EFFECTS OF OZONE ON PROTEASES AND PROTEASE INHIBITORS OF THE HUMAN AND RAT LUNG

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ABSTRACT

Animal studies have clearly shown that 0_3 has the potential to cause irreversible connective tissue alteration in the form of pulmonary fibrosis. However, it is not known if exposure of humans to ambient Southern California levels of 0_3 in a chronic episodic pattern is likely to cause connective tissue damage. The objective of this project was to perform a detailed analysis of the biochemical events that are believed to precede connective tissue disruption, i.e. changes in connective tissue proteases and protease inhibitors, in the lung lavage fluid of animals episodically exposed to moderate-to-low levels of 0_3 over long periods of time. Similar measurements were made on the lung lavage fluid of humans following a single exposure to near ambient levels of 0_3. Since nitric acid ($HN0_3$) also has oxidant properties, the effect of $HN0_3$ exposure (alone and in combination with 0_3) on lung lavage fluid protease activity was also examined.

An important feature of this project is that the lung lavage fluid samples used in most of these studies were obtained from three other ARB-funded studies. The use of lavage fluid samples from other studies resulted in considerable savings of resources for this project and allowed the animal use of the previously funded studies to be maximized. The principal investigators generously agreed to supply surplus lung lavage fluid from both chronic animal and acute human exposures. In addition, a short-term exposure of rats was used to aid in extrapolation of the results from the acute human exposures to the chronic animal exposures. The 0_3 concentrations ranged from 0.15-0.40 ppm. An episodic exposure pattern consisting of 4 hrs of exposure/day on 3 consecutive days per week was used for all the animal exposure studies. The duration of the episodic exposure studies ranged from 1-40 weeks. The specific endpoints that were examined and characterized in lung lavage fluid were protein content, elastase-like activity, collagenase activity, and neutrophil elastase inhibitory capacity. In addition, lung lavage fluid was also assayed for low molecular weight elastase inhibitors.

Acute, subacute, and chronic episodic exposure to 0_3 did not result in increased levels of free neutrophil elastase or collagenase in lung lavage fluid even though acute 0_3 exposure was associated with increased numbers of neutrophils in the lavage fluid. In contrast, acute exposure of humans and rats to 0_3 resulted in substantial increases in the elastase inhibitory capacity of lavage fluid. Although this result may seem to indicate that acute 0_3 exposure had a beneficial effect on the protease/antiprotease balance, the increase in elastase inhibitory capacity was caused by increased lung permeability and serum transudation and thus is indicative of lung injury. Acute exposure of humans to 0_3 had no effect on the activity of the low molecular weight elastase inhibitors. Acute exposure studies in humans indicated that sequential exposure to $HN0_3$ followed by 0_3 did not enhance the 0_3-induced increases in lavage fluid protein or elastase inhibitory capacity.

Chronic and subchronic episodic exposure of laboratory animals to 0_3 had no effect on any of the parameters measured. These negative results may be due in part to the low concentration of 0_3 and the relative insensitivity of animals exposed at rest (as opposed to humans exposed during exercise) to the effects of 0_3 exposure. However, a concentration of 0_3 that was only slightly higher than that used in some of the chronic studies had striking effects on lavage fluid parameters during a short-term one week exposure. Thus, it is likely that attenuation of sensitivity (adaptation response) occurred during the longer exposures despite the episodic nature of the exposure.

EXECUTIVE SUMMARY

I. BACKGROUND

Exposure to O_3 is known to cause connective tissue damage in the form of pulmonary fibrosis. It is not known if exposure of humans to ambient Southern California levels of O_3 in a chronic episodic pattern can cause connective tissue damage. In this study, the biochemical events that precede connective tissue damage, specifically the balance of proteases and protease inhibitors, was monitored in humans after a single O_3 exposure and in rats that were episodically exposed to low levels of O_3 for many months.

II. METHODS

Fischer 344 rats and rabbits were exposed to O_3 in an episodic pattern consisting of 4hrs of exposure per day for 3 consecutive days. Human exposures to O_3 were for 4 hrs with exercise. Following the exposures, the lungs of human subjects and animals were lavaged using well-established procedures and the lavage fluid was used in the analysis of proteases and protease inhibitors.

III. RESULTS

No increase in the levels of elastase or collagenase were detected in lung lavage fluid from animals and humans in acute subchronic or chronic exposure to O_3. A substantial increase in elastase inhibitory capacity was seen in acute human and animal exposures to O_3, although chronic animal exposures resulted in no increase in elastase inhibitory capacity. No increase in low molecular weight antiproteases was seen in the acute human exposures. No effect was seen in the animal lungs on any of the measured parameters after chronic or subchronic episodic exposure to O_3.

IV. CONCLUSIONS

Acute, subacute, and chronic episodic exposure to O_3 did not result in increased levels of free neutrophil elastase or collagenase in lung lavage fluid even though acute O_3 exposure was associated with increased numbers of neutrophils in the lavage fluid. Acute exposure of humans and rats to O_3 resulted in substantial increases in the elastase inhibitory capacity of lavage fluid. Although this result may seem to indicate that acute O_3 exposure had a beneficial effect on the protease/antiprotease balance, the increase in elastase inhibitory capacity was caused by increased lung permeability and serum transudation and thus is indicative of lung injury. Chronic and subchronic episodic exposure to O_3 had no effect on the elastase inhibitory capacity of lung lavage fluid. A novel method was developed to directly measure the activity of low molecular weight antiproteases in lung lavage fluid. Acute exposure of humans to O_3 had no effect on low molecular weight elastase inhibitory activity in this study. The pattern of episodic O_3 exposure used in the animal exposure studies prevented short-term (within one week) sensitivity attenuation (adaptation response). However, attenuation of sensitivity to the acute injurious effects of episodic O_3 exposure did occur within 8 weeks. Acute exposure

studies in humans indicated that sequential exposure to HNO_3 followed by O_3 did not enhance the O_3-induced increases in lavage fluid protein or elastase inhibitory capacity.

I. INTRODUCTION

The purpose of this project was to examine the effect of 0_3 exposure on connective tissue proteases and protease inhibitors in the lung lining layer of rats and humans. Animal studies have clearly shown that 0_3 has the potential to cause irreversible connective tissue alteration in the form of pulmonary fibrosis (Hesterberg and Last 1981, Reiser et al. 1987). However, it is not known whether exposure of humans to ambient Southern California levels of 0_3 in a chronic episodic pattern is likely to cause connective tissue damage. In this project, the biochemical events that precede connective tissue damage, i.e. an imbalance of proteases and protease inhibitors, was monitored in humans after a single 0_3 exposure and in rats that were episodically exposed to low levels of 0_3 for many months. The effect of $HN0_3$ exposure, alone and in combination with 0_3, was also examined because there is evidence that acidic atmospheres may increase the potential of 0_3 to cause connective tissue damage.

An important feature of this project is that the lung lavage fluid samples used in these studies were obtained from three other ARB-funded studies. The principal investigators generously agreed to supply surplus lung lavage fluid from the exposures listed in Table 1. The exposures are listed in order of increasing duration of exposure. Since the emphasis of the project is on chronic effects of 0_3 exposure, it is preferable to group the data by exposure duration rather than by species, co-pollutant, or 0_3 concentration. The use of lung lavage fluid from previously funded projects resulted in considerable savings of exposure, animal purchase, and animal care cost for this study. In addition, the use of animal tissues and fluids in multiple studies represents a maximization of animal resources. These projects were selected not only for the savings in resources that they provided, but to allow the investigators to examine the chronic and acute effects of ozone on connective tissue proteases and protease inhibitors in both animal models and human subjects. Animal models offer the unique opportunity to study chronic lung damage effects in a controlled environment. While animal models do demonstrate some differences in their lungs compared to humans, the basic structure and functions remain quite similar, making rats and rabbits an excellent model for the effect of 0_3 on lung proteases and protease inhibitors.

A short-term exposure of rats to a moderate level of 0_3 was done as part of this project to bridge the gap between the chronic animal exposures and the acute human exposure studies.

The data from this exposure aids in extrapolation of results from the chronic repeated episodic animal exposures to the single acute human exposures and provides information on the effects of episodic 0_3 exposure which is a more realistic exposure pattern than a continuous chronic exposure. Rats were exposed to I cycle of the episodic pattern that was repeated in the chronic exposures, i.e., 4 hours per day for 3 consecutive days of exposure to 0_3, followed by 4 days of no exposure. Rats were sacrificed at 4 time points; (1) the day after the first consecutive exposure (2) immediately after the third consecutive daily exposure; (3) following four days of recovery in clean air, and (4) following the first day of the next round of daily 4 hour exposures. This experiment answered the following questions: Do acute changes in lavage fluid parameters increase or decrease when the exposure is repeated? What happens if there is a recovery period followed by re-exposure? This information is needed to compare data from the acute human studies to the chronic animal exposures and to evaluate the potential of repeated episodic 0_3 exposure to cause disease in humans.

The specific endpoints that were examined and characterized in lung lavage fluid were protein content, elastase-like activity, collagenase activity, and neutrophil elastase inhibitory capacity. In addition, lung lavage fluid was also assayed for low molecular weight elastase inhibitors. There is growing evidence that a low molecular weight elastase inhibitor, secretory leukocyte

protease inhibitor (SLPI), plays a critical role in the lung defense system against neutrophil-mediated tissue damage (Burnett and Stockley 1993).

II OBJECTIVES.

The objective of this project was to perform a detailed analysis of the biochemical events that are believed to precede connective tissue disruption, i.e. changes in connective tissue proteases and protease inhibitors, in the lung lavage fluid of animals episodically exposed to moderate-to-low levels of 0_3 over long periods of time. Similar measurements were made on the lung lavage fluid of humans following a single exposure to near ambient levels of 0_3. Since $HN0_3$ also has oxidant properties, the effect of $HN0_3$ exposure (alone and in combination with 0_3) on lung lavage fluid protease activity was also examined.

The specific aims of the project were:

1. determine the effects of 0_3 on elastase activity of lung lavage fluid and on the elastase inhibitory capacity;

2. determine the effect of 0_3 on collagenase-like activity of lung lavage fluid;

3. determine the effect of 0_3 on low molecular weight elastase inhibitors in lung lavage fluid;

4. determine how the timing of repeated episodic 0_3 exposure affects the above parameters of the protease/antiprotease balance.

III. MATERIALS AND METHODS

Exposures

Lavage fluid was generously provided by other investigators from three different projects. The first study was preformed at the Air Pollution Health Effects Laboratory at the University of California, Irvine with Dr. William J. Mautz as the principle investigator. In this study, Fischer 344 rats were exposed to 0.3 ppm 0_3, air and a five component mixture of 0.3 ppm 0_3, 0.2 ppm $N0_3$, 50 $\mu g/m^3$ $HN0_3$, 100 $\mu g/m^3$ NH_4HSO_4, and 60 $\mu g/m^3$ carbon particles. Rats were exposed for 4 hr/d, 3 d/wk, for up to 6 months with lavage being performed at 8 and 26 weeks.

The second project involved the exposure of Fischer 344 rats and rabbits to air, 0.15 ppm 0_3, 50 $\mu g/m^3$ $HN0_3$, and 0.15 ppm 0_3 in combination with 50 $\mu g/m^3$ $HN0_3$ for 12 and 40 weeks. The principle investigator Dr. William J. Mautz, at the Air Pollution Health Effects Laboratory at the University of California, Irvine, was responsible for the exposures to rats. The rabbit exposures were performed by Dr. Christine E. Nadziejko at New York University.

The acute human exposures were performed at the University of California, San Francisco under the direction of Dr. John Balms. In this investigation, healthy male adults were exposed to 0.2 ppm 0_3 and to 0.2 ppm 0_3 in combination with 500 $\mu g/m^3$ $HN0_3$ for 4 hours with exercise. An additional group of healthy adult males was exposed to 0.4 ppm 0_3 with and without 500 $\mu g/m^3$ $HN0_3$ for 4 hours while exercising.

An additional group of Fischer 344 rats were exposed at UC Irvine to 0.4 ppm 0_3 for 4hr/day for up to 3 days. This study served to bridge the gap between the acute human exposures and the chronic animal exposures.

Bronchoalveolar Lavage

Bronchoalveolar lavage was performed on all animals using standard procedures. Briefly, the animals were anesthetized with sodium pentobarbital, killed by exsanguination by the abdominal aorta, and the thoracic cavity opened. The lungs were lavaged through a small opening in the trachea with phenol red-free Hank's balanced salt solution, without Ca^{++} or Mg^{++}. The lavage fluid was instilled and withdrawn 3 times and pooled. The investigators made at least 2-3 mL of surplus lavage fluid available for this study.

The bronchoalveolar lavage procedures for human subjects are well established by Dr. Balms research group. The lavage was preformed using flexible, fiberoptic bronchoscopy 18 hours postexposure. Intravenous access was maintained and arterial oxygen saturation and heart rate monitored during the procedure. Midazolam was administered intravenously and lidocaine was administered topically to aid in maintaining subject comfort. Supplemental oxygen was used during the procedure. Isolated airway lavage was performed using a custom-designed, double-balloon catheter with 12 mL of warmed normal saline in eight 1.5 mL aliquots. In addition, bronchoalveolar lavage was preformed using 150 mL of warmed normal saline in three 50 mL aliquots. After the procedures, the subjects were observed for 1-2 hours. The investigators made 2-3 mL of surplus lavage fluid available for this study.

Reagents

Purified neutrophil elastase (from human sputum 800-900 units/mg using the substrate N-succinyl-ala-ala-ala *p*-nitroanilide), porcine pancreatic elastase (120-145 units/mg using elastin orcein as a substrate) and fluorescein-labeled elastin were purchased from Elastin Products Co., Owensville, MO. Partially purified α_1 AT (from human plasma), α chymotrypsin

(Type VII from bovine pancreas, 40-60 units/mg), enzyme substrates, and cetyl trimethyl ammonium bromide (CTAB) were purchased from Sigma Chemical Co, St Louis MO.

Lavage Fluid Assays

Total Protein: The protein content of the lavage fluid was measured using a BCA kit (Pierce, Rockford IL) using the protocol supplied by the manufacturer. Bovine serum albumin was used as a standard.

Elastase-like Activity: Elastase-like activity of lung lavage fluid was measured by incubating I 00 μL of lung lavage fluid with 100 μL of 2mM succinyl, alanine, alanine *p* nitroanilide (SLAPN) at pH 7.8. Absorbance at 410 nm was measured immediately and after 18 hrs; incubation at 37 °C. Data were expressed as change in absorbance/18 hrs. Neutrophil elastase was used as a positive control.

Neutrophil Elastase Activity: Neutrophil elastase activity of lung lavage fluid was measured using N-methoxy-succinyl-ala-ala-pro-val-*p*-nitroanilide using the protocol described above.

Total EIC (rat and rabbit lavage samples): This assay was performed as described previously (Nadziejko et al., 1992). In brief, 0.5-1 μg of neutrophil elastase in 500 μL of DPBS was added to 350 μL of lavage fluid and maintained at room temperature for 5 min. Residual elastase activity was determined by adding 2 mg of fluorescein-labeled elastin in 150 μL DPBS. The enzyme-substrate mixtures were placed on a rotating platform at room temperature for 30, 60, or 90 mins, then centrifuged at 8800 x G for 2-4 min. The fluorescence of the supernatant fluid was measured at excitation and emission wave lengths of 495 and 520 nm, respectively, using an LS-5 fluorescence spectrophotometer (Perkin Elmer, Norwalk, CT). A standard curve was obtained by measuring the fluorescence of mixtures containing neutrophil elastase and fluorescein-labeled elastin in the absence of lavage fluid. EIC is expressed as μg neutrophil elastase inhibited/350 μL lavage fluid.

Total EIC (human lavage samples) : One hundred μL of lavage fluid was added to 50 μL N-methoxy-succinyl-ala-ala-pro-val-*p*-nitroanilide (3.0 mM) followed by 50 μL neutrophil elastase (2.0-12.0 μg/mL). All solutions were prepared in 0. 1 M HEPES buffer, pH 7.4, and 0. 1 M NaCl. The stock solutions of neutrophil elastase contained 200 μg bovine serum albumin/mL to prevent inhibition resulting from binding of elastase to the microtiter plates. At least three concentrations of neutrophil elastase were tested per sample. The rate of change in absorbance at 410 nm of the samples was compared to standards containing 100 μL HEPES buffered saline, 50 μL substrate and 50 μL neutrophil elastase. The maximum elastase inhibitory capacity of each lavage fluid sample was calculated and expressed as μg neutrophil elastase inhibited/mL lavage fluid.

CTAB-resistant EIC: One vol of 1. 12 mg/mL CTAB was added to 9 vol lavage fluid, mixed well, and incubated at 37 °C for 16-20 hrs. Fifty μL of 3.0 mM N-methoxy-succinyl-ala-ala-pro-val-*p*-nitroanilide were added to 100 μL of the CTAB-treated lavage fluid followed by 50 μL neutrophil elastase (0.8-3.2 μg/mL). At least two concentrations of neutrophil elastase were tested per sample. The rate of change in absorbance of the samples was compared to

standards containing 100 μL of 112 mg/mL CTAB in HEPES buffered saline, 50 μL substrate and 50 μL neutrophil elastase. The maximum amount of elastase inhibited by each CTAB-treated sample was calculated.

Individual antiprotease activities of elafin and SLPI: The antiprotease activities of elafin and of SLPI were determined by measuring the inhibition of pancreatic elastase and α chymotrypsin, respectively by CTAB-treated human lavage fluid. The assay procedure was similar to that described above except that the CTAB-treated lavage fluid samples were incubated with the enzymes for 10 min at room temperature prior to adding substrate. The substrate for pancreatic elastase was N-succinyl-ala-ala-ala *p*-nitroanilide and the substrate for α chymotrypsin was N-succinyl-ala-ala-pro-phe *p*-nitroanilide.

Collagenase activity: A gelatin-degrading assay described by Davis and Martin (1990) was used. Gelatin was biotinolated by adding 100 μg/mL gelatin to 100 μg/mL biotinamidocaproate N-hyroxysuccinimide ester for 1 hr at room temperature. The biotinolated gelatin was added to a low-binding 96 well microtiter plate at a concentration of 0.25 μg/well. The wells were blocked with 0.1 % Tween 20 in 0.9% saline solution. The samples were diluted 1: 1 (v:v) with 50 mM Tris-HCl, 0. 1 % Tween 20 and 5 mM CaCl, and added to the microtiter plate. Following a 24 h incubation at 37 °C, the wells were washed, blocked with 1 % bovine serum albumin in 0. 1 % Tween 20 and 0. 9 % saline and incubated with avidin-peroxidase (1 μg/mL) for 30 min. The microtiter plate was washed and peroxidase activity was determined using 2,20-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma, St Louis MO). The absorbance was determined using a microtiter plate reader with a 410 nM filter.

IV. RESULTS AND DISCUSSION

Characterization of Specific Endpoints

Elastase-like Activity

Elastase-like activity in lung lavage fluid of humans, rats and rabbits was measured using the synthetic substrate SLAPN (succinyl-trialanine-nitro anilide). Assays were performed by incubating aliquots of lung lavage fluid, SLAPN and buffer in microtiter plates for 16 hrs. Elastase activity was detected as an increase in absorbance at 410 nm, which was monitored using a microtiter plate reader, and compared to standards prepared from neutrophil elastase. This method of detecting elastase-like activity in the lavage fluid of all three species proved to be very sensitive. However, we suspected that the assay was non-specific because significant levels of elastase-like activity were detected in the lavage fluid of all the humans and animals tested regardless of exposure status. Additional experiments (outlined in Figure 1) were performed to better characterize the enzyme in lavage fluid that was responsible for hydrolysis of SLAPN. Lavage fluid samples from rats exposed to air or 0.3 PPM 0_3 for 8 weeks (Table 5) were incubated with fluorescein-elastin for up to 24 hrs. No elastolytic activity was detected indicating that the hydrolysis of SLAPN was not due to free neutrophil elastase. However, it was possible that the elastase-like activity detected using SLAPN was due to neutrophil elastase trapped within α_2 macroglobulin. When rat lung lavage fluid was fractionated using a molecular weight cut-off filter, all of the elastase-like activity was found in the > 300 KD fraction (Table 6) which is consistent with the idea that SLAPN reacts with neutrophil elastase trapped within α_2 macroglobulin. However, when a small peptide substrate (N-methoxy-succinyl-alanine- alanine-proline-valine-*p*-nitroanilide) that is more specific for neutrophil elastase than SLAPN was used, lavage fluid samples from rats, rabbits and humans had no detectable neutrophil elastase activity. These experiments indicate that the elastase-like activity detected in lavage fluid using SLAPN is not due to elastase but rather reflects the activity of a high molecular weight enzyme or enzyme complex that has esterase activity. Although SLAPN is often used to measure elastase activity in lavage fluid, our results show that N-methoxy-succinyl-alanine-alanine-proline-valine-*p*-nitroanilide is a more appropriate choice of substrate because it is more specific for neutrophil elastase.

Collagenase activity

Lavage fluid samples were tested for collagenase activity using biotin-labeled gelatin (see Methods). Using this method we were able to detect bacterial collagenase at concentrations as low as 0.001 μg/mL and salivary collagenase in human saliva at dilutions of 1:100. Despite the sensitivity of the assay, no collagenase activity was detected in any sample of rat or rabbit lung lavage fluid.

Elastase Inhibitory Capacity

The lung lavage fluid of rats and humans was found to contain substantial elastase inhibitory capacity as expected. However, lung lavage fluid of rabbits had no detectable inhibitory activity towards either human neutrophil elastase or porcine pancreatic elastase. Thus, in studies involving rabbits, no data are reported for elastase inhibitory capacity or for low molecular

weight inhibitors (see below). Since the active site of α_1 antitrypsin (α_1AT) is highly conserved among various species including rabbits, it is unlikely that rabbit α_1AT lacks inhibitory activity toward human neutrophil elastase or porcine pancreatic elastase. The most likely explanation for our findings is that the capillary endothelium of the rabbit lung may block transport of plasma proteins into the lung lining layer.

Low molecular weight elastase inhibitors

At the time that the proposal for this project was submitted, it was known that the human lung produces a low molecular weight neutrophil elastase inhibitor called secretory leukocyte proteinase inhibitor (SLPI). After the proposal was submitted, a second structurally similar inhibitor, named elafin, was isolated from airway secretions. We had planned to measure the low molecular weight inhibitors in lung lavage by measuring the elastase inhibitory capacity of the fraction of lavage fluid that was less than 30,000 in molecular weight. However, since SLPI binds to high molecular weight molecules such as mucin and heparin (Faller et al. 1992, Van-Seuningen et al. 1989), it was necessary to use a different approach to distinguish the elastase inhibitory activity of SLPI and elafin from that of α_1 AT. We developed a simple method for direct measurement of the antiprotease activity of SLPI and elafin in lung lavage fluid that is based on the resistance of these antiproteases to inactivation by a cationic detergent, cetyltrimethylammonium bromide (CTAB). Detergents are known to denature many proteins including α_1 AT. Alpha I antitrypsin is much more easily denatured than SLPI or elafin because its three dimensional conformation is maintained by two intramolecular salt bridges whereas the structures of SLPI and elafin are stabilized by internal disulfide bonds. The individual antiprotease activities of elafin and of SLPI were determined by measuring the inhibition of pancreatic elastase and α chymotrypsin, respectively by CTAB-treated human lavage fluid. In a separate study, we used this method to determine the contribution of low molecular weight inhibitors to the total neutrophil elastase inhibitory capacity of bronchoalveolar lavage samples obtained from 23 normal subjects (Nadziejko et al. 1995). The low molecular weight antiproteases accounted for 22 ± 2% (mean ± S.E.M., n=23) of the total neutrophil elastase inhibitory capacity of human bronchoalveolar lavage fluid (BAL) of normal human subjects. SLPI activity (measured by inhibition of a chymotrypsin) accounted for 72 ± 4% (mean + S.E.M., n=23) of the low molecular weight antiprotease activity . Elafin activity was below the limit of detection. Rat lung lavage fluid was found to have very low but detectable levels of low molecular weight antiprotease activity. It was beyond the scope of this project to measure SLPI by ELISA and to correlate our measurements of SLPI activity with levels of SLPI protein measured by ELISA. Therefore in this report we have used the term CTAB-resistant elastase inhibitory capacity (EIC) rather than the term SLPI activity. However, studies in which we have added known amounts of SLPI or elafin to lavage fluid indicate that CTAB-resistant EIC is a very accurate index of SLPI activity in lung lavage fluid of humans and rats (data not shown).

Effect of acute exposure of humans to 0_3

As shown in Table 2, a 4 hr exposure of 12 subjects to 0.2 PPM 0_3 while exercising resulted in a significant increase in lavage fluid protein content and total elastase inhibitory capacity (EIC). These biochemical changes are indicative of increased lung permeability caused by the damaging effects of 0_3. CTAB-resistant EIC was unchanged by exposure to 0_3 in this

experiment. However, we have analyzed lavage fluid samples from another study in which 23 subjects were exposed to 0.2 ppm 0_3 or to filtered air for 4 hrs in a random-order, single-blinded crossover study. In this study, CTAB-resistant EIC decreased significantly ($p < 0.05$) from 9.0 ± 0.8 to 7.0 ± 0.6 pmoles neutrophil elastase inhibited/mL (mean ± S.E.M., n = 23) following acute 0_3 exposure (Nadziejko et al. 1995).
Exposure to $HN0_3$ (500 μg/m) had no effect on any parameter measured. Sequential exposure to $HN0_3$ followed by 0_3 did not enhance the 0_3-mediated increase in lavage fluid protein and EIC. Statistical testing using a linear regression model with dummy variables indicated that there were no significant antagonistic interactions between $HN0_3$ and 0_3. A smaller study in which 5 subjects were exposed to 0_3 and 4 subjects were exposed to both 0_3 and $HN0_3$ failed to show any differences between the two groups (Table 3).

Effect of short term episodic exposure of rats to 0.4 ppm 0_3

The subchronic and chronic animal exposure studies included in this project all had exposure patterns of 4 hrs per day on 3 consecutive days followed by 4 days of no exposure. This pattern was repeated for 8-40 weeks. This pattern of exposure was chosen because it resembles human exposure patterns in Southern CA. It was expected that the relatively brief duration of exposure and long recovery period would prevent tolerance and/or adaptation to 0_3-mediated injury. The effect of the pattern of exposure on 0_3-induced injury was examined by sacrificing groups of rats as follows: (1) the day after the first daily exposure ; (2) immediately after the third consecutive daily exposure; (3) following fours days of recovery in clean air, and (4) following the first day of the next round of daily 4 hr exposures. As shown in Table 4, only total EIC was elevated after the first day of exposure. Lavage fluid protein total EIC, and % neutrophils in lavage fluid were all increased following 3 consecutive daily exposures suggesting that 0_3-induced injury was cumulative over the 3 days. Lavage fluid protein and % neutrophils returned to control levels after 4 days of recovery but total EIC remained elevated, suggesting that recovery was not fully complete. Lavage fluid protein EIC and neutrophils were all elevated above control values when the animals were re-exposed for one day following the 4 day recovery period. This experiment suggests that the exposure pattern did not induce adaptation and/or tolerance to 0_3 over the short term.
Table 4 also shows that low levels of CTAB-resistant elastase inhibitory activity were detected in rat lavage but in contrast to human lavage fluid, the CTAB-resistant EIC was less than I % of the total EIC (data not shown). No statistically significant change was seen in CTAB-resistant EIC following any of the exposure days. Elastase-like activity decreased below control levels following day I and day 3 of exposure even though neutrophils were increased in lavage fluid after day 3 of exposure. Changes in elastase-like activity are difficult to interpret because, as mentioned above, this assay appears to detect esterase activity and is unrelated to neutrophil elastase activity.

Effects of an 8 week.-exposure of rats to 0.3 ppm 0_3
This exposure was compromised by a slowly progressing pneumonitis in some of the exposure animals. Subtle signs of pneumonitis were seen in quality control animals sacrificed immediately after arrival from the vendor. Subsequent exposures described in this report utilized animals from a different vendor and no further cases of pneumonitis or other respiratory diseases were seen. Lavage fluid samples from animals from the 8 week exposure that had minimal or no signs of pneumonitis (lavage fluid cells contained less than 10% neutrophils)

were used in this project for method development. As shown in Table 5, the 0_3-exposed rats showed no increase in lavage fluid protein or EIC, even though these endpoints were strikingly elevated following acute and short-term exposures. Elastase-like activity was elevated in the lavage fluid of 0_3-exposed rats. Additional studies showed-that most of the elastase-like activity was due to a high molecular weight enzyme or enzyme complex and was not due to free neutrophil elastase.

Effects of subchronic and chronic episodic exposure of rats and rabbits to 0_3 with and without $HN0_3$.

Tables 6A and 6B indicate that 0.15 PPM 0_3, 50 $\mu g/m^3$ $HN0_3$ or the mixture of 0_3 and $HN0_3$ had no effect on any of the parameters measured in either rats or rabbits. These negative results may be due in part to the low concentration of 0_3 and the relative insensitivity of animals exposed at rest (as opposed to humans exposed during exercise) to the effects of 0_3. However, as shown in Table 7, a 26 week exposure of rats to 0.3 ppm 0_3 caused only a slight increase in lavage fluid protein and elastase like-activity. The increased elastase-like activity was not due to neutrophil elastase and probably reflects nonspecific esterase activity. Exposure to 0.3 ppm 0_3 in combination with other components of air pollution had no effect on any parameter measured. Since 0.4 ppm 0_3 had striking effects on lavage fluid parameters during a short term one week exposure, it is likely that adaptation occurred during the longer exposures despite the episodic nature of the exposure. As shown in Tables 8A and 8B, a 40 week exposure of rats and rabbits to 0.15 ppm 0_3, 50 $\mu g/m^3$ $HN0_3$, or the mixture Of 0_3 and $HN0_3$ had no effects with the exception that elastase-like activity was slightly elevated in the lavage fluid of rats exposed to 0_3 plus $HN0_3$.

V. SUMMARY AND CONCLUSIONS

In this study, the biochemical events that precede connective tissue damage, specifically the changes in connective tissue proteases and protease inhibitors, was monitored in humans after a single 0_3 exposure and in rats that were episodically exposed to low levels of 0_3 for many months. Acute, subacute, and chronic episodic exposure to 0_3 did not result in increased levels of free neutrophil elastase or collagenase in lung lavage fluid even though acute 0_3 exposure was associated with increased numbers of neutrophils in the lavage fluid. Acute exposure of humans and rats to 0_3 resulted in substantial increases in the elastase inhibitory capacity of lavage fluid. Although this result may seem to indicate that acute 0_3 exposure had a beneficial effect on the protease/antiprotease balance, the increase in elastase inhibitory capacity was caused by increased lung permeability and serum transudation and thus is indicative of lung injury. Chronic and subchronic episodic exposure to 0_3 had no effect on the elastase inhibitory capacity of lung lavage fluid. A novel method was developed to directly measure the activity of low molecular weight antiproteases in lung lavage fluid. Acute exposure of humans to 0_3 had no effect on low molecular weight elastase inhibitory activity in this study. The pattern of episodic 0_3 exposure used in the animal exposure studies prevented short-term (within one week) sensitivity attenuation (adaptation response). However, attenuation of sensitivity to the acute injurious effects of episodic 0_3 exposure did occur within 8 weeks. Acute exposure studies in humans indicated that sequential exposure to $HN0_3$ followed by 0_3 did not enhance the 0_3-induced increases in lavage fluid protein or elastase inhibitory capacity.

VI. RECOMMENDATIONS

1. Although the episodic 0_3 exposure pattern used in the long term animal exposure studies (4hrs/day, 3days/week) appeared to prevent sensitivity attenuation (adaptation response) during the first week of exposure, sensitivity attenuation developed by eight weeks of exposure. It is recommended that future studies of the effects of chronic episodic 0_3 exposure incorporate a longer time period between exposure episodes.

2. During this study, a novel assay for low molecular weight elastase inhibitors was developed. Although the results of this study indicated that acute 0_3 exposure had no effect on these inhibitors, it is likely that the number of subjects in the study was too low to detect any changes. It is recommended that this endpoint be included in any future studies on the effects of human exposure to 0_3 on lavage fluid parameters.

3. Acute exposures of humans and rats to relatively low concentrations of 0_3 resulted in striking increases in the elastase inhibitory capacity of the bronchoalveolar lavage fluid. We have previously shown that this phenomenon is due to increased alveolar capillary permeability and serum transudation (Nadziejko and Chen, 1994). While this increase in lung permeability appears to be well tolerated by healthy humans, increased pulmonary permeability may cause decreased oxygenation and/or cardiovascular problems in individuals with congestive heart failure or pulmonary hypertension. It is recommended that future studies examine the acute effects of 0_3 on animal models of common human diseases.

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Figure 1. Biochemical Characterization of Elastase-like Activity.

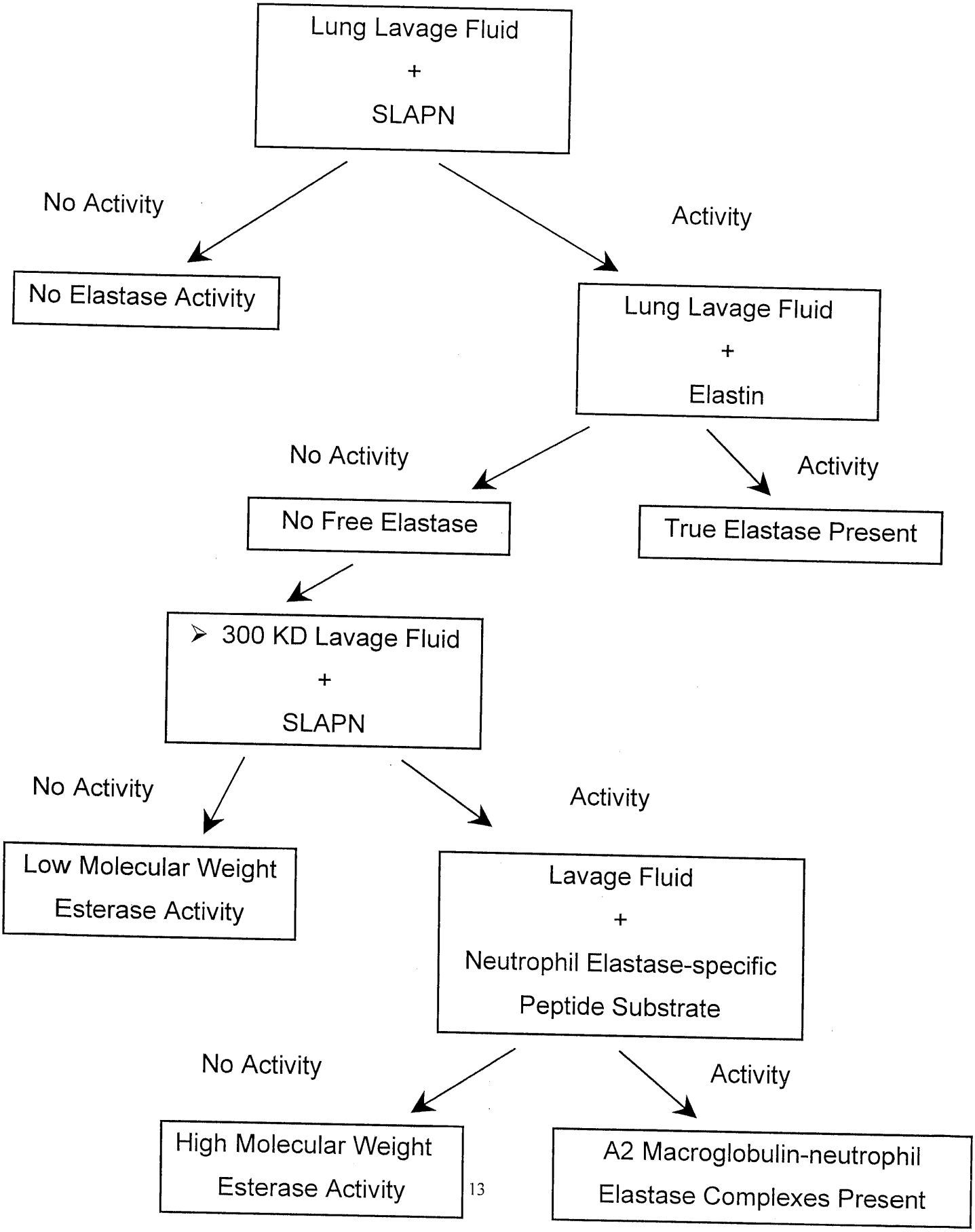

Table 1. Exposure Summary

Species	Exposure Groups and Concentration	Exposure Pattern	Lung Lavage Time Points	Table Number
Human	1. Air 2. 0.2 ppm O_3 3. 0.5 mg/m^3 HNO_3 4. 0.2 ppm O_3 + 0.5 mg/m^3 HNO_3	4 h + exercise	8 h post exposure	Table 2
Human	1. 0.4 ppm O_3 2. 0.4 ppm O_3 + 0.5 mg/m^3 HNO_3	4 h + exercise	8 h post exposure	Table 3
Rat	1. Air 2. 0.4 ppm O_3	4 h/d, 3 d/week	1 d, 3 d, 4 d post-exposure, 1 d re-exposure	Table 4
Rat	1. Air 2. 0.3 ppm O_3	4 h/d, 3 d/week	8 weeks	Table 5
Rat Rabbit	1. Air 2. 0.15 ppm O_3 3. 50 μg/m^3 HNO_3 4. 0.15 ppm O_3 + 50 μg/m^3 HNO_3	4 h/d, 3 d/week	12 weeks	Table 6A, 6B
Rat	1. Air 2. 0.3 ppm O_3 3. 0.3 ppm O_3 + complex mixture	4 h/d, 3 d/week	26 weeks 4 weeks post-exposure	Table 7
Rat Rabbit	1. Air 2. 0.15 ppm O_3 3. 50 μg/m^3 HNO_3 4. 0.15 ppm O_3 + 50 μg/m^3 HNO_3	4 h/d, 3d/week	40 weeks	Table 8A, 8B

Table 2. Acute Exposure of Humans to Air, O_3, HNO_3, or HNO_3 Plus O_3 For 4 Hours While Exercising. Data are shown in terms of % of control (upper line) as well as the measured values (lower line) and are expressed as mean ± S.E.M.

Exposure	Air	500 μg/m^3 HNO_3	0.2 ppm O_3	0.2 ppm O_3 + 500 μg/m^3 HNO_3
Number of Subjects	13	10	12	9
Protein (μg/ml)	100 ± 11 125 ± 14	83 ± 10 105 ± 12	157 ± 15* 198 ± 19*	134 ± 41 169 ± 52
Elastase-like activity (A_{410})	100 ± 17 0.012 ± 0.002	75 ± 17 0.009 ± 0.002	92 ± 25 0.011 ± 0.003	67 ± 17 0.008 ± 0.002
Neutrophil Elastase Activity	0	0	0	0
Total EIC (μg NE inhibited)	100 ± 8 0.49 ± 0.04	87 ± 6 0.43 ± 0.03	211 ± 44* 1.00 ± 0.22*	167 ± 14 0.81 ± 0.3
CTAB-resistant EIC (μg NE inhibited)	100 ± 13 0.19 ± 0.02	108 ± 12 0.20 ± 0.02	118 ± 34 0.22 ± 0.06	69 ± 14 0.13 ± 0.03

*Mean significantly different from control.

Table 3. Acute Exposure of Humans to 0.4 ppm O_3 With and Without HNO_3 for 4 hrs While Exercising. Data are expressed as mean ± S.E.M. Group means were not significantly different for any of the parameters measured.

Exposure	0.4 ppm O_3	0.4 ppm O_3 + 500 μg/m^3 HNO_3
Number of Subjects	5	4
Protein (μg/ml)	150 ± 76	151 ± 44
Elastase-like activity (A_{410})	0.029 ± 0.018	0.020 ± 0.002
Total EIC (μgNE inhibited)	1.19 ± 0.28	1.33 ± 0.30
CTAB-resistant EIC (μgNE inhibited)	0.15 ± 0.04	0.16 ± 0.02

Table 4. Effects of Short Term Episodic Exposures of Rats to 0.4 ppm O_3 for 4 hrs/day. All data are expressed as mean ± S.E.M. Biochemical data are expressed in terms of % control (upper line) as well as the measured values (lower line).

Exposure	Air	O_3 X 1 day	O_3 X 3 days	O_3 X 3 days + 4 days air	O_3 X 3 days + 4 days air + 1 day O_3
Number of Animals	39	10	10	10	10
Protein	100 ± 2	98 ± 5	123 ± 4*	103 ± 4	123 ± 5*
(μg/ml)	199 ± 4	196 ± 10	245 ± 7*	206 ± 8	244 ± 10*
Elastase-like	100 ± 5	111 ± 5	61 ± 2*	86 ± 5*	116 ± 5
Activity (A_{410})	0.192 ± 0.01	0.213 ± 0.01	0.117 ± 0.004*	0.166 ± 0.01*	0.223 ± 0.01
Total EIC	100 ± 7	159 ± 13*	210 ± 11*	131 ± 14*	141 ± 12*
(μg NE inhibited)	0.29 ± 0.02	0.46 ± 0.04*	0.61 ± 0.03*	0.38 ± 0.04 *	0.41 ± 0.03 *
CTAB-resistant EIC	100 ± 18	98 ± 33	59 ± 31	112 ± 29	69 ± 21
(ng NE inhibited)	24.3 ± 4.3	23.8 ± 8.0	14.4 ± 7.4	27.5 ± 7.1	16.9 ± 5.1
% Macrophage	98.5 ± 0.2	98.2 ± 0.5	96.4 ± 0.5*	99.3 ± 0.4*	96.8 ± 0.6*
% Neutrophil	0.8 ± 0.1	1.2 ± 0.5	2.9 ± 0.6*	0.5 ± 0.4	2.5 ± 0.7*
% Monocytes	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
% Lymphocytes	0.5 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	0.1 ± 0.1	0.5 ± 0.2

* Mean significantly different from control.

Table 5 8-Week Episodic Exposure of Rats to 0.3 ppm O_3. Biochemical data are expressed as percent of control (upper line) as well as the measured values (lower line). All data are expressed as mean ± S.E.M.

Exposure	Air	0.3 ppm O_3
Number of Animals	6	6
Protein	100 ± 8	98 ± 7
(μg/ml)	130 ± 10	128 ± 9
Elastase-like activity (A_{410})	100 ± 11	140 ± 13*
(μg elastase)	4.7 ± 0.5	6.7 ± 0.6*
High molecular wt	100 ± 6	132 ± 6*
Elastase-like activity (A_{410})	0.62 ± 0.04	0.82 ± 0.04*
Total EIC	100 ± 21	84 ± 11
(ng NE inhibited)	0.19 ± 0.04	0.16 ± 0.02

*Mean significantly different from control.

Table 6A. 12-Week Episodic Exposure of Rats to 0.15 ppm O_3 and 50 $\mu g/m^3$ HNO_3 Alone and In Combination. All data are expressed as mean ± S.E.M. Biochemical data are expressed in terms of % control (upper line) as well as the measured values (lower line).

Exposure	Air	0.15 ppm O_3	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3
Number of Animals	10	10	10	10
Protein	100 ± 2.8	105 ± 3	96 ± 4	107 ± 3
(μg/ml)	174 ± 5	183 ± 6	167 ± 7	187 ± 6
Elastase-like activity	100 ± 6	109 ± 7	110 ± 7	108 ± 4
(μg elastase)	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
(A_{410})				
Total EIC	100 ± 9	108 ± 8	97 ± 9	89 ± 8
(μg NE inhibited)	0.29 ± 0.02	0.31 ± 0.02	0.28 ± 0.03	0.25 ± 0.02
CTAB-resistant EIC	100 ± 35	64 ± 41	52 ± 28	88 ± 27
(ng NE inhibited)	43.6 ± 15.1	27.1 ± 17.5	22.3 ± 11.9	37.7 ± 11.5
Collagenase activity	0	0	0	0

Table 6B. 12-Weeks Episodic Exposure of Rabbits to 0.15 ppm O_3 and 50 $\mu g/m^3$ HNO_3 Alone and In Combination. All data are expressed as mean ± S.E.M. Biochemical data are expressed in terms of % control (upper line) as well as the measured values (lower line).

Exposure	Air	0.15 ppm O_3	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3
Number of Animals	9	8	7	9
Protein	100 ± 9	116 ± 9	86 ± 6	82 ± 10
($\mu g/ml$)	139 ± 3	161 ± 12	120 ± 8	114 ± 14
Elastase-like activity	100 ± 18	127 ± 18	91 ± 18	109 ± 36
(A_{410})	0.11 ± 0.02	0.14 ± 0.02	0.10 ± 0.02	0.12 ± 0.04
Collagenase activity	0	0	0	0

Table 7. 26 Week Episodic Exposure of Rats to 0.3 ppm O_3, or a Mixture of 0.3 ppm O_3, 0.2 ppm NO_3, 50 $\mu g/m^3$ HNO_3, 100 $\mu g/m^3$ NH_4HSO_4, and 60 $\mu g/m^3$ Carbon Particles. Data are expressed in terms of % control ± S.E.M(n) (upper line) and as the measured values ± S.E.M. (lower line).

Matched Analysis		Analysis 1		Analysis 2	
Exposure		Air	O_3	Air	Mixture
	Sacrifice Time				
Protein ($\mu g/ml$)	Immediate	100 ± 3(12) 185 ± 5	113 ± 3(12)* 209 ± 6*	100 ± 4(13) 107 ± 4	101 ± 3(15) 108 ± 4
	1 Mo. Post-Exposure	100 ± 5(12) 180 ± 9	103 ± 2(12) 186 ± 3	100 ± 4(12) 111 ± 4	101 ± 3(12) 112 ± 3
Elastase-like activity (μg elastase) (A_{410})	Immediate	100 ± 4(12) 0.19 ± 0.01	120 ± 7(12) 0.23 ± 0.04*	100 ± 6(13) 0.16 ± 0.01	94 ± 6(15) 0.15 ± 0.01
	1 Mo. Post-Exposure	100 ± 5(12) 0.25 ± 0.04	104 ± 7(12) 0.26 ± 0.04	100 ± 5(12) 0.21 ± 0.01	110 ± 5(12) 0.23 ± 0.01
Neutrophil Elastase (A_{410})	Immediate	0	0	0	0
	1Mo. Post-Exposure	0	0	0	0
Total EIC (μg NE inhibited)	Immediate	100 ± 25(12) 0.15 ± 0.04	133 ± (12) 0.20 ± 0.05	100 ± 71(13) 0.07 ± 0.05	86 ± 43(15) 0.06 ± 0.03
	1 Mo. Post-Exposure	100 ± 11(12) 0.29 ± 0.03	117 ± 11(12) 0.34 ± 0.03	100 ± 7(12) 0.16 ± 0.01	81 ± 57(12) 0.13 ± 0.04
CTAB-resistant EIC (ng NE inhibited)	Immediate	100 ± 17(12) 27.8 ± 4.6	91 ± 19(12) 25.3 ± 5.4	100 ± 17(13) 27.8 ± 4.6	93 ± 26(15) 25.6 ± 7.2
	1 Mo. Post-Exposure	100 ± 19(12) 39.4 ± 7.4	107 ± 15(12) 42.2 ± 5.7	100 ± 19(12) 39.4 ± 7.4	127 ± 10(12) 50.0 ± 3.9
Collagenase activity	Immediate	0	0	0	0
	1 Mo. Post-Exposure	0	0	0	0

*Mean significantly different from control.

Table 8A. 40-Week Episodic Exposure of Rats to 0.15 ppm O_3 and 50 $\mu g/m^3$ HNO_3 Alone and In Combination. All data are expressed as mean ± S.E.M. Biochemical data are expressed in terms of % control (upper line) as well as the measured values (lower line).

Exposure	Air	0.15 ppm O_3	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3
Number of Animals	12	12	12	12
Protein	100 ± 4	99 ± 4	98 ± 3	110 ± 6
($\mu g/ml$)	280 ± 11	278 ± 12	276 ± 9	308 ± 17
Elastase-like activity	100 ± 3	101 ± 4	104 ± 4	118 ± 5*
(μg elastase) (A_{410})	0.160 ± 0.01	0.162 ± 0.01	0.166 ± 0.01	0.189 ± 0.01*
Total EIC	100 ± 8	88 ± 10	89 ± 12	88 ± 9
(μg NE inhibited)	0.31 ± 0.03	0.28 ± 0.03	0.28 ± 0.03	0.27 ± 0.03
CTAB-resistant EIC	100 ± 18	91 ± 22	51 ± 20	49 ± 13
(ng NE inhibited)	43.7 ± 8.0	39.9 ± 9.4	22.4 ± 8.8	21.2 ± 5.6
Collagenase activity	0	0	0	0

*Mean significantly different from control.

Table 8B. 40-Week Episodic Exposure of Rabbits to 0.15 ppm O_3 and 50 $\mu g/m^3$ HNO_3 Alone and In Combination. All data are expressed as mean ± S.E.M. Biochemical data are expressed in terms of % control (upper line) as well as the measured values (lower line).

Exposure	Air	0.15 ppm O_3	50 $\mu g/m^3$ HNO_3	0.15 ppm O_3 + 50 $\mu g/m^3$ HNO_3
Number of Animals	9	8	7	9
Protein	100 ± 8	88 ± 5	86 ± 7	88 ± 7
($\mu g/ml$)	213 ± 6	187 ± 11	183 ± 15	187 ± 16
Elastase-like activity	100 ± 26	54 ± 8	83 ± 30	64 ± 10
(A_{410})	0.27 ± 0.07	0.14 ± 0.02	0.22 ± 0.08	0.18 ± 0.07
Collagenase activity	0	0	0	0