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Guinea Pig Ozone-Induced Airway Hyperreactivity is Associated with Increased N-Acetyl-β-D-Glucosaminidase Activity in Bronchoalveolar Lavage Fluid

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Abstract. High level ozone exposure is known to cause acute, neutrophil-independent airway hyperreactivity in the guinea pig. The precise biochemical mechanisms involved remain unclear. Because of its potential pathophysiologic importance, we examined whether a lysosomal hydrolase, N-acetyl-β-D-glucosaminidase (NAGA) was released from the airways in vivo and from bronchoalveolar cells, specifically macrophages. Muscarinic reactivity was determined by measuring specific airway resistance (sRaw) in response to increasing doses of aerosolized acetylcholine in guinea pigs that were either exposed to air or to ozone (3.0 ppm, 2 h). The ozone-exposed animals showed substantial muscarinic hyperreactivity 30 min after exposure. In addition, both total and percent released NAGA in bronchoalveolar lavage fluid obtained immediately after reactivity testing were significantly greater in the ozone-exposed group. It was also found that substantially more NAGA was released from mixed bronchoalveolar lavage cells in response to 20 μM A23187. Moreover, bronchoalveolar macrophages of ozone-exposed animals secreted more NAGA upon stimulation in vitro by either 20 μM A23187 or 200 μg/ml opsonized zymosan. We conclude that ozone-induced airway hyperreactivity in guinea pigs is associated with the presence of increased NAGA activity in bronchoalveolar fluid. Our data suggest that bronchoalveolar macrophages may, at least in part, be responsible for release of this enzyme into the airways after ozone exposure.

Key words: Asthma—Bronchial reactivity—Hexosaminidase—Lysosomal hydrolase—Oxidants.

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Introduction

Ozone, an oxidant gas found in photochemical smog, causes acute and chronic airway injury in a variety of species, including man [2, 4, 12, 15, 23, 29]. In guinea pigs, exposure to high levels of ozone is associated with airway hyperreactivity which is neutrophil independent [24]. The biochemical mechanisms of this disorder are not well understood at present.

Lysosomes of phagocytes are known to be critical in the lung's response to inhaled particulates. Lysosomal hydrolases, in particular, may be pathophysiologically important in various diseases due to their destructive effects on tissues [19, 28]. Their potential damaging effect on airway cells, especially those of the respiratory mucosa, may be germane to the pathogenesis of airway hyperreactivity [23].

The precise changes in lung hydrolase activity that occur after acute, high level ozone exposure are unclear based upon past work. Hurst et al. [17] described a shift of hydrolases in rabbit alveolar macrophages exposed to ozone (0.25–7.0 ppm, 3 h) from lysosomal to cytosolic compartments such that the lysosomal fraction lost and the soluble cellular extract gained activity. In addition, the authors showed that hydrolase activities (acid phosphatase, lysozyme and B-glucuronidase) in whole cells from ozone exposed animals were depressed as much as 50% from baseline. The maximal effect, which lasted for 24 h, was shown to occur after an exposure to 3 ppm for 3 h. Whether or not the depressed intracellular activity was due to secretion of hydrolases into the extracellular space was not addressed in the study. Other investigators have described an increase in hydrolase (acid phophatase and N-acetyl-β-D-glucosaminidase; NAGA) activity of whole lungs from rats or mice after both brief, lower level exposures and continuous, higher level exposures [5, 8, 32]. Airway hydrolase activity was not evaluated in these reports. We were particularly interested in whether there was an association between bronchoalveolar hydrolase activity and the development of airway hyperreactivity in animals exposed to ozone. Therefore, we assessed NAGA activity in bronchoalveolar lavage fluid and the airway reactivity of ozone-exposed guinea pigs compared to room air-exposed animals. To relate changes in hydrolase activity in lavage fluid to that of bronchoalveolar cells, we also measured the release of NAGA post-ozone from bronchoalveolar cells in vitro and specifically from bronchoalveolar macrophages in response to opsonized zymosan (OPZ) and to the calcium ionophore A23187.

Methods

Protocol

Fourteen male Hartley strain guinea pigs (600–700 g, body wt) were evaluated physiologically and by bronchoalveolar lavage initially. Animals were exposed while awake, resting, and spontaneously breathing at rest to either 3.0 ppm of ozone (experimental) or to room air (control) for 2 h. Specific

airway resistance (sRaw) and bronchial reactivity to acetylcholine (ACh) were measured 30 min after exposure in the 7 pairs of animals. Immediately thereafter, the guinea pigs were anesthetized with ketamine/zylazine IM (80/15 mg/kg) and bronchoalveolar lavage was done through a tracheostomy using warmed (37°C), endotoxin-free phosphate buffered saline (PBS) in a total volume of 50 ml per each animal (10 ml × 5) under sterile hood. From each animal, lavage fluids and bronchoalveolar cells were separated by centrifugation at 300 g for 15 min at 25°C. A fraction of bronchoalveolar cells were stained with crystal violet as well as Wright-Giemsa stain for total and differential cell counts by hemocytometer. Both the bronchoalveolar cell population and the macrophages separated from these were evaluated in vitro thereafter.

Airway Studies in vivo

Specific airway resistance (sRaw) and muscarinic reactivity were measured as previously described in intact, unanesthetized, spontaneously breathing guinea pigs [26]. Briefly, each animal was positioned in a two-compartment lucite chamber designed to keep its head fixed (with mouth closed) and isolated from its body and the plethysmograph. Flow at the snout was measured using a pneumotachograph (no. 0, Fleish Instruments, Pres Lausanne, Switzerland) connected to a differential pressure transducer (model MP45-1, Validyne, Northridge, CA). The animal in the chamber was placed in the plethysmograph, which was equipped with another transducer for measuring changes in box pressure. The pneumotachograph was calibrated using a rotometer (model 1355-01C1AAA, Brooks Instrument, Hatfield, PA) to pass known airflows through it. Changes in box pressure were calibrated (with the box tightly sealed) by rapidly delivering boluses of air from a syringe. Airflow and box pressure signals were displayed simultaneously on an X-Y oscilloscope (model 502A, Textronix, Portland, OR). The angle described during the rapid in the respiratory phase of the animal's breathing was measured, and sRaw was calculated from it as previously described [1].

The animals were exposed awake and spontaneously breathing at rest in a 1 m^3 Rochester chamber. Ozone was generated by passing 100% O_2 through an ozonizer (type III, Sander, Frankfurt, West Germany) regulated by a variable voltage supply. The ozone level was monitored using a ultraviolet ozone analyzer (model 1003 AH, Dasibi, Glendale, CA) calibrated by comparison to a reference source from the Tennessee Public Health Department.

Bronchial reactivity was assessed by measuring sRaw as a function of concentration of ACh inhaled. Thirty minutes before testing, each animal was pretreated with propranolol (10 mg/kg in 0.9% normal saline injected intraperitoneally) to decrease variation in airway bronchoconstrictor responsiveness within and between animals due to endogenous beta-adrenergic stimulation [10, 27]. Reactivity to inhaled ACh was determined using a nebulizer (model 1700, Hudson, Temecula, CA) driven by a compressed-air source at 28 psi (nebulizer output: 290 ± 8 μl/min; aerosol particle size: 4.28 ± 1.62 μm aerodynamic mass median diam.). After sRaw was determined before and after 30 breaths of PBS, 30 breaths of 0.01% ACh (in PBS) were administered, and the peak sRaw over the next 5 min was recorded. The concentration of ACh was then approximately doubled, and the delivery was repeated. This process was repeated until at least a doubling of the baseline sRaw occurred. Cumulative ACh aerosol dose-response curves were constructed by plotting, on semilogarithmic paper, base-line sRaw and the peak values of sRaw for each dose of ACh administered. The effective dose of ACh that produced a doubling of base-line sRaw (ED200ACh expressed as percent) was determined by interpolation. Animals showing a 50% decrease in ED200ACh after ozone exposure were considered hyperreactive. Mean values for the ED200ACh were expressed as the geometric mean and standard error. Changes in log values of ED200ACh for each animal pair (ozone- and air-exposed) were compared using the Student t test. In all cases, differences were considered significant for $p < 0.05$.

Bronchoalveolar Macrophage Separation

Alveolar macrophages were separated by Ficoll-Hypaque density gradient (s.g. 1.077). Fifteen ml of Ficoll-Hypaque was underlaid through 16 G spinal needle and cells were centrifuged at 400 g for

30 min at 25°C. The macrophage layer (top layer) was recovered, washed once in endotoxin-free PBS by centrifugation (400 g × 30 min), and suspended in medium M199 with penicillin 100 u/ml, streptomycin 0.25 μg/ml, amphotericin 0.25 μg/ml, L-glutamine and 10% fetal bovine serum (FBS, v/v). The cell suspension was plated on 16 mm well tissue culture plates (0.5×10^6 cells/well) and incubated for 1 hr at 37°C in a humidified incubator containing 5% CO_2 and 95% air. After an hour adherence, nonadherent cells were removed and monolayers were washed with Hanks' balanced buffer solution (HBSS) twice before adding fresh M199 without FBS for cell stimulation in vitro.

Stimulation of Bronchoalveolar Lavage Cells

A23187 was dissolved in DMSO to a final concentration < 0.1%. Opsonized zymosan particles were prepared by boiling zymosan for 2 h in endotoxin-free saline followed by 3 washes by centrifugation (400 g × 15 min) and incubation with pooled human serum for 30 min. Mixed cells were stimulated with A27187 (20 μM) and macrophages were stimulated with either OPZ (200 μg/ml) or A23187 (20 μM) [3, 21] for 4 h in a humidified CO_2 incubator. For the latter, macrophages from 10 additional animals (exposed to either ozone or air) were evaluated. At the end of 4 h stimulation, media were harvested and cell lysates were prepared by adding 1 ml of 0.05% Triton-X100 to each well.

Enzyme Assays

Culture supernatants and cell lysates were analyzed for activities of a hexosaminidase (N-Acetyl-β-Glucosaminidase) as previously described by Lew et al. [20]. The lysosomal enzyme activities were determined in 96-well microtiter plates by mixing 50 μl of sample with 100 μl of prewarmed buffered substrate. After incubation at 37°C, the reactions were stopped by the addition of 100 μl of 0.5 M borate buffer, pH 10.6. Hexosaminidase activity was detected using p-nitrophenyl-2-acetamido 2-deoxy-β-D-glucosaminide (5 mM) in 0.1 M citrate/phosphate buffer, pH 4.5, with an incubation time of 1 h. The p-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically using Biotek Instruments enzyme immunoassay plate reader fitted with a 405-nm wavelength filter. Absorbance (Abs) was converted into micromoles of substrate cleaved using the Beer-Lambert Law (Molar concentration = Abs 405 nm/E × 1 (light path in cm)) and the molar extinction coefficient for p-nitrophenol (E = 18,700). Units of specific activity were defined as micromoles of substrate cleaved per hour:

$$1 \text{ unit}/10^6 \text{ cells} = 1 \mu\text{mol/h}/10^6 \text{ cells}$$

$$= \frac{\text{Abs 405 nm}}{18700 \times 0.5} \times \frac{1 \mu\text{mol}}{1 \mu\text{l}} \times 250 \mu\text{l} \times \frac{1000 \mu\text{l}}{50 \mu\text{l}} \div 1 \text{ h} \times \frac{10^6 \text{ cells}}{\text{cell ct/well}}$$

To assess cell viability, a cytosolic enzyme lactase dehydrogenase (LDH) activity in both cells and culture media was assayed by measuring the oxidation of lactic acid to pyruvic acid in the presence of NAD^+ at pH 9.0 as previously described [6]. Briefly, pyruvic acid was coupled with 2.4-dinitrophenylhydrazine (0.5 mg/ml in 1 M HCl), and absorbance at 490 nm was measured after alkalinization of the solution with 1 M NaOH. LDH release of 5% or less was considered noncytotoxic. LDH release (%) = (culture media/cells + culture media) × 100.

Drugs

Acetylcholine, propanolol and A23187 were purchased from Sigma Chemical Co., St. Louis, MO and zymosan was purchased from ICN, Inc., Cleveland, OH. Ficoll-Hypaque (s.g., 1.077), endotoxin-free PBS, A23187, antibiotic-antimycotic solution, L-glutamine and p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucosaminide were obtained from Sigma Chemical Co., St. Louis, MO.

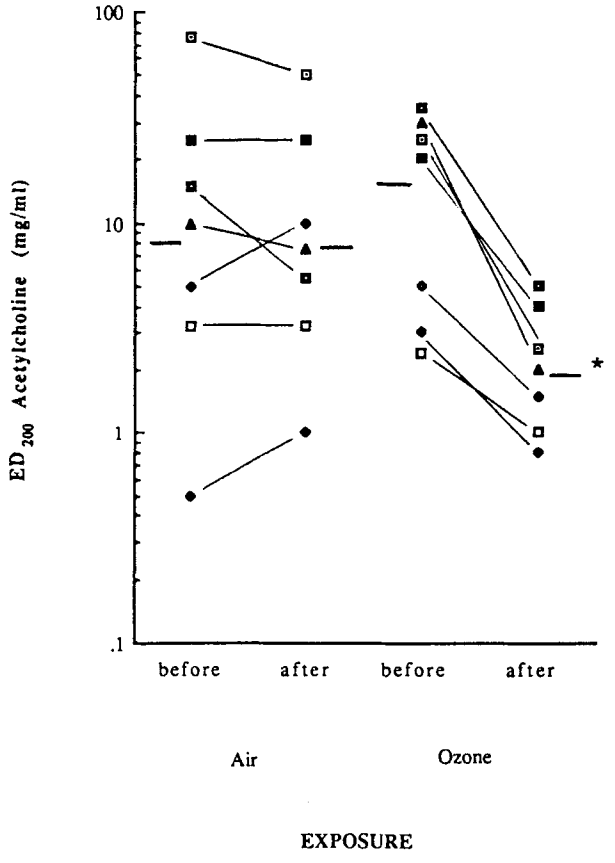

Fig. 1 Effect of exposure on muscarinic airway reactivity. sRaw in response to increasing doses of aerosolized acetylcholine were measured as described in the Methods. The ED200ACh of each case before and after exposure is shown. Lines to sides of data points indicate geometric means for the groups. In contrast to the absence of changes post-air exposure, there was a significant decrease in the ED200ACh of ozone-exposed animals. *$p < 0.05$.

Medium M199 was obtained from Whitaker MA Bioproducts, Walkersville, MD and fetal bovine serum was obtained from Hyclone Inc., Logan, UT. All reagents were tested for endotoxin content using Limulus amoebocyte lysate (Sigma) and showed less than 0.01 ng per ml.

Results

The ED200ACh of air and ozone-exposed animals before and 30 min post-exposure are shown in Figure 1. There were no significant changes in ED200ACh before and after exposure to air. In contrast, the ED200ACh of ozone-exposed animals was decreased to 12% of the baseline value (1.8 + 1.0 vs. 13.5 + 5.5 mg/ml), a substantial increase in muscarinic reactivity after ozone exposure ($p < 0.05$, $n = 7$). Hyperreactivity was present in all animals post-ozone.

The results of analysis of bronchoalveolar lavage fluid as well as bronchoal-

Table 1. Bronchoalveolar lavage fluid findings

	Animal group	
	Air-exposed (n = 7)	Ozone-exposed (n = 7)
Total cell recovery ($\times 10^6$)	8.3 ± 1.0	10.1 ± 3.5
Differential (% MØ/PMN)	89/11	72/28
Lavage yield (ml)	34.4 ± 1.9 (50)	34.8 ± 2.1 (50)
Cell viability	>95%	>95%
NAGA activity (U/10^6 cells)		
Total	0.67 ± 0.35	1.35 ± 0.15*
Cells	0.40 ± 0.05	0.50 ± 0.05
BAL fluid	0.27 ± 0.05	0.85 ± 0.15*
%Release	39.6 ± 2.3	61.0 ± 6.3*

1 unit = 1 μmol/h; MØ = macrophages; PMH = polymorphonuclear leukocytes

*$p < 0.05$

veolar cells from air- and ozone-exposed animals are shown in Table 1. Data concerning cell yield, differentials, and viability are included. Enzyme activity in the bronchoalveolar lavage fluid of ozone-exposed animals was 0.85 ± 0.15 units per 10^6 cells compared to 0.27 ± 0.05 for the air-exposed animals ($p < 0.005$, n = 7). Total activity (cells + BAL fluid) was also increased in the ozone group ($p < 0.005$, n = 7). Intracellular levels of NAGA activity in cells from ozone-exposed guinea pigs were comparable to those of cells from air-exposed animals (0.50 ± 0.05 vs. 0.40 ± 0.05 units per 10^6 cells). Cell viability of lavage fluid cells as assessed by LDH release were at least 95% in all experiments.

The results of stimulation of mixed bronchoalveolar lavage cells in vitro with A23187 (20 μM) are shown in Figure 2. Cells from ozone-exposed animals released more NAGA into the media in response to A23187 than did control animals. Mean changes in NAGA (as % of total) were 9.9 ± 2.4 for the ozone-exposed and 3.6 ± 0.8 for the control group ($p < 0.01$, n = 7).

Bronchoalveolar macrophages, separated by Ficoll-Hypaque density gradient (s.g. 1.077) and incubated for 60 min to facilitate their adherence, were stimulated in serum-free media. Results of their stimulation with OPZ (200 μg/ml) as well as A23187 (20 μM) are shown in Figure 3. There was a statistically significant elevation in NAGA release from macrophages of ozone-exposed animals compared to those of air-exposed animals in response to both A23187 and OPZ. The mean changes in NAGA in media (as % of total) were significant ($p < 0.05$). The changes were 7.1 ± 1.3 (ozone) vs 4.1 ± 0.5 (air) in response to OPZ (n = 7); and 6.3 ± 2.3 (ozone) vs. 1.7 ± 0.9 (air) in response to A23187 (n = 12). LDH release in all A23187 stimulated samples were less than 5% of total activity indicating that stimulation of cells in vitro was not cytotoxic.

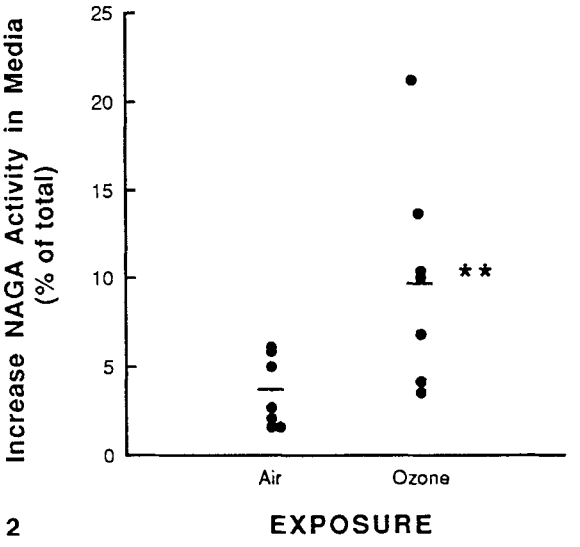

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Fig. 2 Response of mixed BAL cells to A23187 stimulation. Increased NAGA activity in media (% total = media + cell activity) above baseline release (unstimulated cells) in air- and ozone-exposed animals (n = 7 for each group). NAGA was measured in culture media and cell extracts after 4 h stimulation with A23187 (20 μM) in vitro. Mean values are represented by horizontal lines. Cells from ozone-exposed animals released more NAGA into the media in response to A23187 than did control animals. ** = $p < 0.01$. Basal secretion of NAGA were 0.019 ± 0.004 and 0.026 ± 0.004 U/10^6 cells for the control and ozone groups, respectively.

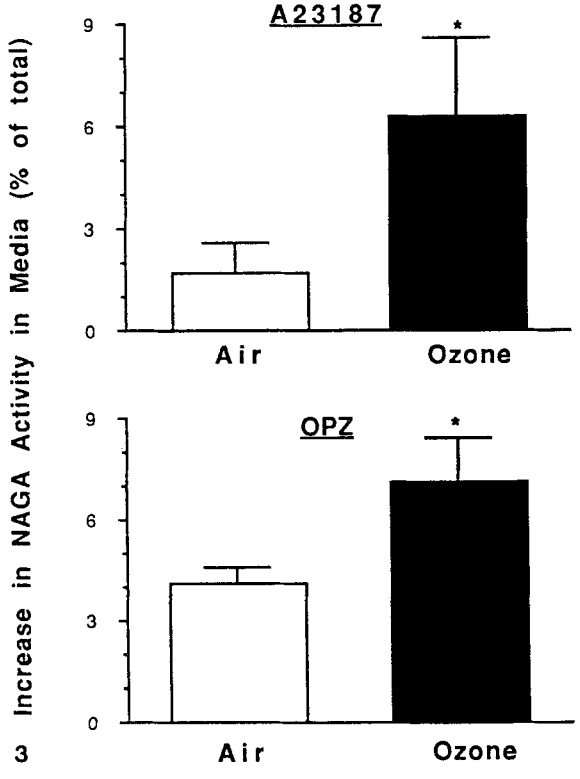

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Fig. 3 Response of bronchoalveolar macrophages to stimulation in vitro. NAGA activity in media (% total = media + cell activity) above baseline release (unstimulated cells) in response to A23187 (upper panel) and OPZ (lower panel). Bronchoalveolar macrophages were separated as described in Methods and were stimulated by either A23187 (20 μM) or OPZ (200 μg/ml). NAGA assays were done 4 h later. (□ = air exposure; ■ = ozone exposure). There was a statistically significant elevation in NAGA release from macrophages of ozone-exposed animals compared to those of air-exposed animals in response to both A23187 and OPZ. * = $p < 0.05$ for both. Basal secretion of NAGA were 0.022 ± 0.002 and 0.029 ± 0.003 U/10^6 cells for the control and ozone groups, respectively.

Discussion

We have demonstrated in this study that NAGA activity of bronchoalveolar lavage fluid from ozone-exposed guinea pigs is markedly elevated. This was associated with the presence of airway muscarinic hyperreactivity from ozone exposure. It was also found that NAGA activity of mixed bronchoalveolar cells in vitro, and specifically bronchoalveolar macrophages, from ozone-exposed animals was increased. This evidence suggests that these macrophages are, at least in part, a source of the increased NAGA in the airways post-ozone.

In previous studies, there has been considerable variation in the reported effects of ozone exposure on lung macrophage function [5, 9, 11, 14]. This variability may derive from the macrophage function(s) analyzed, the dose and duration of ozone exposure in vivo or in vitro, and the species evaluated. In this study, we found that both mixed bronchoalveolar lavage cells, and specifically bronchoalveolar macrophages isolated acutely from lungs of ozone-expressed guinea pigs released more NAGA than did cells from control animals in response to either a phagocytic stimulus OPZ, or to the calcium ionophore A23187 which seem to act through different mechanisms. Opsonized zymosan is known to cause a selective secretion of granule enzymes from macrophages whereas calcium ionophore is believed to cause membrane perturbation and possibly cell lysis as evidenced by the release of LDH, a cytosolic enzyme [13]. The possible damaging effect of ozone exposure on lysosomal membranes of various lung cells has been previously suggested by Castleman et al. [5]. This increased release of NAGA from ozone-exposed macrophages may derive from macrophage lysosomal and cell membrane damage that a number of investigators have previously demonstrated ultrastructurally and/or cytochemically [5, 9, 30].

Our finding that NAGA release is increased after ozone exposure complements past studies of Hurst et al. These authors showed that monolayers of rabbit macrophages exposed in vitro to 2 ppm of ozone for 3 h significantly increased their secretion of lysosomal enzymes (acid phosphatases, lysozymes, and β-glucuronidase) compared to their basal activity [18]. In addition, a shift in hydrolytic activity of ozone-exposed pulmonary macrophages from the lysosomal to the cytosolic compartment was also identified by these investigators. This suggested that macrophage lysosomal enzymes may be mobilized and secreted into the extracellular space during ozone exposure [17]. It should be noted, however, that Hu and co-workers failed to show an increased activity of acid hydrolases in ozone-exposed animals [16]. The differences in our results may relate, in part, to the much higher levels of ozone employed in our study.

The fact that total NAGA activity (bronchoalveolar cells plus lavage fluid) from ozone-exposed animals is markedly elevated is intriguing. Following release of preformed NAGA, intracellular activity would be expected to be decreased initially based on our previous work [20]. In the present study, intracellular enzyme activity from bronchoalveolar cells of both air- and ozone-exposed groups were comparable (0.40 ± 0.05 vs. 0.50 ± 0.05 U/10^6 cells, respectively) (Table 1), while NAGA activity in lavage fluid markedly increased after ozone exposure (0.85 ± 0.15 U/10^6 cells compared to 0.27 ± 0.05). This suggests to

us that ozone exposure may either 1) increase specific enzyme activity, 2) cause influx of macrophages with high NAGA content, or 3) induce rapid de novo synthesis of enzyme. The latter possibility does not seem likely because of the short time frame of our experiments. Results of the cytochemical study by Castleman et al. [5] are consistent with each of these possibilities.

Our finding of increased total NAGA activity after ozone exposure (1.35 ± 0.15 U/10^6 cells (ozone group) compared to 0.67 ± 0.35 (control)) coincides with the findings of Dillard et al. [8]. These investigators found that specific activities of lysosomal hydrolases in rat lung homogenate increased after ozone exposure (0.79 ppm for 7 days: NAGA, 163%; acid phosphatase, 161%; and cathepsin C, 346% of control values). Aside from pulmonary alveolar macrophages, which our data suggest may be at least one source of the increase in bronchoalveolar NAGA activity post-ozone, it is also possible that the increased NAGA may have originated from 1) bronchoalveolar lining cells rather than from cells in the fluid, 2) due to a cytotoxic effect of ozone on lavage cells increasing NAGA relatively by decreasing cell number, and/or 3) from leakage of NAGA from the intravascular space into the airways due to oxidant-induced lung microvascular damage [16]. The former is a possibility which is provocative and which requires consideration in future studies. The second possibility can be excluded in that bronchoalveolar cell number did not decrease post-ozone. Of the latter possibility, it seems unlikely to us that such a change accounts for all of the increase in bronchoalveolar NAGA activity seen in our study as NAGA activity in the ozone-exposed group increased much more than did total protein content of these lavage fluids (215 ± 37 vs. 100 ± 21% increase over control values for NAGA vs. protein content, respectively). The contribution of bronchoalveolar macrophages to this increased enzyme activity is supported by our observation that subsequent stimulation in vitro of ozone-exposed macrophages caused an increased release of NAGA compared to macrophages from air-exposed animals.

This increased acid hydrolase activity in the airways after ozone exposure may have a potential causal relationship with development of muscarinic airway hyperreactivity. Our data is consistent with the possibility that bronchoalveolar macrophages release enzymes which contributes to the pathogenesis of airway reactivity. One mechanism at play may be the destructive effects of such hydrolases on cell membranes of the airway mucosa. These effects may influence the function of mucosa-derived factors influencing bronchomotor tone [25, 31]. Such damage of the respiratory mucosa may be linked to the generation of oxidants such as hypohalous acids [22] which may enter the airway microenvironment and augment smooth muscle responsiveness to endogenous neurotransmitters [23, 25].

We conclude that ozone-induced airway hyperreactivity in the guinea pig is associated with the presence of increased NAGA in bronchoalveolar fluid. Our data suggests that alveolar macrophages are, at least in part, responsible for the release of this enzyme into the airways after ozone exposure. The potential role of such macrophage-derived enzymes in the pathogenesis of oxidant-induced airway hyperreactivity requires further study.

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