



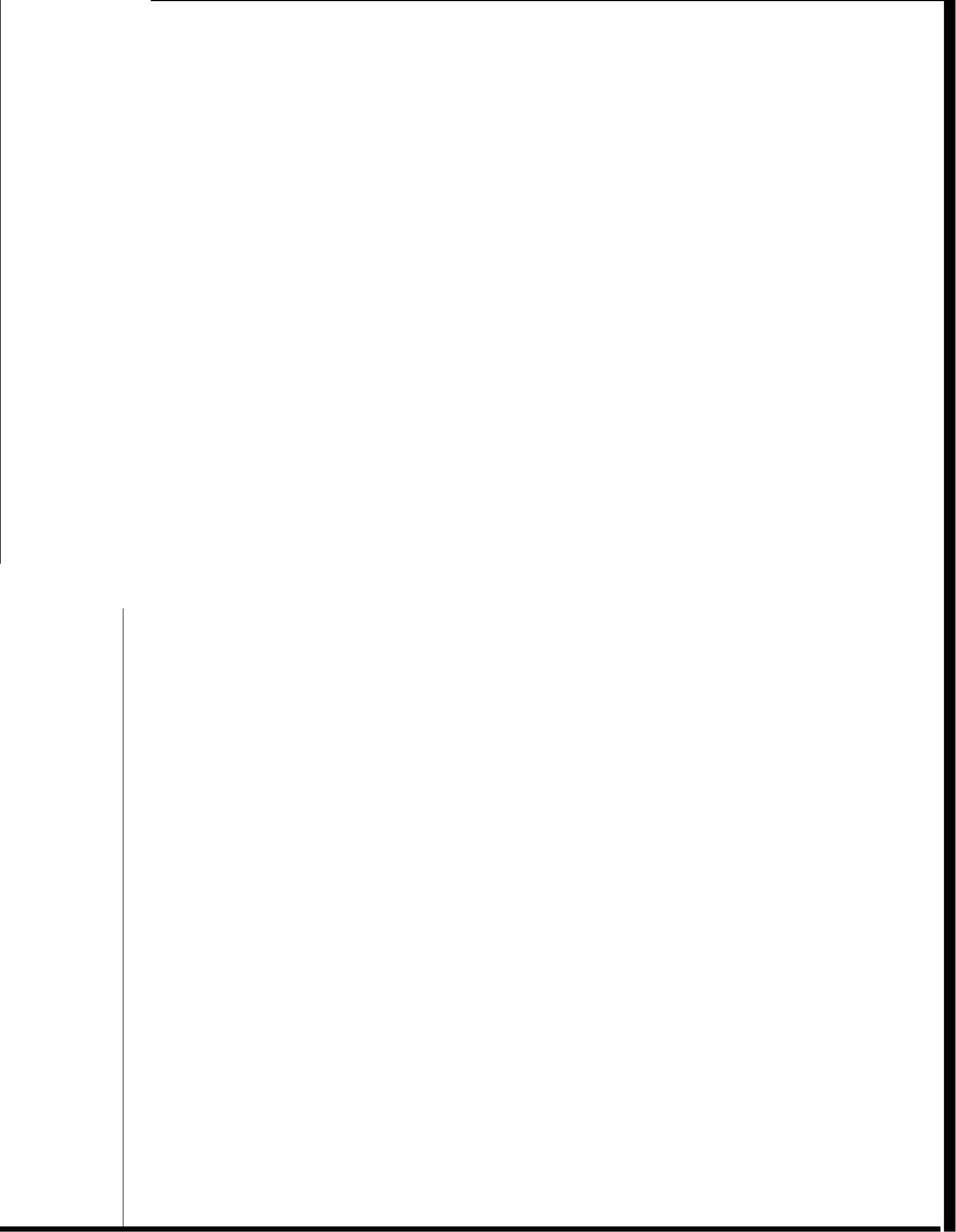
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# Effects of Acidity and Ozone on Airway Epithelium

CALIFORNIA ENVIRONMENTAL PROTECTION AGENCY



AIR RESOURCES BOARD  
Research Division



# EFFECTS OF ACIDITY AND OZONE ON AIRWAY EPITHELIUM

**Final Report  
Contract No. A933-075**

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## Abstract

This contract was designed to utilize an *in vitro* exposure system to assess the early biochemical responses of the airway epithelium to two of the major pollutants found in California outdoor air: acid and ozone. Thus far, there has been an apparent disparity between epidemiologic findings suggesting an important role for airborne acidity in causing adverse health effects and human exposure studies suggesting little effect of acidic atmospheres on standard tests of lung function. One possible explanation for this disparity would be that acidic pollution causes adverse effects that are not detected by lung function testing. If this were the case, the airway epithelium would be the most likely target of such effects, because it would be the major site of deposition of nitric acid vapor and acidic fogs, the two principal forms of acidic pollution in California. In this contract we examined the effects of acidity on four major aspects of epithelial function: mucin secretion, fibronectin secretion, secretion of the potent cytokine transforming growth factor  $\beta$  (TGF $\beta$ ) and protein synthesis. Surface acidification to pH 6 or pH 5/ caused an overall reduction in protein synthesis and specifically induced the synthesis of two well-known stress-induced proteins: hsp 72 and grp 78. However, no such effect was produced by *in vitro* exposure of epithelial cells to any concentration of nitric acid vapor, including concentrations ranging from 50 to 18,000  $\mu\text{g}/\text{m}^3$ . No exposure to acid caused any significant evidence of cytotoxicity or any effect on glycoconjugate, fibronectin or TGF $\beta$  synthesis or secretion. In contrast, exposure of the same cells to ozone caused concentration-dependent cytotoxicity, even at the lowest concentration tested (0.05 ppm). Surprisingly, ozone exposure produced no detectable effect on cellular metabolism other than inhibition of protein synthesis and cell death. Epithelial cells exposed to ozone did not demonstrate a stress-response, and prior induction of stress proteins did not protect airway epithelial cells from the lethal effects of ozone.

### Acknowledgements

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Disclaimer

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

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## Conclusions

The project completed under this contract permits the following conclusions:

1. In vitro exposure of airway epithelial cells to surface acidification causes a pH-dependent reduction of overall protein synthesis and induction of the synthesis of the stress-induced proteins hsp 72 and grp 78. The reduction in protein synthesis occurs after exposures to pH 6.3 and below, and the induction of stress proteins occurs after exposures to pH 6 and below.
2. Surface acidification over the range studied had no effect on the secretion of glycoconjugates, fibronectin or TGF $\beta$ , and did not cause detectable cell lysis.
3. In vitro exposure of airway epithelial cells to nitric acid vapor in concentrations up to 18,000  $\mu\text{g}/\text{m}^3$ , had no detectable effect on protein synthesis or any of the other endpoints studied.
4. In vitro exposure of airway epithelial cells to ozone in concentrations ranging from 0.05 to 0.2 ppm caused concentration-dependent cell lysis as assessed by LDH release or by the release of  $^{51}\text{Cr}$ , but did not induce synthesis of stress proteins or increase expression of fibronectin mRNA.
5. Positive findings from in vitro exposures of a single cell type (e.g. the identification of hsp induction by acid and the finding of ozone-induced cell lysis) are useful for generating hypotheses that can be tested in vivo, but negative findings are of limited value because single cell exposures do not allow identification of effects that require interactions among different populations of lung cells and because cultured airway epithelial cells are not necessarily representative of all of the cell types normally present in the epithelium in vivo. Negative findings in studies of the effects of nitric acid could be due to adsorption of nitric acid onto tissue culture plasticware, with a resultant inability to actually expose the epithelial cells.

## Recommendations

1. Future studies of the in vivo effects of inhaled acid on expression of hsp 72 and grp 78 indistinct cell populations are required to determine whether these proteins are sensitive indicators of environmentally-relevant acid exposure. These studies should ideally involve techniques such as immunocytochemistry or in situ hybridization that will allow for identification of expression in individual sub-populations of airway cells.
2. Future studies should also focus on ozone-induced changes in the expression of early mediators of inflammation and fibrosis in sub-populations of airway cells in vivo.
3. Given the largely negative findings from our studies of acid exposure and the significant cell lysis induced by even the lowest concentration of ozone we examined, further studies of the in vitro effects of these pollutants on cultured airway epithelial cells are probably of only limited value.

## BODY OF REPORT

### Introduction

Acidity is widely suspected as an important factor contributing to the adverse effects of air pollution on human health. The best support for this suspicion comes from epidemiologic evidence suggesting that acid and/or acid precursors are predictors of mortality and hospital visits in patients with lung disease (0,2). Concern about these adverse health effects has led the Clean Air Scientific Advisory Committee of the United States Environmental Protection Agency to recommend evaluation of a standard specifically regulating airborne acidity.

Because the most dramatic effects attributed to airborne acidity (e.g., death and hospital visits) occur over a relatively short time frame (hours to days), several laboratories, including our own, have undertaken a series of studies examining the acute effects of exposure to acid on human subjects. Such studies have now been performed on normal subjects and subjects with asthma and have examined the effects of acid fogs and submicronic acid aerosols on most standard tests of lung function. Although a few studies have found small decrements in FEV<sub>1</sub> or small increases in total pulmonary resistance in subjects with asthma (3,4), these effects have not been consistently observed and none have been of sufficient magnitude to be clinically significant. Furthermore, increasing the concentration of inhaled acid by up to two orders of magnitude (from 100 ug/m<sup>3</sup> up to 10 mg/m<sup>3</sup>) has not produced more consistent or larger effects (5,6). These results strongly suggest: 1) that airborne acidity by itself is not an important cause of bronchoconstriction; 2) that the small changes in lung function reported may have other explanations; and 3) that standard tests of lung function may not be the most sensitive or appropriate way to assess the acute effects of inhaled acid on the lung.

In California, acidic pollution is most often present either as nitric acid vapor (or as acid fog). Indeed, the concentrations of nitric acid vapor in the Los Angeles basin are among the highest in the world (7). Fog water in California can have up to 100 times the acidity usually seen in acid rain, with pH values as low as 1.7 (8). Because of its high solubility in water, inhaled nitric acid vapor that reaches the lung is likely to deposit along the epithelium of the central conducting airways.

Because most fog water is present in relatively large particles, acid fogs are also likely to deposit primarily in the upper and central airways. The conducting airways are lined by an impermeant layer of epithelial cells. These cells provide a protective barrier that completely separates other airway tissues from inhaled materials. Thus, if inhaled nitric acid vapor or acid fog cause adverse effects on the lung, the airway epithelium is highly likely to be the initial target.

It has long been recognized that airway epithelial cells play a major role in protecting the lung from adverse effects of inhaled materials. Well-known functions of airway epithelial cells include: providing the ciliary motor that drives mucociliary clearance, contributing to the production of mucus and to the underlying layer of airway lining fluid, and providing a barrier against inhaled allergens and infectious microorganisms. Disruption of these functions by local mucosal injury, edema, or an alteration in mucus secretion might produce little or no change in standard tests of lung function, but could profoundly increase the susceptibility of the airways to inhaled viruses, bacteria, allergens or other air pollutants.

In addition to performing these barrier functions, it is now known that the airway epithelial cell is highly metabolically active and manufactures a number of products involved in protecting itself and its neighbors from environmental injury, and in the processes of airway injury and repair. The synthetic and secretory functions of airway epithelium could be relevant to studies of acidic air pollution in three ways. First, the identification of changes in the normal synthetic or secretory function of these cells in response to surface acidification could provide sorely needed markers for use in subsequent studies of the effects of inhaled acid on human subjects. Second the identification of specific patterns of injury in response to acid might help to generate testable hypotheses about adverse effects of acid in vivo. Third, characterization of the nature and timing of synthetic and secretory effects of acid and of other pollutants (e.g., ozone) could be important for designing rational and efficient studies examining possible interactions between pollutants.

In this contract we examined the in vitro effects of acid and of ozone on the overall pattern of protein synthesis and on the synthesis and secretion of three specific products of airway epithelial cells. The three specific products: mucus glycoproteins, fibronectin, and transforming growth factor beta (TGF $\beta$ )

were chosen because each is known to play an important role in the normal defensive function and/or the response to injury of airway epithelial cells.

## Methods

### 1. Effects of surface acidification on airway epithelial cells.

Guinea pig airway epithelial cells were harvested from excised tracheas of male Hartley-outbred guinea pigs (Charles River) obtained from a caesarian originated, barrier-sustained colony and housed in a laminar flow isolator. Epithelial cells were separated by digestion in 0.1 % pronase in calcium and magnesium free Ham's F-12 at 37°C and scraped from the underlying trachea with a knife blade. The cells were further disaggregated with a glass pipet, washed twice and resuspended in supplemented Ham's F-12 medium containing 5% fetal calf serum. They were plated onto 25mm collagen coated filter supports (Costar) at a plating density of  $3 \times 10^5$  cells and grown to confluence over 10-14 d. The apical surface of confluent monolayers was exposed to either culture medium at pH 7.4 or MES buffer adjusted to pH 7, 6.5, 6.5, or 5.0 for periods of time ranging from 10 minutes to 3 hours. For all experiments, a 50 ul sample of the fluid above each monolayer was used to measure LDH release after each control and experimental exposure.

#### a) Mucin Secretion

For these experiments glycoconjugates were labeled by incubating confluent monolayers with 20 uCi/ml of <sup>3</sup>H glucosamine overnight. Each monolayer was washed 5x top and bottom to remove unincorporated label and then 1.2 ml of unsupplemented Ham's F-12 medium was added to the bottom compartment (basal surface) and 0.5 ml to the top compartment (apical surface) of each well. After a control period equal to the duration of surface acidification, each sample was harvested to quantify baseline secretion from the apical and basal cell surfaces. The bottom medium was replaced with fresh Ham's F-12 and the top medium was replaced with either medium or MES buffer at one of the selected pHs noted above.

After the chosen incubation time the medium and buffer on both surfaces was again harvested. Medium and buffer pH from each surface was measured before and after

each incubation period. In order to remove any unincorporated label remaining, harvested samples were loaded onto 10-DG desalting columns (**Bio-Rad**) and the 2 ml fraction corresponding to the void volume was saved. A 100 ul aliquot was sampled, added to 1 ml scintillant, and the radioactivity of the sample counted.

b) Protein synthesis and stress protein production

In these experiments, cells were exposed to various surface pHs for periods of time ranging from 30 minutes to 2 hours and were then pulse labeled with 50 uCi/ml <sup>35</sup>S methionine at various time points (from immediately to 24 hours after exposure) to assess the effects of acidification on protein synthesis. Immediately after labeling the cells were lysed in Laemmli sample buffer with added nuclease and boiled for 10 minutes to degrade intracellular proteases. Labeled proteins were then separated by SDS-PAGE on 12.5% gels and autoradiographed. To more precisely determine the nature of any changes in protein synthesis labeled proteins were further separated by two dimensional gel electrophoresis. Effects on overall protein synthesis were assessed by comparing gels from samples containing equal amounts of total protein. Effects on relative synthesis of various proteins were assessed by comparing gels from samples containing equal amounts of incorporated radioactive label. Induction of known stress proteins was assessed from Western blots using specific monoclonal antibodies to hsp 72 and 73.

c) Fibronectin synthesis and secretion

For studies of fibronectin secretion, baseline samples were obtained from the top and bottom wells of <sup>35</sup>S methionine-labelled monolayers as described above. Monolayers were then be exposed to medium or MES buffer. At the end of each incubation period, top and bottom samples were again obtained and the cells were lysed in Laemmli sample buffer as above. Denaturing immunoprecipitations were performed using conditioned media or cell lysates boiled in Laemmli sample buffer and subsequently diluted in immunoprecipitation buffer.. All samples were preabsorbed with protein A-Sepharose beads (Pharmacia/LKB) and then incubated with a rabbit anti-rat fibronectin polyclonal antibody (Cal Biochem, 1:1000 dilution). Samples were remixed with protein A-Sepharose beads. After washing, the final sample was eluted by boiling the beads in Laemmli sample buffer. Rabbit anti-mouse IgG (Nordic) was used as a negative control. Immunoblotting was performed

by transferring the proteins onto nitrocellulose (Shleicher & Scheel, Inc., Keene, NH) using a Hoefer transfer apparatus, followed by blotting with the rabbit anti-rat fibronectin polyclonal antibody (Calbiochem, 1:1000 dilution) or a mouse anti human fibronectin monoclonal antibody (1ST-9) directed against the EIIIA domain (gift of Dr. L. Zardi, 1:200 dilution).

d) TGF $\beta$  synthesis and secretion

TGF $\beta$  secretion was assessed in apically and basally conditioned medium by the mink lung cell bioassay. Medium from control filters and filters exposed to surface acidification for various time periods was added to subconfluent dishes of mink lung cells before and after activation by incubation for 30 min at pH 2.

e). Cytotoxicity

LDH release was measured spectrophotometrically (LD-L Kit, Sigma) from a 0.5 ml surface wash obtained by incubating the apical cell surface with 0.5 ml of Ham's F-12 medium for 5 min. immediately after exposure. The LDH released was divided by the maximal releasable LDH (after cell lysis with 2% Triton) to obtain the percent release. SICr release assays were performed by labelling cells with SICr (50 IICi/dish for 1.5 hr) and then washing both the apical and basolateral surfaces for 5 min after exposure with 0.5 ml of Ham's F-12. Release was measured in a gamma counter and compared to total releasable SICr determined by Triton lysis as above.

2. In vitro effects of nitric acid vapor on airway epithelium

Overall Strategy

In these studies, we focused on the effects of nitric acid on the synthesis of stress proteins, because that was the major effect of surface acidification in the studies described above. In addition, we performed a small number of studies examining the effects of nitric acid on each of the other endpoints to ensure that nitric acid did not produce effects independent of acidification.

### Nitric acid generation

Concentrated  $\text{HN03}$  is quite volatile. At the same time the vapor absorbs onto any available moisture. We took advantage of the former property to generate nitric acid from the headspace above a concentrated solution. To insure that the nitric acid formed was not absorbed by water in the system, the generator was maintained at a constant temperature below the temperature of our incubators and all tubing not inside the incubators was wrapped with heating tape. Nitric acid vapor was generated by passing a metered flow of clean dry air through the head space of a midjet impinger containing concentrated nitric acid maintained at a constant temperature of  $33^{\circ}\text{C}$ . The nitric acid vapor thus generated was diluted with a metered flow of a humidified mixture of 95% air and 5%  $\text{CO}_2$ . The total airflow rate was 1 l/minute. The nitric acid concentration in the generator and the generation airflow was adjusted to attain a range of nitric acid concentrations from 50 to  $18000 \text{ ug}/\text{m}^3$ . Confluent epithelial cell monolayers from a single harvest equilibrated without surface fluid for 24 hours were exposed in pairs to humidified clean air or to nitric acid vapor. The concentration of nitric acid in the exposure chamber was monitored at 15 min intervals during exposure.

### Nitric acid monitoring and quality assurance

Nitric acid concentration was measured by sampling a metered flow of chamber air directly from the chamber into a glass midjet impinger containing 10 ml of water. Initially, we performed sampling with 2 impingers in series to ensure that all of the nitric acid could be sampled with a single impinger. The nitric acid concentration was then calculated by measuring the concentration of nitrate ion by ion chromatography (Dionex). To confirm that all of the nitrate ion in our chambers was in the form of nitric acid, we performed differential filter sampling with extraction of acid vapor through a coated, acid-etched, glass tube and measured residual nitrate on a nylon filter (Gelman, Nylasorb). We did not find any detectable particulate nitrate in these experiments. The chromatograph was calibrated before each series of samples and after each hour of operation with 4 standards that were made up fresh each day. As in the experiments described above measured medium pH, LDH release, and cell counts at the end of each exposure.

### 3. Effects of ozone on airway epithelium

#### Specific methods

Ozone exposures were performed in the same exposure chambers used for nitric acid exposure. Ozone was generated by passing a metered flow of air through a specially designed generator consisting of a one-inch-long ultraviolet light bulb within a custom-built stainless steel tube. This system was built for us by Jel-light corporation and is a modification of the calibrator unit built for the Dasibi model #1008-RS ozone meter. Ozone was measured continuously within the chamber by a Dasibi V.V. photometric analyzer.

In these experiments, we examined the same endpoints and range of time periods described for acid exposures above. We studied ozone concentrations ranging from 0.04 to 0.2 ppm. Because ozone, even at the lowest concentration studied caused significant cell lysis (as demonstrated both by release of LDH and of 51Cr) we did not think that assays of glycoconjugate or fibronectin secretion were meaningful. We thus performed additional studies to assess any effects of ozone on fibronectin mRNA expression. For these experiments Northern analysis was performed using equal amounts of total RNA separated by formaldehyde/agarose electrophoresis and transferred to nylon filters. 32P-labeled probes specific for total guinea pig fibronectin and for guinea pig fibronectin containing the alternatively spliced EIII domain were constructed using the random primer method (20). Hybridizations were performed in SX SSE, 40% formamide, SX Denhardt solution, 20 mM Tris pH 7.4, 10% dextran sulfate, and 100 mg/ml salmon sperm DNA overnight at 50°C. Filters were washed in 2X SSE and 0.1 % SDS at 50°C, and then exposed to film for 16 hours at -70°C with an intensifying screen.

#### Results

##### 1. Surface acidification

Surface acidification with MES buffer caused a pH-dependent reduction in overall protein synthesis of metabolically-labelled guinea pig tracheal epithelial cells (figure 1). In parallel, there was a pH-dependent induction of the synthesis of two specific proteins, one with an apparent molecular mass of 72 kD, and the other 78 kD (figure 2). Two dimensional gel analysis confirmed the induction of both proteins by acidification (figure 3). The acid-induced proteins exhibited similar electrophoretic patterns to the previously described stress-induced proteins hsp 72 and grp 78. The identity of the 72 kD protein as hsp 72 was further confirmed by Western blotting with a monoclonal antibody (C92) specific for hsp 72 (figure 2). These effects of surface acidification were detectable after periods as short as 60 min, but were maximal after 4 hours of acidification.

Glycoconjugate secretion was assessed by measuring the incorporation of <sup>3</sup>H glucosamine into macromolecules present in apically and basally conditioned medium after periods of surface acidification from 30 min to 4 hours with a range of pHs down to 5.0. None of the exposures to surface acid caused significant increases or decreases in glycoconjugate secretion into either compartment (Table 1). These observations are consistent with the recently published observation that exposure of hamster tracheal epithelium to acid caused a slight decrease in glycoconjugate secretion, but only after exposure to pH 4 (12). Surface acidification had no effect on total fibronectin synthesis or secretion or on the synthesis and secretion of fibronectin containing the alternatively-spliced EIIIA region.

The concentration of TGF $\beta$  in apically and basally conditioned medium from control cultures was always below the lower limit of detection of the mink lung bioassay. In an effort to increase the baseline signal, we made numerous changes in the culture conditions, including omission of each of the growth factors present in our supplemented growth medium, a range of concentrations of retinoic acid, and a variety of plating densities. Despite these manipulations, we were never able to reproducibly detect TGF $\beta$  secretion from our cells. To determine whether surface acidification increased TGF $\beta$  secretion, we assessed the effects of surface acidification for up to 4 hours at pHs down to 5.0 on both activated and inactive TGF $\beta$  (acid-activatable). We were unable to detect any TGF $\beta$  secretion from these cells after any exposure to acid.

Cell viability was assessed after exposure to each condition of acidification by measurement of LDH release. None of the conditions tested caused release of significantly more LDH than that seen after control exposures (Table 2). In addition, the potential cytotoxic effect of the most extreme exposure (to pH 5 for 4 hours) was assessed by measurement of SICr release. Again, no cytotoxicity attributable to acidification was detected.

#### 4. Exposure to nitric acid vapor

Because the major effect of surface acidification was on the pattern of protein synthesis, we focused our initial studies of the effects of nitric acid vapor on this endpoint. In preliminary studies we established the generation conditions required to produce stable chamber atmospheres containing nitric acid vapor concentrations ranging from 50 to 1000  $\mu\text{g}/\text{m}^3$ . Repeated 10 min samples at 15 min intervals demonstrated that the chamber concentration varied by less than 5%. In experiments performed with two impingers in series, no nitrate was ever detectable in the second impinger. All of the nitrate could be captured in a single acid-etched, coated tube, confirming the absence of particulate nitrate in our system. To avoid any effects due to surface drying, exposures to nitric acid vapor were all performed for 2 hours or less. However, to ensure that we did not miss any delayed effects of nitric acid on protein synthesis, pulse labelling with  $^{35}\text{S}$ -methionine was performed either immediately or at 1 hour, 3 hours, 5 hours, 7 hours or 20 hours after exposure. Initial studies revealed no effects of any concentration of nitric acid vapor on either overall protein synthesis or on the induction of hsp 72 or grp 78.

Because we were surprised by the failure of nitric acid vapor to mimic the effects of surface acidification, we next assessed the effects of progressively increasing concentrations of nitric acid vapor, up to a maximal concentration of  $18,000 \mu\text{g}/\text{m}^3$ , on the same endpoints. In these studies, none of the concentrations of nitric acid vapor tested had any reproducible effect on protein synthesis or on stress-protein induction.

In additional experiments, we also examined the effects of a range of nitric acid concentrations on glycoconjugate secretion, fibronectin synthesis and secretion, and secretion of  $\text{TGF}\beta$ , and found no significant effects. No

concentration of nitric acid vapor, up to 18,000  $\mu\text{g}/\text{m}^3$ , had any effect on cell viability.

### 3. Effects of ozone

In contrast to acidification, ozone caused a concentration-dependent increase in cytotoxicity, as revealed by both an increase in LDH release and in the release of SICr. As shown in figure 3, exposure to 0.05 ppm ozone caused a 5.4% increase in specific SICr release, and exposure to 0.2 ppm caused a 19.2% increase.

Metabolic labelling during and for 30 minutes after 0.05, 0.10, or 0.20 ppm ozone exposure demonstrated no significant changes in the pattern of protein synthesis by tracheal epithelial cells (figure 4). Additional experiments with intermittent pulse-radiolabelling up to 22 hours after ozone exposure revealed no major changes in protein synthesis. Higher resolution analysis of radiolabelled proteins by 2-D gel electrophoresis also did not demonstrate significant induction of stress protein synthesis in these cells (figure 5).

Consistent with results from metabolic labelling, western immunoblotting of celllysates from air and ozone exposed tracheal epithelial cells demonstrated no significant induction of hsp 72 or hsp 73 after ozone exposure, whereas there was marked induction of hsp 72 (and to a lesser extent hsp 73) after heat shock treatment (figure 6). Interestingly, a significant amount of hsp 72 was consistently observed under normal growth conditions. This does not represent an *in vitro* artifact since epithelial cells immediately isolated from the trachea of sacrificed guinea pigs also exhibited low level constitutive expression of hsp 72.

To examine the possibility that ozone may somehow prevent the induction of stress protein synthesis, tracheal epithelial cell monolayers were simultaneously exposed to 0 or 0.20 ppm ozone and 5 or 75  $\mu\text{M}$  sodium arsenite followed by pulse-radiolabelling with [ $^{35}\text{S}$ ]methionine. In the presence of ozone, sodium arsenite still resulted in increased stress protein synthesis (figure 7). In a parallel experiment, simultaneous epithelial cell heat shock treatment and ozone exposure elicited synthesis of heat shock proteins. Therefore, in the presence of ozone, respiratory cells are still capable of increased stress protein synthesis in response to heat-shock.

Since previous studies in many cell types have demonstrated that induction of heat shock proteins is associated with enhanced cell survival after a subsequent stress treatment (9, 10, 11), we examined whether a prior heat shock treatment would confer protection against a subsequent ozone exposure. Tracheal epithelial cells were incubated at either 37° or 43° C for one hour, allowed to recover overnight at 37° C, and then exposed to air or 120 ppm ozone. SDS-PAGE analysis confirmed the increased synthesis of the 72, 73, 90, and 110 kD heat-shock proteins in the 43° C treated cells. Despite the increased concentration of stress proteins in the heat-shock treated cells, there was no difference in the magnitude of ozone-induced <sup>51</sup>Cr release between the control and the previously heat-shocked cells.

Because we were unable to generate any concentration of ozone that did not produce cell lysis, we were unable to directly examine the effects of ozone, if any, on secretion of glycoconjugates, fibronectin, or TGFβ. One dimensional SDS-PAGE of apically and basally conditioned medium from metabolically labelled epithelial cells exposed to ozone, always revealed marked increases in essentially all intracellular proteins, including cytoskeletal proteins such as actin. Thus, we designed and constructed a cDNA probe specific for guinea pig fibronectin and another specific for the alternatively spliced E11A domain of guinea pig fibronectin, and used these probes to assess the effect of ozone exposure on the concentration of mRNA encoding the two principal forms of fibronectin in guinea pig tracheal epithelial cells. No concentration of ozone, from 0.05 to 0.2 ppm, significantly increased the expression of either form of fibronectin mRNA.

## Discussion

The experiments performed under this contract were exploratory in nature, intended to determine whether or not exposure to nitric acid and/or ozone would produce important effects on a range of biological endpoints. These studies demonstrated two significant positive findings: that surface acidification of tracheal epithelial cells induces stress protein synthesis without causing cytotoxicity, and that exposure of the same cells to concentrations of ozone as low as 0.05 ppm causes cytotoxicity but does not induce a stress-response. I will discuss the significance of each finding separately.

The stress-response is characterized by a general inhibition of overall protein synthesis and by specific induction of synthesis of a small number of proteins thought to play a role in the protection of cells from the effects of protein denaturation. This response is now widely recognized as a general mechanism by which most cells defend themselves against a wide range of adverse environmental conditions. The identification of such a response to surface acidification suggests that conditions which lead to prolonged acidification of the airway luminal surface could be deleterious to airway epithelial cells. However, the absence of any evidence of cell death after acidification suggests that these cells may be well defended against acidification, perhaps in part due to the increased concentrations of stress proteins.

The direct relevance of these findings to environmentally-relevant exposures to atmospheric acidity is uncertain. There is no direct data available about the expected effects of acid pollution on airway luminal pH. Furthermore, we were unable to produce the same effect with in vitro exposure to any concentration of nitric acid vapor, even concentrations at least 2 orders of magnitude higher than the concentrations found in the environment. However, since it is not possible to precisely mimic in vivo exposure conditions in vitro, these results do not exclude any potential in vivo effect of inhaled acid on stress protein production by airway epithelial cells. For example, it is conceivable that under the low flow conditions present in our exposure chamber, nitric acid vapor was adsorbed by the tissue culture plasticware, and therefore not present in the expected concentration on the surface of the epithelial cells.

Neither nitric acid vapor, nor surface acidification produced any detectable effect on any of the other biochemical endpoints we examined in this study. One of these endpoints, glycoconjugate secretion, was recently examined by other investigators using hamster tracheal epithelial cells. In that study, the range of pHs we examined were also without effect, but exposure to pH 4 caused a slight but statistically significant decrease in secretion.

Previous studies examining the effects of ozone on respiratory cells in culture demonstrated little or no cytotoxic effect of ambient concentrations of ozone, despite in vivo evidence to the contrary. Earlier in vitro exposure methods utilizing rocker platforms or tilted rotating plates interposed a layer of medium between the cells and gaseous ozone for all or part of the exposure

period. Our laboratory and others have cultured airway epithelial cells on filter supports at an air-liquid interface to allow direct contact with the gas phase, similar to *in vivo* conditions (12). Using this *in vitro* exposure method, we have demonstrated ozone-induced cytotoxicity in both epithelial cells and alveolar macrophages at ozone concentrations as low as 0.05 ppm, which correlates well with *in vivo* evidence of ozone-induced cytotoxicity and inflammation at concentrations as low as 0.08 ppm (13). These concentrations of ozone are well within the current National Ambient Air Quality Standard of 0.12 ppm and reflect ambient concentrations in many communities in the United States (14).

No significant acute or delayed ozone-induced changes in the pattern of protein synthesis were detected by 1-D or 2-D gel analysis. Although ozone exposure caused significant cytotoxicity, the lack of stress protein synthesis after ozone exposure was not a consequence of cellular cytotoxicity itself because induction of stress protein synthesis was observed after other stresses (e.g. heat shock treatment) associated with significant cellular cytotoxicity.

Previous studies in many cell types have demonstrated that induction of heat shock proteins is associated with enhanced cell survival after a subsequent stress treatment (9, 10, 11). However, the stressors examined after an earlier heat shock treatment (e.g. a second heat shock treatment or sodium arsenite exposure) were all capable of independently inducing stress protein synthesis. The absence of stress protein synthesis after exposure to ozone, and the finding that prior induction of heat shock proteins did not decrease the cytotoxicity of a subsequent ozone exposure, suggest that induction of stress proteins may not serve an important role in protecting respiratory cells from oxidant stress. Since the primary site of ozone-induced cellular injury is thought to be the plasma membrane, ozone-induced plasma membrane lipid peroxidation (and to a lesser extent protein peroxidation) may lead to rapid membrane disruption and cell lysis in the absence of major alterations in intracellular protein synthesis (15). If stress proteins exert their protective effects by interacting with damaged intracellular proteins, they may not prevent cell death caused by plasma membrane lysis.

Interestingly, we were not able to demonstrate any important effects of *in vitro* exposure to ozone on cellular metabolism. The principal effect appeared to simply be cell lysis. This observation raises the possibility that some of the effects of ozone seen *in vivo* may be initiated by products

released from lysed epithelial cells rather than by effects of ozone on the synthesis or secretion of specific mediators by these cells.

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## The Response of Guinea Pig Airway Epithelial Cells and Alveolar Macrophages to Environmental Stress

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Cells lining the respiratory tract form an interface between the organism and the external environment and are repeatedly exposed to physical, chemical, and metabolic stresses. We examined the response of cultured guinea pig tracheal epithelial cells and alveolar macrophages to various forms of stress, including clinically and environmentally relevant metabolic stresses such as ozone and acid exposure. Classic stress treatments such as heat shock and sodium arsenite treatment induced the synthesis of 28, 32, 72, 73, 90, and 110 kD stress proteins similar to those observed in other cell types. In contrast, no significant changes in the pattern of protein synthesis were detected after exposure to ambient concentrations of ozone, although ozone exposure caused significant cytotoxicity to both cell types. Another potent oxidant, hydrogen peroxide, similarly did not induce appreciable stress protein synthesis. However, surface acidification of tracheal epithelial cells and alveolar macrophages caused the induction of 72 and 78 kD stress proteins. While stress proteins may play a role in the response of respiratory cells to certain injuries such as hyperthermia and surface acidification, they may not be important in the defense against ozone or other forms of oxidative injury.

Cells lining the respiratory tract are situated at an air-tissue interface where the organism encounters the external environment. These cells represent the organism's "first line of defense" against inhaled or aspirated particles, organisms, and pollutants such as ozone (1, 2). Alterations in gene expression following environmental stress in respiratory epithelial cells and alveolar macrophages have not been well characterized.

Most organisms defend themselves against adverse changes in their environment by altering their patterns of gene expression. One remarkably well-conserved cellular response to abrupt changes in local environmental conditions consists of the increased and selective synthesis of a small group of proteins referred to as heat shock or stress proteins. The transient synthesis of this family of proteins after heat shock treatment or after other physical, chemical, and metabolic insults has been observed with only minor variations in most cell types studied to date (3-5). Stress proteins perform important metabolic functions in the unstressed cell and appear to function in a protective or reparative manner after stress (6-11). Environmental stresses

relevant to specific cell types, such as the effects of ozone or acid exposure on respiratory cells, have not been studied in the context of the stress response.

Studies in both animals and humans have demonstrated significant functional and structural respiratory tract abnormalities after exposure to ambient concentrations of ozone (2, 12-14). The effects of ozone on specific lung cell types have been difficult to study due to difficulties in establishing an *in vitro* exposure system that mimics *in vivo* conditions. Previous *in vitro* studies reported minimal or no cytotoxic effects of ambient concentrations of ozone on cultured epithelial cells and alveolar macrophages (15-17). In contrast, a recent *in vivo* study in human subjects described increased lactate dehydrogenase (LDH) release into bronchoalveolar lavage fluid after exposure to as little as 0.08 parts per million (ppm) of ozone, suggestive of ozone-induced cytotoxicity (18, 19). Although the basis for this difference between *in vitro* and *in vivo* findings is unclear, previous *in vitro* exposure systems utilized rocker platforms or tilted rotating plates that interposed a layer of medium between the cells and gaseous ozone for all or part of the exposure period. This liquid barrier may have resulted in decreased contact between the ozone gas and the cultured cells under study.

Airway epithelial cells and alveolar macrophages are frequently exposed to acidic conditions due to the aspiration of acidic gastric contents into the respiratory tract during deep sleep (20). Although acid aspiration has been associated with severe lung injury and significant mortality in certain clinical situations (21, 22), most people do not develop lung injury after nocturnal acid aspiration. Inhalation of acid aerosols into the respiratory tract as a result of acidic air pol-

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Abbreviations: heat shock proteins, hsp; lactate dehydrogenase, LDH; phosphate-buffered saline, PBS; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE.

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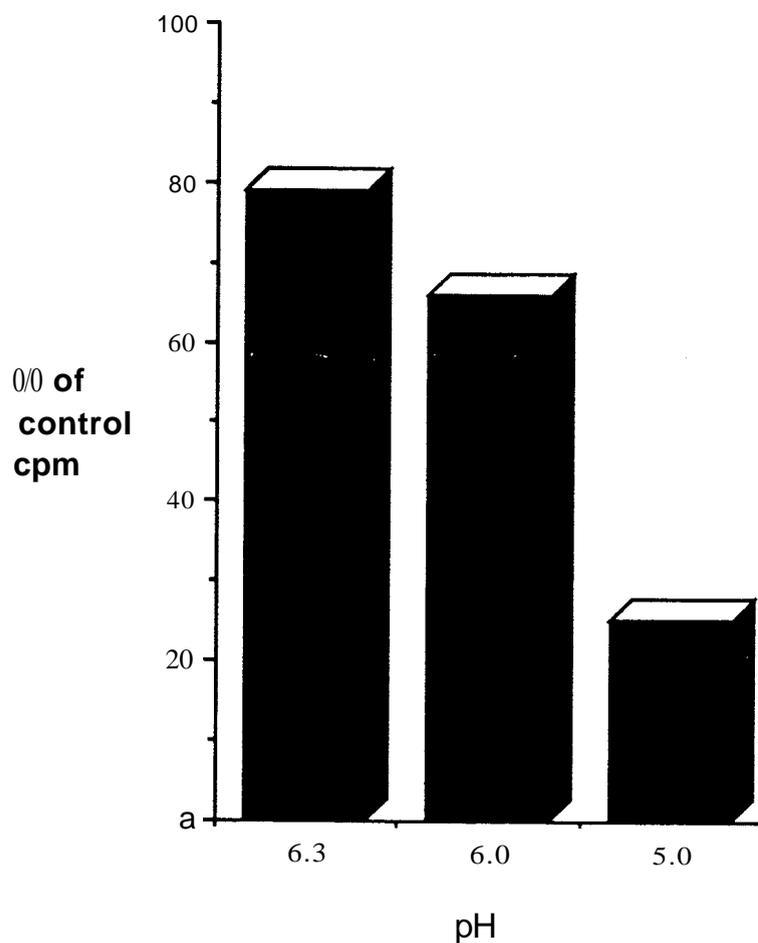


Figure 1 Effect of surface acidification on protein synthesis. The apical surface of confluent monolayers of tracheal epithelial cells were apically exposed to isotonic 0.05M MES pH 5.0, 6.0, 6.3, or 7.0 for four hours while being radiolabelled from below with [<sup>35</sup>S]methionine. Cells were then harvested and a portion of the lysates was analyzed in a liquid scintillation counter to determine the [<sup>35</sup>S]methionine activity. Activity in lysates of cells exposed to pH 7.0 were used as control.

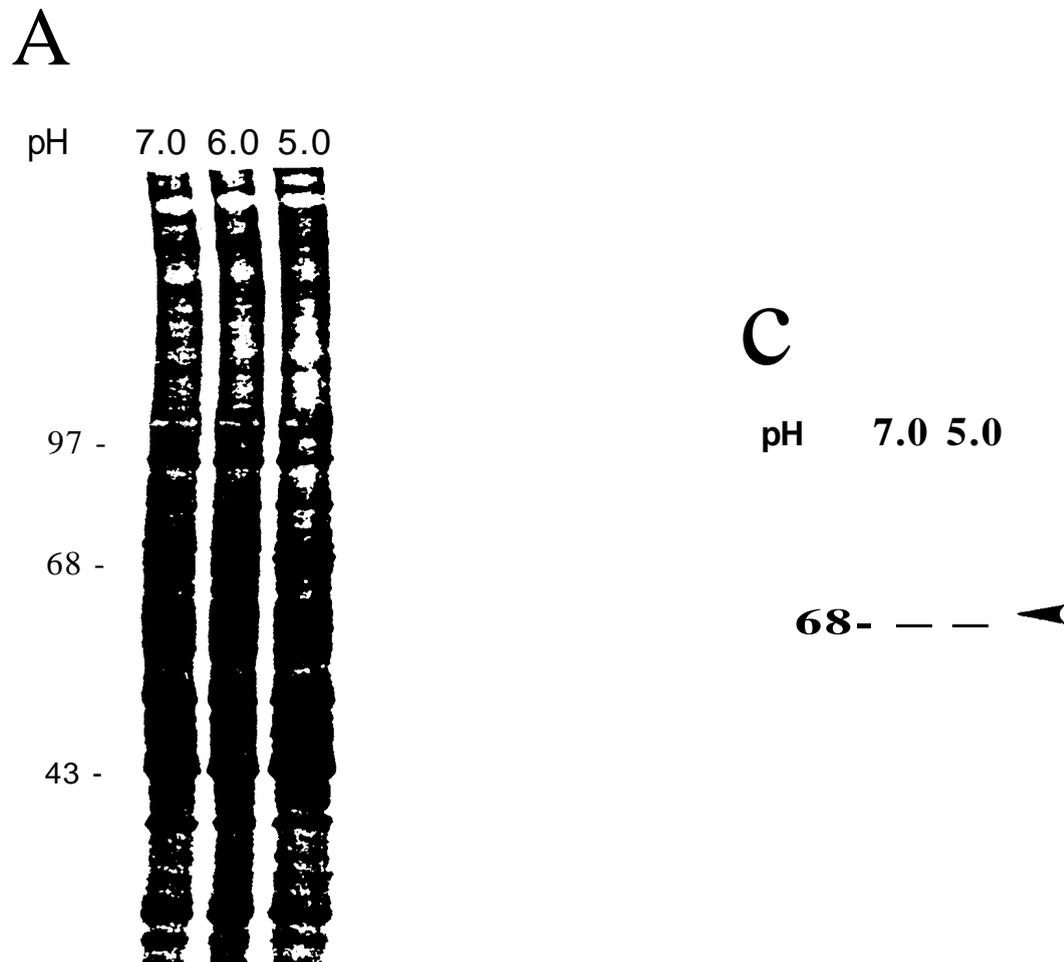


Figure 2 Surface acidification induces stress protein synthesis in respiratory cells. Tracheal epithelial cells (A and C) were apically exposed to isotonic 0.5M MES pH 5.0, 6.0, or 7.0 for four hours, washed, and subsequently radiolabelled with [ $^{35}$ S]methionine for 1.0 hour under normal pH conditions. After labelling, cells were harvested and the radiolabelled proteins analyzed by SDS-PAGE. Shown in panel A is the autoradiogram with molecular mass markers indicated at the left. The major acid-induced stress proteins of 78, and 72 kD are indicated by arrowheads at the right. Shown in panel C is a Western immunoblot of tracheal epithelial cell lysate after exposure to pH 7.0 and pH 5.0. The blot was probed with antibody (C92) specific for hsp 72.

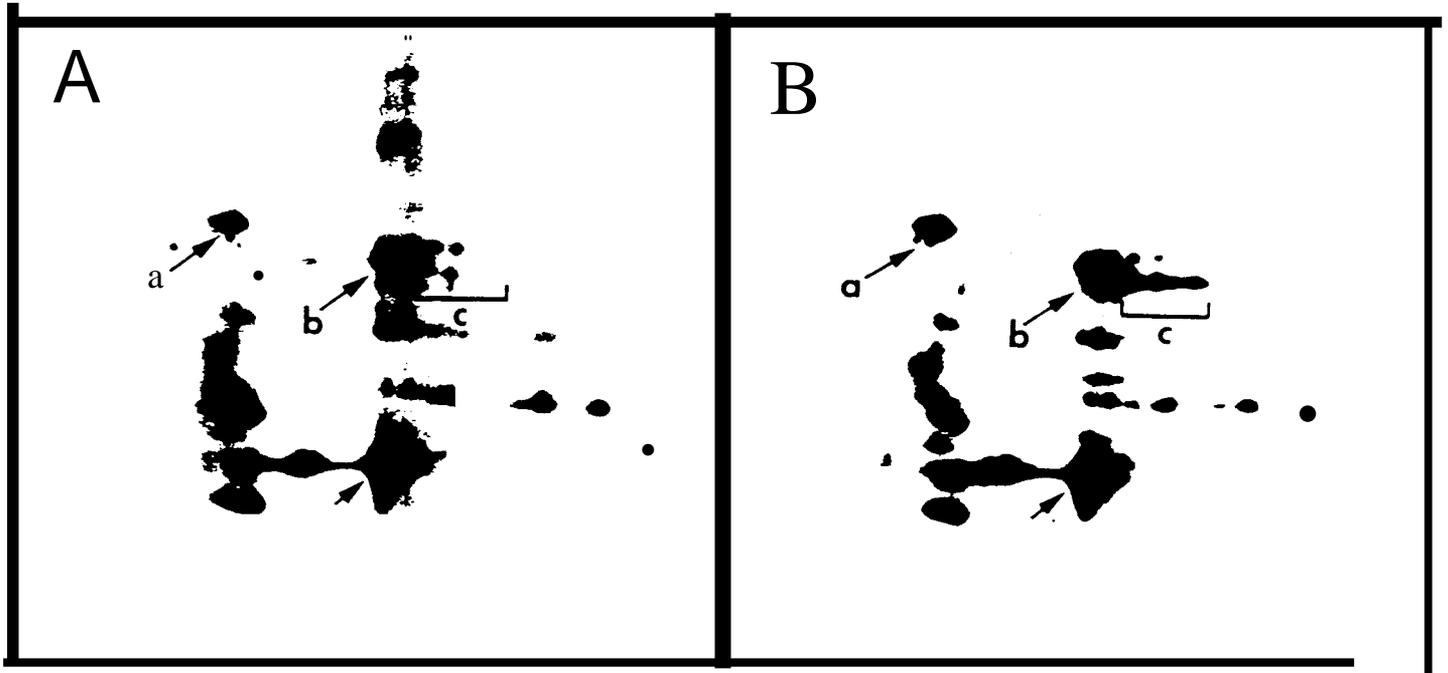


Figure 3 Two-dimensional gel analysis of acid-induced proteins in respiratory cells. Tracheal epithelial cells were apically exposed to isotonic 0.05M MES of pH 7.0 (A) or pH 5.0 (B) for four hours, washed, and subsequently radiolabeled with [ $^{35}$ S]methionine for 1.0 hour under normal pH conditions. Celllysates were analyzed by two-dimensional gel electrophoresis (acidic end of the gels is to the left). The acid-induced stress proteins are indicated by an arrow and designated as: a, 78 kD; b, 73 kD; and c, 72 kD (multiple isoforms are indicated). The position of actin is indicated by an unlabelled arrowhead.

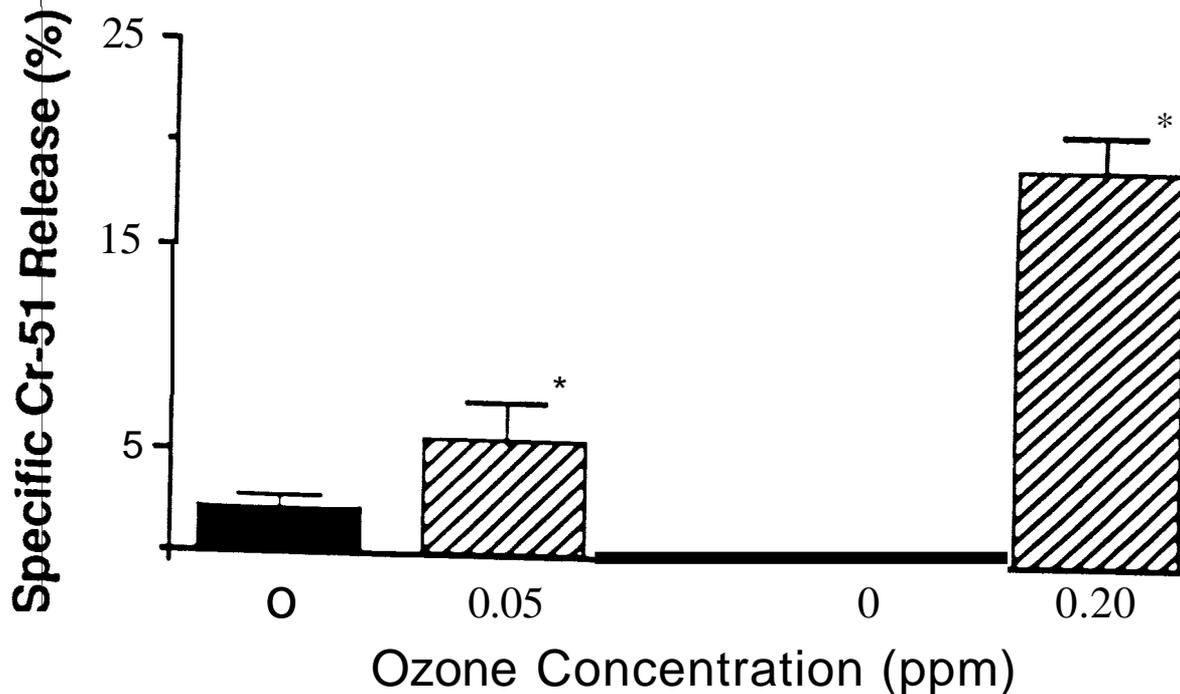


Figure 4. Ambient concentrations of ozone cause dose-dependent cytotoxicity as determined by  $^{51}\text{Cr}$  release assays. Tracheal epithelial cells cultured on porous filters were exposed to 0, 0.05, or 0.20 ppm ozone for 1 hour after prior radiolabelling with  $^{51}\text{Cr}$ . Immediately post-exposure, the apical cell surfaces were incubated with 0.5 ml of medium for 5 minutes for subsequent gamma counting to quantitate the amount of  $^{51}\text{Cr}$  released. All exposure conditions were repeated in triplicate. Specific  $^{51}\text{Cr}$  release (%) was calculated using the formula  $[(E-S)/(T-S)] \times 100$ , where E =  $^{51}\text{Cr}$  released under experimental conditions, S =  $^{51}\text{Cr}$  released spontaneously, and T = total releasable  $^{51}\text{Cr}$  after cell lysis with 2% triton. \* $p < .005$ .

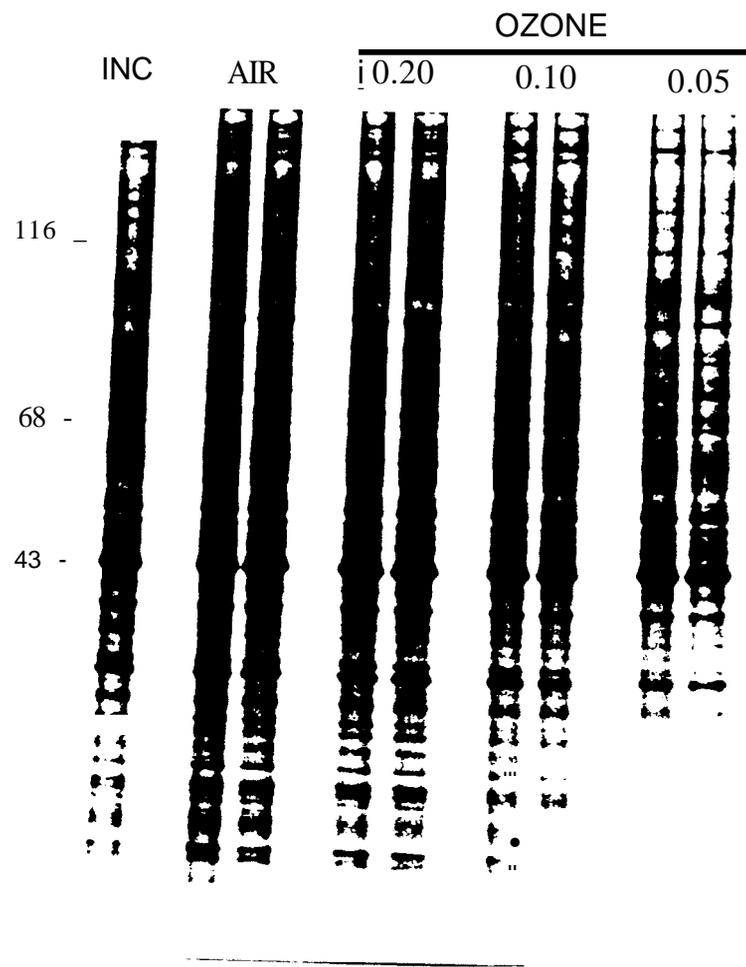


Figure 5. Ambient concentrations of ozone do not induce stress protein synthesis in airway epithelial cells. Tracheal epithelial cells cultured on microporous filters were exposed to 0,0.05,0.10 and 0.20 ppm ozone at 37° C for 1 hour and radiolabelled with [<sup>35</sup>S]methionine during ozone exposure and subsequently for 30 minutes. Control cells (not exposed to ozone) were either maintained in a standard 37° C incubator (INC) or exposed to humidified filtered air in a 37° C exposure incubator (AIR). After labelling, the cells were harvested and the radiolabelled proteins analyzed by SDS-PAGE. Shown are the autoradiograms of the gels with molecular mass markers indicated at the left of each autoradiogram.

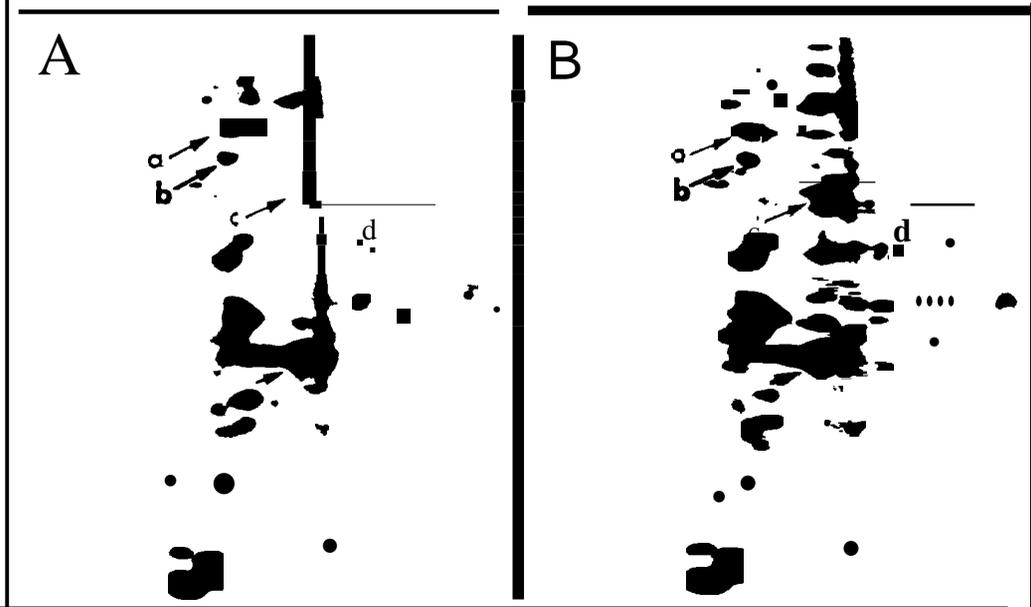


Figure 6. Two-dimensional gel analysis of radiolabelled proteins in airway epithelial cells ozone exposure. Tracheal epithelial cells cultured on microporous filters were exposed to 0.20 ppm ozone (B) or filtered air (A) at 37°C for 1 hour and allowed to recover in a standard 37°C incubator. Cells were pulse-radiolabelled with [35S]methionine for 1.5 hours beginning 6 hours after ozone or air treatment. After labelling, the cells were harvested and the radiolabelled proteins analyzed by two-dimensional gel electrophoresis (acidic end of the gels is to the left). Shown are regions of the fluorographed gels illustrating the major stress proteins. The individual stress proteins are indicated by a letter to the lower left and designated as: a, 90 kD; b, 77 kD; c, 73 kD; d, 72 kD; and e, 32 kD. The position of actin is indicated by an unlabeled arrowhead. Minor differences in the total cpm loaded on autoradiogram B as compared to autoradiogram A result in the appearance of slightly increased intensity of proteins on autoradiogram B.

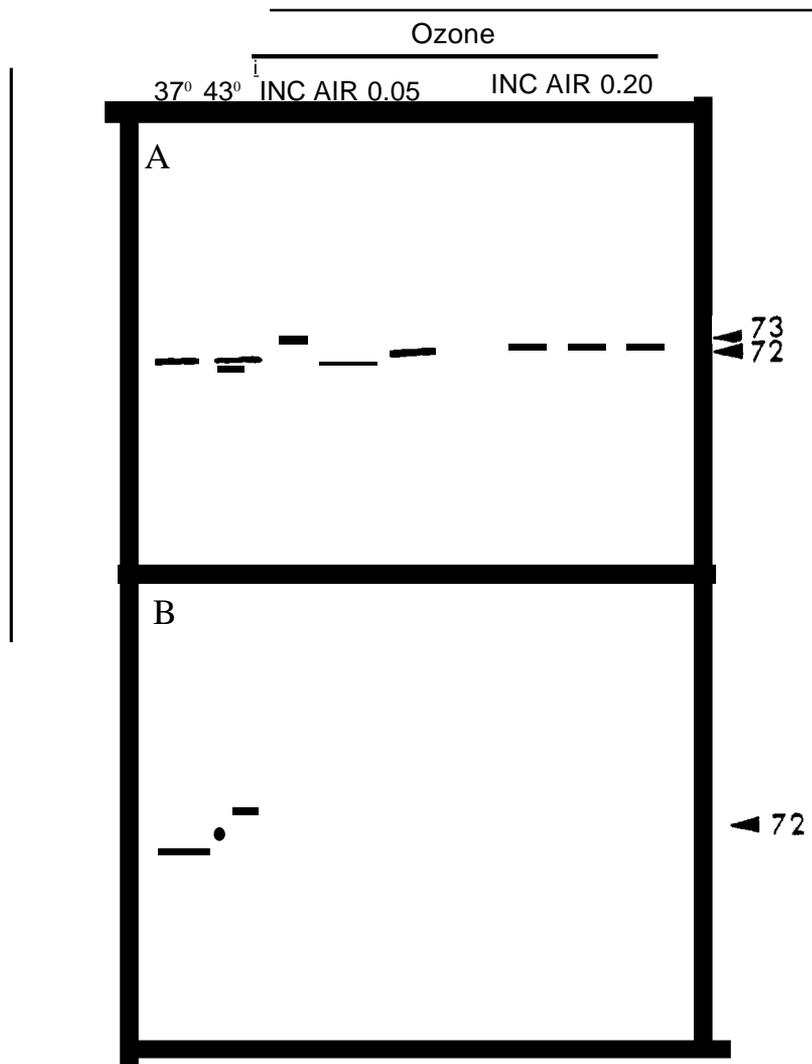


Figure 7 Ozone exposure does not induce synthesis of the major 72 kD inducible heat shock protein in airway epithelial cells. Tracheal epithelial cells cultured on microporous filters were exposed to heat shock or ozone as previously described. Celllysates of epithelial cells incubated at 37° C (lane 1) or 43° C (lane 2) or of epithelial cells maintained in a standard 37° C incubator (lanes 3 and 6), exposed to humidified filtered air (lanes 4 or 7), or exposed to ozone concentrations of 0.05 ppm (lane 5) or 0.20 ppm (lane 8) were resolved by SD5-PAGE and transferred to nitrocellulose. Matched samples from 0.05 ppm and 0.20 ppm ozone experiments are represented in lanes 3-5 and 6-8 respectively. After incubation with antibody (N27) immunoreactive with both constitutive and inducible members of the hsp 70 family (panel A), or antibody (C92) immunoreactive only with the inducible hsp 72 (panel B), the bound antibodies were visualized by alkaline-phosphatase-mediated color development. The 72 and 73 kD stress proteins are indicated by arrowheads.

TABLE 1

	pH			
	7.40	7.00	6.00	5.00
Glycoconjugate Secretion				
Top (cpm)	19580	17680	17040	16280
Bottom (cpm)	4660	6240	5820	6500

Effect of surface acidification on glycoconjugate secretion. Tracheal epithelial cells were labelled overnight with [3H]glucosamine hydrochloride. Cell surfaces were washed and isotonic 0.05M MES buffer pH 7.4, 7.0, 6.0, and 5.0 was placed on the apical surface. After 30 minutes of incubation, samples were collected from top and bottom compartments and desalted. A portion of the samples was counted by liquid scintillation.

TABLE 2

35

	pH			
	7.40	7.00	6.00	5.00
% of Total Cellular IDH Released	0.8%-1.5%	0.9%-1.4%	1.6%-2.4%	2.4%-4.1 %
Intact Cells (in millions)	2.6	2.3	2.8	2.2

Effects of surface acidification on cellular viability were assessed by placing isotonic 0.05M MES buffer at various pH values on the apical surface of confluent cell layers. After four hours of incubation, LDH activity in the apical fluid was spectrophotometrically assayed (LD-L Kit, Sigma Chemicals) and intact cells were counted in a hemocytometer after removal from membranes with trypsin. Total cellular LDH was determined by lysing several dishes of cells in 0.2% Triton X-1 DO/PBS, assaying total LDH in the lysates, and averaging the results.

TABLE 3 TABULAR SUMMARY OF DATA

Endpoint	Surface Acidification	Nitric Acid Vapor	Ozone
Cytotoxicity			increased
Overall Protein Synthesis	decreased		decreased
Stress Protein Synthesis	increased		
TGF $\beta$ Secretion			
Glycoconjugate Secretion			
Total Fibronectin mRNA			
EIIIA Fibronectin mRNA			