, *in vivo* oxidation of lung α_1-PI might be favored because of lipid peroxidation (Pryor et al., 1982), phagocyte stimulation (Matheson et al., 1979; Weiss et al., 1983) or some yet unknown mechanism that might be potentiated by inhaling O_3.

In vitro experiments have shown clearly that α_1-PI is quite sensitive to oxidants, but reports of *in vivo* experiments examining this process are mixed. Initially, Gadek et al. (1979) found that the elastase inhibitory activity of human lung lavage fluid from smokers was reduced relative to non-smokers. At the same time, Janoff et al. (1979) found that cigarette smoke inactivated α_1-PI in the lungs of rats, and that the α_1-PI in serum samples from human smokers was 20% less active than that of normals. Subsequently, Carp et al. (1982) also found inactive α_1-PI in lung lavage from human smokers relative to non-smokers. Supporting these findings are the reports of Beatty et al. (1982) and Chowdhury et al. (1982) of reduced inhibitory activity in plasma samples from smokers. In contrast to these observations, Stone et al. (1983) found no difference in the activity of lung lavage α_1-PI from smokers versus non-smokers. Boudier et al. (1983) reported that the α_1-PI in lavage fluid from smokers and non-smokers was only about 50% active, but that there was no statistical difference between the two groups. This group also examined α_1-PI in the plasma of smokers and non-smokers and found it 100% active in both cases. Thus, some groups have presented data supporting the hypothesis that inhaled oxidants (cigarette smoke) inactivate α_1-PI *in vivo*, but data from other investigators failed to detect inactivation.

This question recently was discussed in *Science* (vol. 224, p. 755-763, 18 May 1984), by representatives from both sides of the issue, but the cause of these different findings was not immediately obvious. The varied results may be due to differences in lavage techniques, assay methods, and/or standards employed. Interestingly, the "pure" α_1-PI standard used by one group was only about 60% active, while our α_1-PI is consistently 100% active. One group (R. Abboud), which was supplied with α_1-PI from this laboratory, found that α_1-PI in bronchoalveolar lavage fluids from smokers was 90% active. Although the use of impure or partially inactive α_1-PI may cause some errors in quantitation, this should not affect the comparison of data from smokers versus non-smokers, but it does subject the data to question. All discussants felt that further studies were needed to clarify this situation.

Ozone is a far more reactive oxidant than cigarette smoke, and is a single molecule in contrast to the complicated mixture of components comprising cigarette smoke. Therefore, O_3 is an ideal oxidant for the study of α_1-PI inactivation *in vivo*. Simply put, if O_3 doesn't inactivate α_1-PI *in vivo*, it seems unlikely that other compounds will.

Detection of *in vivo* α_1-PI inactivation by O_3 may be difficult. The amount of α_1-PI exposed to O_3 via inhalation may be a small percentage of the total pool and, therefore, may not be easily quantitated by assaying blood plasma samples. The methods used to assay α_1-PI should have allowed the detection of a 4% change in α_1-PI inhibitory activity. Although no significant changes were observed, undetectable changes may have been as high as 4%. The amount of α_1-PI in lung surfactant is estimated to be 0.2% of that in the blood, which would make detection of changes in the activity of lung α_1-PI almost impossible to measure in blood plasma samples. Inactivation of α_1-PI also may occur indirectly via enzymatic oxidation by leukocyte myeloperoxidase (Matheson et al., 1979). Thus, the recruitment of PMN leukocytes to the lung would not only increase the elastase burden, but it also would contribute to α_1-PI inactivation. Other factors probably complicate the system further. For example, the rate at which lung α_1-PI equilibrates with the plasma is not known.

Although O_3 does not cause emphysema in animals, the lesion produced by O_3 is in the centriacinar region of the lung at the junction between the mucus and surfactant layers (Menzel, 1984). Emphysema due to smoking also is a centriacinar lesion, and this area of the lung probably has the lowest concentration of proteinase inhibitors. Blood plasma α_1-PI would be in surfactant, and BLPI is a mucus component.

IN VITRO EFFECTS OF OZONE ON LEUKOCYTE PROTEINASE INHIBITORS

Ozone is probably one of the most powerful oxidants encountered, the toxic effects of which, on men and animals, have been reviewed by Menzel (1984). Since the lung is the point of greatest exposure to the atmospheric environment, studing the effect of O_3 on lung proteins seemed appropriate. Oxidant inactivation of α_1-PI with cigarette smoke (Janoff and Carp, 1977) and phagocyte derived oxidants (Matheson et al., 1979) was suggested as an important step in the pathogenesis of emphysema. Ozonation of α_1-PI resulted in the total loss of inhibitory activity against HNE and the oxidation of Met, Trp, Tyr, and His residues. Specific oxidation of the inhibitory site Met is known to cause a 2,000-fold decrease in the association rate with HNE (Beatty et al., 1980), but preincubation of ozonated α_1-PI with HNE for 1,000 times the normal half-time for association did not cause any increase in HNE inhibition. Therefore, the inactivation caused by O_3 is due to the modification of additional amino acid residues, which may result in an altered conformation. Ozone previously was shown to inactivate α_1-PI (Johnson, 1980), but those exposures were performed in Tris buffered solutions, which react with O_3 (Pryor, 1985). The present study also examined O_3 effects on α_1-Achy, the sputum concentration of which is increased in individuals with chronic bronchitis (Ryley and Brogan, 1973). BLPI accounts for 70% of the elastase inhibitory activity of sputum (Hochstrasser et al., 1972), and recent data suggests that BLPI is the major elastase inhibitor in bronchoalveolar lavage fluid, as well (Idell et al., 1985; Stockley et al., 1984).

The inhibitory site of α_1-Achy contains a leucine rather than Met, which makes it resistant to mild oxidation (Beatty et al., 1980). It should be noted that exposure to O_3 only reduced α_1-Achy's Cat G inhibitory activity to 50% of normal. Maximum inactivation occurred at 50 moles of O_3 per mole of α_1-Achy, which is the same ratio that caused total inactivation of α_1-PI, but additional O_3 to 250 moles/mole of inhibitor did not result in further losses of inhibitory activity. This effect may be the result of minor conformational changes. However, such changes might be expected to influence association rates. Increasing enzyme-inhibitor preincubation periods to 1,000 times normal association half-times did not result in more inhibition. The Met and Trp residues of α_1-PI seem more accessible to O_3 than those of α_1-Achy, but the Tyr residues of α_1-Achy were more extensively oxidized than those of α_1-PI. The reasons for these differences are not clear, but may become obvious when the crystallographic structures of these two homologous inhibitors are known.

Both BLPI and Eglin C were inactivated by O_3 with the loss of HNE and Cat G inhibitory activities. The inactivation of both inhibitors was essentially linear to 30 moles of O_3 per mole of inhibitor, and very little decrease in inhibition occurred above 40 moles of O_3 per mole of BLPI and Eglin C, respectively. Amino acid analyses at these points showed extensive oxidation of Met, Trp, and Tyr in BLPI, which does not contain His. Although Eglin C does not contain Met or Trp, over 90% of its Tyr residues and 80% of its His residues were oxidized. Met and Trp are the most susceptible amino acids to O_3 oxidation (Mudd et al., 1969), and since Eglin C is lacking these two residues, we expected Eglin C to be resistant to inactivation by low concentrations of O_3. The O_3 inactivation plots for BLPI and Eglin C show similar rates of inactivation, even though their amino acid compositions are quite different. It would appear that O_3 damages proteins in nonspecific ways. The oxidation of several different amino acid residues disrupts protein structure. Although Eglin C does not have oxidizable residues in its inhibitory site, the oxidation of other residues was sufficient for inactivation. All four inhibitors were inactivated at similar rates, which suggests that O_3 exposure, rather than protein structure, is the rate limiting step. Additional studies with other proteins will be needed to see if this is a general rule or a mere coincidence. Conversely, BLPI contains seven disulfide bridges (Smith and Johnson, 1985), which might be expected to increase its stability. Disulfides obviously afford little, if any, protection from O_3, because α_1-PI and Eglin C, which lack disulfide bridges, were inactivated with about the same amount of O_3. Also, α_1-Achy does not contain disulfide bridges, but it was the most O_3-resistant inhibitor studied.

Methionine-specific modification of BLPI with NCS resulted in a greater loss of Cat G inhibitory activity than HNE inhibitory activity. At two moles of NCS per mole of protein Met, 80% of BLPI's activity against Cat G was lost, but only 40% of its HNE inhibitory activity was lost. Since two moles of NCS/mole Met only oxidizes surface Met residues (Schechter et al., 1975), these results indicate that Met is involved in the Cat G inhibitory site and suggest some role for Met in the HNE inhibitory site.

Antioxidants, at normal physiological concentrations, provided only minimal protection from O_3. Vitamin C, Trolox C, and mannitol (free radical scavengers) were more protective of α_1-PI than of BLPI, suggesting that free radical attack is a greater factor in α_1-PI inactivation than in BLPI inactivation. Trolox C, which repairs amino acid free radicals (Bisby, 1985), was the best protectant. These data indicate that vitamin E (α-tocopherol) may protect lung proteins from O_3 oxidation. Higher concentrations of Troxol C (100 μg/ml) resulted in 88% protection of α_1-PI (unpublished data), but this concentration was 10 times the equivalent physiological level of vitamin E. It should be pointed out that Trolox C is water

soluble and that *in vivo* fat-associated vitamin E may not protect α_1-PI. Vitamins C and E protected animals from O_3 (Menzel, 1984), but these effects are probably due to free radical- and lipid peroxide-mediated toxicity related to cell membrane oxidation.

The study's findings indicate that inhaled O_3 may damage the proteinase inhibitors of the lung, but the possible extent of this effect *in vivo* is difficult to estimate. Ozone reacts with other biological molecules and the inactivation of α_1-PI and BLPI by inhaled O_3 may be negligible. Previous *in vitro* studies showed the inactivation of α_1-PI in blood plasma requires about five times as much O_3 per mole of α_1-PI as compared to solutions of the pure inhibitor (Johnson, 1980). Thus, other plasma components afford some protection, but not as much as expected. No decrease in the inhibitory activity of blood plasma α_1-PI could be measured in individuals exposed to 0.5 ppm O_3 for four hours on two consecutive days (Johnson et al., 1986). Additional studies are needed to answer questions about the location of oxidized amino acids in relation to protein tertiary structure and the effects of oxidation on protein conformation. Such information might aid the design of oxidant resistant proteins.

INHIBITION KINETICS OF BLPI

Based on association rate data, there is little doubt that BLPI preferentially inhibits HNE relative to cathepsin G. The K_i determined for HNE with BLPI is one order of magnitude higher than the value of 1.2 X 10^{-11} M reported by Gauthier et al. (1982). In both cases, K_i was determined by calculating the ratio of k_{off}/k_{on}. The k_{on} determined by Gauthier et al. (1982) (1.1 X $10^7 M^{-1}s^{-1}$) is two orders of magnitude higher than the value obtained in this study, while the k_{off} is one order of magnitude lower than the previously reported value (1.3 X $10^{-4}s^{-1}$). These discrepancies may be explained as follows. The kinetic approach employed by Gauthier et al. (1982) involved competition experiments in which HNE was added to mixtures of α_1-PI and BLPI. This method was an adaptation of Vincent and Lazdunski's (1972) competition experiments employing one inhibitor and two enzymes. Attempts to apply the approach of Gauthier et al. (1982) for the measurement of BLPI-cathepsin G association rates resulted in failure because plots of log (a/a-x) versus log (b/b-y) (where a = initial concentration of α_1-PI, x = concentration of α_1-PI-cathepsin G complex, b = initial concentration of BLPI, and y = concentration of BLPI-cathepsin G complex) did not have a positive slope or pass through the origin as required by theory. Re-examination of the data presented by Gauthier et al. (1982) revealed a similar problem; i.e. plots of log (a/a-x) versus log (b/b-y) using their data yielded a line parallel to the log (a/a-x) axis. The inconsistency of these data with the theory is indicative of an erroneous result. These findings were suggestive of an association of porcine pancreatic elastase with BLPI, but no association could be detected under conditions such that the association rate of BLPI and porcine pancreatic elastase must be less than $10^2 M^{-1}s^{-1}$. The reason why the approach of Gauthier et al. (1982) failed is not immediately clear, but may be related to the competition of a reversible inhibitor (BLPI), with an irreversible one (α_1-PI), and/or the introduction of a second enzyme to measure the irreversible inhibitor (α_1-PI).

In contrast, Hochstrasser et al. (1981) have reported a K_i of 2.5 x 10^{-9}M for BLPI and HNE using the method of Green and Work (1953). This report also contained a K_i value of 2.7 x 10^{-8}M for BLPI and cathepsin G, which is one order of magnitude higher than the present result. Values of 4.0 x 10^{-9}M and 7.0 x 10^{-10}M were listed for bovine trypsin and chymotrypsin, respectively. The K_i of Hochstrasser et al. (1981) for bovine trypsin is in close agreement with present data, but the K_i for chymotrypsin obtained in the present study is two orders of magnitude higher than the previously reported value. These discrepancies seem to be due to the methods employed. Data resulting from the present studies are probably more correct because the approach of Vincent and Lazdunski (1972) is more rigorous than the method of Green and Work (1953), which is subject to considerable error at the equivalence point, and which was employed by Hochstrasser et al. (1981).

For an inhibitor to be physiologically efficient, two requirements must be met. First, k_{on} must be sufficiently fast to completely remove a potentially damaging proteinase from a susceptible tissue. The delay time of inhibition, as described by Bieth (1980), is the time required for complete inhibition of a proteinase *in vivo* and is given by the equation,

$$\text{delay time} = \frac{5}{k_{on}\,[I_o]} \qquad (4)$$

The BLPI concentration of sputum has been reported to be as high as 5.87 x 10^{-6}M (Kramps et al., 1984). Based on this concentration and determined association rates for HNE and cathepsin G, delay times of 8 seconds and 25 seconds can be calculated respectively for the control of these proteinases in the upper respiratory tract. The second requirement is that the complexes formed must be stable. For inhibitors such as α_1-PI and α_1-Achy, this requirement is easily fulfilled since these inhibitors bind covalently to their respective enzymes and are non-reversible (Travis and Salvesen, 1983). BLPI, however, exhibits reversible binding and, therefore, dissociation rates must be slow enough to provide a stable complex.

The half-time of dissociation ($t_{1/2,\,off}$) is given by the equation (Bieth, 1980):

$$t_{1/2\ off} = \frac{0.693}{k_{off}} \qquad (5)$$

Using the determined k_{off} values for HNE and cathepsin G the half-times of dissociation for these proteinase complexes are 10 hours and 2 hours, respectively. Fryksmark et al. (1983) have further documented the stability of the BLPI-HNE complex by showing that these complexes are stable for at least

24 hours. The proportion of free enzyme at equilibrium also is dependent on the ratio of $[E_o]/k_i$ (Bieth, 1974). Since BLPI concentrations generally are much greater than the amount of HNE and cathepsin G that may be present in the bronchial mucus (Ohlsson et al., 1983), $[E_o]$ may be replaced by $[I_o]$, and the overall reaction is essentially first order with respect to $[I_o]$ under these conditions. If the concentration of BLPI within the upper respiratory tract is 5.87×10^{-6}M, as reported by Kramps et al. (1984), the ratios of $[I_o]/K_i$ for HNE and cathepsin G are 3.14×10^4 and 1.40×10^3 respectively, which are more than sufficient for complete proteinase inhibition.

These results indicate that BLPI is primarily an HNE inhibitor, but when HNE is fully inhibited, BLPI will begin to control cathepsin G. The difference in the k_{on} of BLPI with HNE and cathepsin G may not be significant *in vivo* if the concentration of BLPI is sufficiently higher than the sum of HNE and cathepsin G concentrations in bronchial secretions. Present information indicates that this is the case under normal conditions, but the finding of free HNE in secretions from patients with chronic bronchitis (Ohlsson and Tegner, 1975) shows that the system of inhibitors (mainly BLPI) in the upper airways can become saturated. As the concentration of HNE approaches that of BLPI, cathepsin G would be free to attack tissues.

A previous report from this laboratory showed that the metalloelastase from *Pseudomonas aeruginosa* can catalytically inactivate BLPI (Johnson et al., 1982), which also may contribute to uncontrolled proteolysis, both directly and via inhibitor destruction. Additionally, Carp and Janoff (1980) found that cigarette smoke partially inactivates BLPI, but the significance of this is difficult to evaluate in the absence of kinetic data. BLPI is also susceptible to oxidative inactivation by O_3. Thus, a loss of BLPI activity in pathological situations could result in the degradation of host tissue proteins by HNE and cathepsin G.

The role of BLPI with respect to α_1-PI and α_1-Achy within the upper respiratory tract is not clear. The association rates of HNE and Cat G with α_1-PI and α_1-Achy, respectively (Beatty et al., 1980), are faster than with BLPI (the present data). However, the molar concentration of BLPI is approximately 10 times greater than the blood plasma inhibitor concentrations in the upper respiratory tract (Ryley and Brogan, 1973). Based upon the concentration data of Ryley and Brogan (1973) and the association rate data of Beatty et al. (1980), the delay times for inhibition of HNE and cathepsin G by α_1-PI and α_1-Achy in sputum are calculated to be 0.15 seconds and 0.45 seconds, respectively. Thus, the blood plasma inhibitors should be 55 times more efficient in controlling HNE and cathepsin G than BLPI under normal circumstances. However, during acute infection, both HNE and cathepsin G can increase to high levels (Ohlsson and Tegner, 1975), saturating the plasma inhibitors. Ohlsson et al. (1984) showed that BLPI is an acute phase reactant with serum levels reaching 10 times normal. Consistent with this report, Stockley et al. (1984) found that BLPI was responsible for 75% of the HNE inhibitory activity in bronchoalveolar lavage from patients with chronic bronchitis. These findings suggest that BLPI's physiological importance may be to inhibit these enzymes during periods of acute inflammation. Our dissociation rate studies, in agreement with those of Fryksmark et al. (1983), indicate that HNE and cathepsin G bound to BLPI would be transferred to α_1-PI and α_1-Achy, respectively.

Additionally, BLPI may aid the plasma proteinase inhibitors in controlling proteinases bound to their protein substrates. Reilly and Travis (1980) showed that α_1-PI would not fully inhibit HNE pre-absorbed to elastin. Hornebeck and Schnebli (1982) found that the low M_r inhibitor, from the leech, known as Eglin C (M_r 8,100), would inhibit HNE even after pre-absorption of the proteinase to elastin. By analogy BLPI, which is also of low M_r, may serve to inhibit leukocyte proteinases bound to their substrates.

The improved isolation method for BLPI should facilitate additional study of this inhibitor in other secretions and tissues, as well as in bronchial mucus. The kinetic data obtained in this study are intended to provide a benchmark for future experiments designed to examine the effects of amino acid modification, including oxidation, on the function of BLPI.

REGULATORY SIGNIFICANCE

These studies were not designed to provide data for use in setting regulatory standards for O_3 and NO_2. Instead, this project focused on the use of α_1-PI activity as a marker of oxidant injury and the mechanism of O_3 and NO_2 oxidation of protein amino acid residues.

While we had hoped to use the specific activity of blood plasma α_1-PI as a marker for O_3 exposure, our data show that this is not possible. The major difficulty seems to be the tremendous dilution of lung α_1-PI when (and if) it diffuses back into the blood. The activity of α_1-PI may still be a good marker for examining bronchoalveolar lavage fluids. Additionally, BLPI also may serve as a marker.

From a mechanistic viewpoint, our results show that O_3 and NO_2 will damage α_1-PI and BLPI. Ozone oxidation of protein amino acids apparently depends on the three dimensional structure of the protein and its amino acid compositions. These findings suggest that oxidant damage of α_1-PI and BLPI may allow proteinases to attack lung connective tissue proteins, but the extent of such a process is impossible to predict at present. Because emphysema is a slowly developing, chronic disease, its etiology is not easily studied. Although cigarette smoking is clearly linked to the development of emphysema, the components of smoke responsible for disease development have not been identified. If inhaled oxidants are involved, as suggested by present knowledge, the study of O_3 and NO_2 should yield important insights concerning this process. Such investigations also may provide the insight needed to intervene and slow the development of emphysema and similar diseases.

REFERENCES

Afford SC, Stockley RA, Kramps JA, Dijkman JH, Burnett D, 1985. Concentration of bronchoalveolar lavage fluid by ultrafiltration: Evidence of differential protein loss and functional inactivation of proteinase inhibitors. Anal Biochem ; 151:125-30.

Baici A, Seemuller U, 1984. Kinetics of the inhibition of human leucocyte elastase by Eglin from the Leech *Hirudo Medicinalis*. Biochem J; 218:829-33.

Baugh R, Travis J, 1976. Human leukocyte granulocyte elastase: rapid isolation and characterization. Biochemistry; 15:836-41.

Beatty K, Bieth J, Travis J, 1980. Kinetics of association of serine proteinases with native and oxidized α-1-proteinase inhibitor and α-1-antichymotrypsin. J Biol Chem; 225:3931-4.

Beatty K, Robertie P, Senior RM, Travis J, 1982. Determination of oxidized alpha-1-proteinase inhibitor in serum. J Lab Clin Med; 100:186-92.

Bieth JG, 1980. Pathophysiological interpretation of kinetic constants of protease inhibitors. Bull Europ Physiopath Resp; 16 (suppl.): 183-95.

Bieth J, Speiss B, Wermuth C, 1974. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem Med; 11:350-7.

Bisby R, Ahmed S, Cundall R, 1984. Repair of amino acid radicals by a vitamin E analogue. Biochem Biophys Res Comm; 119:245-51.

Boudier C, Pelletier A, Pauli G, Bieth JG, 1983. The functional activity of alpha-1-proteinase inhibitor in bronchoalveolar lavage fluids from healthy human smokers and non-smokers. Clin Chem Acta; 132:309-15.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem; 72:248-54.

Bundy HF, 1963. A new spectrophotometric method for the determination of chymotrypsin activity. Arch Biochem Biophys; 102:416-22.

Carp H, Janoff A, 1978. Possible mechanisms of emphysema in smokers: in vitro suppression of serum elastase-inhibitory capacity by fresh cigarette smoke and its prevention by antioxidants. Am Rev Respir Dis; 118:617-21.

Carp H, Janoff A, 1980. Inactivation of bronchial mucous proteinase inhibitor by cigarette smoke and phagocyte-derived oxidants. Ex Lung Res; 1:225-37.

Casslen B, Rosengren M, Ohlsson K, 1981. Localization and quantitation of a low molecular weight proteinase inhibitor, antileuko protease, in the human uterus. Hoppe-Seyler's Z Physiol Chem; 362:953-61.

Castillo MJ, Nakajima K, Zimmerman M, Powers JC, 1979. Sensitive substrate for human leukocyte and porcine pancreatic elastase: A study of the merits of various chromophonic and fluorogenic leaving groups in assays for serine proteases. Anal Biochem; 99:53-64.

Chase T, Shaw E, 1967. p-Nitrophenyl-p'-guanidinobenzoate HCl: an active site titrant for trypsin. Biochem Biophys Res Commun; 24:508-14.

Chowdhury P, Bone RC, Louria DB, Rayford PL, 1982. Effect of cigarette smoke on human serum trypsin inhibitory capacity and antitrypsin concentrations. Am Rev Respir Dis; 126:177-9.

Cunningham LW, 1954. Molecular and kinetic properties of crystalline diisopropyl phosphoryl trypsin. J Biol Chem; 211:13-19.

Dooley M, Pryor W, 1982. Free radical pathology: inactivation of human α-1-proteinase inhibitor by products from the reaction of NO_2 with H_2O_2 and the etiology of emphysema. Biochem Biophys Res Comm; 106:981-7.

EPA, 1981. Sodium arsenite method for the determination of nitrogen dioxide in the atmosphere. NO. EQN-1277-026.

Erlanger BF, Kokowski N, Cohen W, 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys; 95:271-8.

Freund RJ, Littell RC, 1981. SAS for linear models: A guide to the ANOVA and GLM procedures. Cary North Carolina: SAS Institute, Inc.

Fryksmark U, Ohlsson K, Rosengren M, Tegner H, 1983. Studies of the interaction between leukocyte elastase, antileukoproteinase and the plasma proteinase inhibitors alpha-1-proteinase inhibitor and alpha-2-macroglobulin. Hoppe-Seyler's Z Physiol Chem; 364:793-800.

Gadek JE, Fells GA, Crystal RG, 1979. Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. Science; 204:1315-6.

Gauthier F, Fryksmark U, Ohlsson K, Bieth JG, 1982. Kinetics of the inhibition of leukocyte elastase by the bronchial inhibitor. Biochim Biophys Acta; 700:178-83.

Green NM, Work E, 1953. Pancreatic trypsin inhibitor: II. Reaction with trypsin. Biochem J; 54:347-56.

Gross P, Babyak M, Tolker E, Kaschak M, 1964. Enzymatically produced emphysema. J Occup Med; 6:481-4.

Guilbault GG, Froelich PM, 1973. Rapid, sensitive and selected assay for tryptophan in serum. Clin Chem; 19:1112-4.

Habeeb AFSA, 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. Meth Enzymol; 25:457-64.

Hochstrasser K, Albrecht JG, Schonberger LO, Rasche B, Lempart K, 1981. An elastase specific inhibitor from human bronchial mucus: isolation and characterization. Hoppe-Seyler's Z Physiol Chem; 362:1369-75.

Hochstrasser K, Reichert R, Schwarz S, Werle E, 1972. Isolierung und charakterisierung eines protease inhibitors aus menschlichem bronchialskpet. Hoppe Seyler's Z Physiol Chem; 353:221-6.

Hornbeck W, Schnebli HP, 1982. Effect of different elastase inhibitors on leukocyte elastase pre-adsorbed to elastin. Hoppe-Seyler's Z Physiol Chem; 363:455-8.

Hugli T, Moore S, 1972. Determination of tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysates. J Biol Chem; 247:2828-34.

Janoff A, Carp H, Lee DK, Drew RT, 1979. Cigarette smoke inhalation decreases α_1-antitrypsin activity in rat lung. Science; 206:1313-4.

Janoff A, Chan SK, Abboud RT et al, Stone PJ, 1984. Is α_1-inhibitor inactivated by smoking? Science; 224:755-63.

Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P, 1977. Experimental emphysema induced with purified human neutrophil elastase: Tissue localization of the instilled protease. Am Rev Respir Dis; 115:461-78.

Jeppsson JO, Laurell C-B, Fagerhol M, 1978. Properties of isolated human alpha$_1$-antitrypsins of Pi types M, S and Z. Eur J Biochem; 83:243-53.

Johnson D, 1980. Ozone inactivation of human alpha-1-proteinase inhibitor. Am Rev Respir Dis; 121:1031-8.

Johnson D, Travis J, 1978. Structural evidence for methionine at the reactive site of human α-1-proteinase inhibitor. J Biol Chem; 263:7142-4.

Johnson D, Travis J, 1979. The oxidative inactivation of human alpha-1-proteinase inhibitor: further evidence for methionine at the reactive center. J Biol Chem; 254:4022-6.

Johnson DA, 1983. A procedure for the separation and quantitation of tryptophan and amino sugars on the amino acid analyzer. Anal Biochem; 130:475-80.

Johnson DA, Carter-Hamm B, Dralle WM, 1982. Inactivation of human bronchial mucosal proteinase inhibitor by *Pseudomonas aeruginosa* elastase. Am Rev Respir Dis; 126:1070-3.

Kang S-H, Fuchs MS, 1973. An improvement in the human chymotrypsin assay. Anal Biochem; 54:262-5.

Kramps J, Franken C, Dijkman JH, 1984. ELISA for quantitative measurement of low-molecular-weight bronchial protease inhibitor. Am Rev Respir Dis; 129:959-63.

Laurell CB, Eriksson S, 1963. The electrophoretic α-1-globulin pattern of serum in α-1-antitrypsin deficiency. Scand J Clin Lab Invest; 15:132-40.

Marsh SC, Parikh I, Cuatrecabas P, 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal Biochem; 60:149-52.

Martodam R ,Baugh R, Twumasi D, Liener I, 1979. A rapid procedure for the large scale purification of elastase and cathepsin G from human sputum. Prep Biochem; 9:15-31.

Menzel DB, 1984. Ozone: An overview of its toxicity in man and animals. J Toxicol Envir Health; 13:183-204.

Monthony JF, Wallace EG, Allen DM, 1978. A non-barbitol buffer for immunoelectrophoresis and zone electrophoresis in agarose gels. Clin Chem; 24:1825-7.

Morii M, Travis J, 1983. Amino acid sequence of the reactive site of human alpha-1-antichymotrypsin. J Biol Chem; 258:12749-52.

Mudd JB, Leavitt AO, McManus TT, 1979. Reaction of ozone with amino acids and proteins. Atmosph Environ; 3:669-82.

Ohlsson K, Tegner H, Akesson U, 1977. Isolation and partial characterization of a low molecular weight acid-stable protease inhibitor from human bronchial secretion. Hoppe Seyler's Z Physiol Chem; 358:583-5.

Pryor WA, Dooley M, 1985. Inactivation of human alpha-1-proteinase inhibitor by cigarette smoke: Effect of smoke phase and buffer. Am Rev Respir Dis; 131:941-3.

Reilly CF, Travis J, 1980. The degradation of human lung elastin by neutrophil proteinases. Biochim Biophys Acta; 621:147-57.

Ryley HC, Brogan TD, 1973. Quantitative immunoelectrophoretic analysis of the plasma proteins in the sol phase of sputum from patients with chronic bronchitis. J Clin Pathol; 26:852-6.

Schiessler H, Hochstrasser K, Ohlsson K, 1978. Acid-stable inhibitors of granulocyte neutral proteases in human mucus secretion: biochemistry and possible biological function. In: Havemann K, Janoff A, eds. Neutral proteases of human polymorphonuclear leukocytes. Munich: Urban and Schwarzenberg, Inc. pp. 195-207.

Shechter Y, Burnstein Y, Patchornik A, 1975. Selective oxidation of methionine residues in proteins. Biochemistry; 14:4497-503.

Shotton DM, 1970. Elastase. In: Perlmann, GE, Lorand, L, eds. Methods in enzymology: proteolytic enzymes. New York: Academic Press, Vol 19, pp. 113 – 39.

Sloan B, Abrams WR, Meranze DR, Kimbel P, Weinbaum G, 1981. Emphysema induced *in vitro* and *in vivo* in dogs by a purified elastase from homologous leukocytes. Am Rev Respir Dis; 124:295-301.

Smith CE, Johnson DA, 1985. Human bronchial leucocyte proteinase inhibitor: Rapid isolation and kinetic analysis with human leucocyte proteinases. Biochem J; 225:463-72.

Stockley RA, Morrisson HM, Smith S, Tetley T, 1984. Low molecular mass bronchial proteinase inhibitor in sputum and bronchoalveolar lavage. Hoppe-Seyler's Z Physiol Chem; 365:387-95.

Stone PJ, Calore JD, McGowan SE, Bernardo J, Snider GL, Franzblau C, 1983. Functional α_1-protease inhibitor in the lower respiratory tract of cigarette smokers is not decreased. Science; 221:1187-89.

Thomas, J, Hodes, M, 1981. A new discontinuous buffer system for the electrophoresis of cationic proteins at near neutral pH. Anal Biochem; 118:194-196.

Travis J, Bowen J, Baugh R, 1978. Human alpha-1-antichymotrypsin: Interaction with chymotrypsin-like proteinases. Biochemistry; 17:5651-6.

Travis J, Johnson D, 1981. Human alpha-1-proteinase inhibitor. Meth in Enzymol; 80:754-765.

Travis J, Liener I, 1965. The crystallization and partial characterization of porcine trypsin. J Biol Chem; 240:1962-66.

Travis J, Morie M, 1981. Human alpha-1-antichymotrypsin. Meth in Enzymol; 80:765-74.

Travis J, Salveson GS, 1983. Human plasma proteinase inhibitors. Ann Rev Biochem; 52:655-709.

Vincent JP, Lazdunski M, 1972. Trypsin-pancreatic trypsin inhibitor association: Dynamics of the interaction and role of disulfide bridges. Biochemistry; 11:2967-77.

Wilcox PE, 1970. Chymotrypsinogens-Chymotrypsins. Meth in Enzymol; 19:64-108.

Winer BJ, 1971. Statistical Principles in Experimental Design. 2nd ed. New York: McGraw-Hill.

HEALTH REVIEW COMMITTEE'S REPORT

JUSTIFICATION FOR THE STUDY

In order to obtain a better assessment of human exposure to mobile source emissions, either levels of contaminants or relevant reaction products in body fluids and tissues can be measured, in addition to environmental monitoring. Research that improves our ability to accurately detect injury and damage should also be pursued. Because of their accessibility, further developments are needed to improve the analysis of body fluids (blood, urine, saliva, and broncho-pulmonary secretions) in order to quantify exposure to toxic agents as well as subsequent adverse effects. In addition, early indicators of damage could be used to monitor human populations and to identify sensitive individuals who deserve special protection.

An imbalance of proteinase-antiproteinase activity may contribute to the onset of emphysema. Dr. Johnson proposed to study the effects of oxidants on the human proteolytic enzyme inhibitor system. *In vivo* studies on the functional activity of circulating alpha-1-proteinase inhibitor in humans exposed to oxidants may provide a quantitative measure of risk to lung proteolysis.

In addition to exploring a potential marker of oxidant injury, Dr. Johnson's studies should enrich our understanding of the proteinase-antiproteinase hypothesis. First, *in vivo* confirmation of *in vitro* studies showing inhibitor inactivation by oxidant air pollutants is lacking. Second, the *in vitro* studies are designed to probe the molecular mechanisms by which inhibitors can be inactivated. The use of human proteins (e.g., neutrophil elastase and cathepsin G) should aid in relating these studies to normal *in vivo* function. Finally, additional analysis of bronchial leukocyte proteinase inhibitor is necessary to determine whether or not this inhibitor contributes to the lung's capacity to resist proteolytic attack.

OBJECTIVES OF THE PROPOSED STUDY

The primary goal of Dr. Johnson's project was to assay the functional activity of the proteinase inhibitors, alpha-1-proteinase and bronchial leukocyte proteinase, after exposure of humans to low levels of oxidants. The underlying purpose of these studies was to determine whether or not proteinase inhibitor activity could be used as a marker of oxidant exposure. Additional *in vitro* studies were proposed in order to explore mechanisms of inactivation.

The original aims of the investigator were to:

1. Detect the reduced capacity of serum alpha-1-proteinase inhibitor to inhibit human leukocyte elastase and porcine elastase in human subjects exposed to ozone, nitrogen dioxide, and sulfur dioxide.
2. Determine and define the mechanism of inhibitor inactivation by nitrogen dioxide and ozone *in vitro*.
3. Determine whether bronchial leukocyte proteinase inhibitor is inactivated by nitrogen dioxide and ozone *in vitro*, and if so, by what mechanism.

DESCRIPTION OF THE STUDY DESIGN

Objective #1: For the ozone studies, male volunteers (nonsmokers) were exposed to 0.5 ppm ozone for four hours on two consecutive days. Control subjects were exposed to air. Blood was sampled prior to, and immediately following, each exposure. With respect to nitrogen dioxide and in contrast to the ozone studies, bronchoalveolar fluid was sampled. Initially, volunteers were exposed to 0.6 ppm nitrogen dioxide for three hours and were lavaged at the cessation of exposure. Procedural difficulties have necessitated the repetition of the nitrogen dioxide studies, using peak concentrations of 2 ppm.

Objective #2: Alpha-1-proteinase inhibitor, alpha-1- antichymotrypsin, bronchial leukocyte proteinase inhibitor, and Eglin C, all of which have similar functions but different structures, were exposed to ozone *in vitro* and molecular alterations evaluated. In addition, antioxidants with different protective roles were added to alpha-1-proteinase inhibitor or bronchial leukocyte proteinase inhibitor, and the solution exposed to ozone.

Objective #3: Inactivation of bronchial leukocyte proteinase inhibitor by ozone or nitrogen dioxide was evaluated *in vitro*. In addition, kinetic studies of bronchial leukocyte proteinase inhibitor with human neutrophil elastase and cathepsin G were performed to assess the inhibitor function of this molecule relative to alpha-1-proteinase.

TECHNICAL EVALUATION

ATTAINMENT OF STUDY OBJECTIVES

The studies of sulfur dioxide were deleted at the onset of this project after discussions between the investigator and the HEI. With regard to the effects of ozone in humans *in vivo* and on different antiproteinase species *in vitro*, these objectives have been largely accomplished by suitable methodology. Using different antiproteinases, additional comparative studies that analyze the mechanisms of oxidative inactivation have been performed, and are clearly and appropriately described in the final report.

Studies of the effects of nitrogen dioxide *in vivo* are currently in progress, and are focused on alpha-1-proteinase inhibitor and bronchial leukocyte proteinase inhibitor activity in human bronchoalveolar lavage fluid. *In vitro* nitrogen

dioxide studies were conducted to compare differences in substrate inhibition by oxidized inhibitor. Amino acid analysis of oxidized inhibitor is currently underway in order to better understand the mechansims of inactivation. Work that is ongoing, and for which data were not presented in the investigator's final report, will be reported in the open literature and will not be evaluated in the HEI Health Review Committee Report.

RESULTS AND INTERPRETATION

• *In Vivo* Studies

Dr. Johnson was unable to detect any differences in serum alpha-1-proteinase inhibitor content or activity between ozone- or air-exposed subjects. At the time these studies were undertaken, it had been reported that significant amounts of oxidized alpha-1-proteinase inhibitor were present in the blood of smokers. It was, therefore, reasonable to investigate whether or not oxidized alpha-1-proteinase inhibitor was present in the blood of volunteers who had inhaled ozone, a powerful oxidizing agent. As the investigator points out in his final report, the amount of alpha-1-proteinase inhibitor present in the lung represents only a very small amount relative to the quantities circulating in the blood. Consequently, it might have been expected that dilutional effects would make detection of oxidized alpha-1-proteinase inhibitor in blood very difficult, even if all of the oxidized molecules diffused from the lung into the blood. In retrospect, knowing that at least 50 moles of ozone are required to inactivate one mole of purified alpha-1-proteinase inhibitor *in vitro* and considering the relative molar amounts of the blood protein and inhaled ozone, it seems unlikely that a significant drop in circulating functional inhibitor would be found. Although the difficulty in quantifying oxidized inhibitor could have been predicted based on such calculations, Dr. Johnson's results do provide experimental confirmation of the inability to use serum levels of alpha-1-proteinase inhibitor as a marker of ozone exposure.

Although it appears clear from Dr. Johnson's raw data that differences between air- and ozone-exposed groups in serum alpha-1-proteinase inhibitor activity were not measurable, the choice of the statistical analysis of the data can be questioned. The fixed subject effect model used for the analysis of variance is inappropriate. Since the volunteers had been assigned randomly to the exposure groups and were assumed to be representative of a larger population, the estimation of variance should have taken into account individual variability, which would occur randomly about each measurement. Furthermore, the question of a possible interaction of exposure with the change over time in blood inhibitor levels was not analyzed. If this interaction is significant, the Newman-Kuels multiple comparison is not the proper test. If there were no interactions between the patterns of response over time, the Newman-Kuels procedure provides no information about the form of the time response curve, which could be of interest.

It is conceivable that oxidation of alpha-1-proteinase inhibitor occurs in the lung but is not detectable in the blood with available methodology. It may be possible, however, to detect an excess of oxidized alpha-1-proteinase inhibitor, as well as bronchial leukocyte proteinase inhibitor, in bronchoalveolar lavage fluid after the inhalation of oxidant gases. Such studies are currently in progress on humans exposed to nitrogen dioxide. The description of these studies is presented in the investigator's final report; however, because these experiments are incomplete, the data cannot be evaluated. It should be noted, however, that dilutional effects may still present a problem with bronchoalveolar lavage fluid. Even if oxidation of proteinase inhibitors occurred within microenvironments of the lung, it may be difficult to measure this change in bronchoalveolar lavage fluid.

• *In Vitro* Studies

Dr. Johnson's *in vitro* studies of inhibitor exposure to ozone and nitrogen dioxide are of interest and potential importance. He has confirmed that bronchial leukocyte proteinase inhibitor is a significant inhibitor of human neutrophil elastase and that it is inactivated by ozone and nitrogen dioxide. The kinetic data provided may be used to evaluate the potential role of this inhibitor relative to other inhibitors.

Analysis of ozone-exposed inhibitors suggests that the inhibition of proteolytic enzymes depends on more than just the integrity of the amino acid at the active site of interaction. In addition to methionine, tryptophan, tyrosine, and histidine were oxidized. The susceptibility of these amino acids to oxidation appears to relate to their location within the three-dimensional structure of the protein. Results from the antioxidant experiments imply that ozone damage results from direct attack of the inhibitor protein.

FUTURE RESEARCH

Additional studies of the effects of oxidant gases on particular amino acid residues in inhibitor molecules might eventually lead to the detection of small quantities of these damaged molecules, and could provide an improved approach over measuring decreases in the functional properties of an abundant protein. For example, in this report, the alteration of tryptophan was monitored by a decrease in its 340 nm fluorescence emission peak. The question could be asked as to whether or not a concomitant increase in some other marker, representing the oxidized tryptophan, occurred. Furthermore, could this marker be used quantitatively to monitor the appearance of relatively small amounts of oxidized tryptophan? This approach might provide a useful bioassay for exposure to ozone or nitrogen dioxide. For example, after exposure to a known concentration of ozone, alpha-1-proteinase inhibitor might be isolated from the blood, and the amount of oxidized tryptophan assessed and related to the "dose" of ozone. The methods for such an assessment may not yet exist; nevertheless, it seems appropriate that studies in this direction be considered.

NEW INSIGHTS

Dr. Johnson's project provides some useful, new information that will refine details of the proteinase-antiproteinase hypothesis. His results not only add to our knowledge of mechanisms of oxidant damage to proteins, but they also broaden our perspective on the molecular relationship between proteolytic enzymes and inhibitors. Oxidation of susceptible amino acids other than the active site residue results in a loss of inhibitor function. Biochemical analysis of this sort, when coupled to the knowledge about the three-dimensional structure of inhibitor molecules, could provide insights about the specificity of inhibitors for different enzymes and the susceptibility of these molecules to damage. In addition, these studies demonstrate the relative ease with which *in vitro* data are generated, and the much greater difficulties encountered when trying to demonstrate the *in vivo* significance of *in vitro* observations; this is a major problem facing investigators attempting to validate the proteinase-antiproteinase hypothesis. The new knowledge derived from Dr. Johnson's studies, however, has *no* direct or immediate impact on risk assessment of oxidant exposure, nor has it any implications for public policy.

CONCLUSIONS

Dr. Johnson's studies have been carefully executed, and the results follow from the methods and the study design. Data are illustrated clearly in the graphs and tables. The conclusions and interpretations are reasonable and coincide with the experimental data. It does not appear feasible to use functional activity of alpha-1-proteinase inhibitor in the blood as a marker of ozone exposure or injury. Research work is continuing in an appropriate direction to study the effects of the oxidant nitrogen dioxide locally in the lung through measurements of proteinase inhibitors in bronchoalveolar lavage fluid. This approach, of course, does have its difficulties in that small amounts of proteinase inhibitors are diluted in the bronchoalveolar lavage fluid. The studies of oxidant gas alterations of specific amino acid residues in the inhibitor molecules might eventually be useful in the detection of small amounts of molecular damage. This, in turn, might provide a useful bioassay for exposure to ozone or nitrogen dioxide. In addition to being used for the identification of exposure markers, molecular analysis of oxidant-damaged inhibitors could be applied to improved understanding of the proteinase-antiproteinase hypothesis. Such two-pronged studies about the effects of oxidants on proteinase inhibition in bronchoalveolar lavage fluids and in *in vitro* systems is an appropriate and potentially productive approach.

ABOUT THE AUTHOR

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