Aerosolized neutral endopeptidase reverses ozone-induced airway hyperreactivity to substance P

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MURLAS, CHRISTOPHER G., ZHIHUI LANG, GLENN J. WILLIAMS, AND VIDYASAGAR CHODIMELLA. *Aerosolized neutral endopeptidase reverses ozone-induced airway hyperreactivity to substance P.* J. Appl. Physiol. 72(3): 1133–1141, 1992.—We investigated the effects of ozone exposure (3.0 ppm, 2 h) on airway neutral endopeptidase (NEP) activity and bronchial reactivity to substance P in guinea pigs. Reactivity after ozone or air exposure was determined by measuring specific airway resistance in intact unanesthetized spontaneously breathing animals in response to increasing doses of intravenous substance P boluses. The effective dose of substance P (in μg) that produced a doubling of baseline specific airway resistance ($ED_{200}SP$) was determined by interpolation of cumulative substance P dose-response curves. NEP activity was measured in tracheal homogenates made from each animal of other groups exposed to either ozone or room air. By reverse-phase high-pressure liquid chromatography, this activity was characterized by the phosphoramidon-inhibitable cleavage of alanine-*p*-nitroaniline from succinyl-$(Ala)_3$-*p*-nitroaniline in the presence of 100 μM amastatin. Mean values of the changes in log $ED_{200}SP$ were 0.27 ± 0.07 (SE) for the ozone-exposed group and 0.08 ± 0.04 for the air-exposed group. We found that phosphoramidon significantly increased substance P reactivity in the air-exposed animals ($P < 0.01$), but it had no effect in the ozone-exposed group. This finding was associated with a significant reduction in tracheal homogenate NEP activity of ozone-exposed animals compared with controls: mean values were 18.1 ± 1.9 nmol · min^{-1} · mg protein^{-1} for the ozone-exposed group and 25.1 ± 2.4 nmol · min^{-1} · mg protein^{-1} for air-exposed animals ($P < 0.05$). Inhalation of an aerosolized NEP preparation, partially purified from guinea pig kidney, reversed the substance P hyperreactivity produced by ozone exposure. Inhalation of phosphoramidon post-NEP inhibited this effect. Heat-inactivated NEP aerosol had no influence on ozone-induced hyperreactivity. Our data indicate that ozone exposure decreases airway NEP activity and increases substance P reactivity, which can be reversed by aerosolized NEP.

airway resistance; airway smooth muscle; asthma; bronchoconstriction; bronchial hyperreactivity; enkephalinase; guinea pig; metalloendopeptidase; substance P receptors; tachykinins

INCREASED REACTIVITY of the airways to various stimuli is characteristic of asthma. Airway hyperreactivity has been produced experimentally in animals by several methods, including ozone inhalation (1, 8, 16). Hyperreactivity due to ozone exposure is associated with several signs of airway mucosal injury (15). The effects of mucosal oxidant injury on airway smooth muscle function may be considerable. It has been shown by many investigators that airway mucosal cells produce factors that affect airway muscle tone in a variety of species (6, 12, 21). In particular, the respiratory mucosa (5, 12) may be rich in neutral endopeptidase [NEP; also called enkephalinase (EC 3.4.24.11)], an enzyme that appears to affect guinea pig airway response to substance P in vivo (20) and in vitro (19).

To test whether ozone-induced respiratory injury in guinea pigs was associated with inactivation of airway NEP and an increased reactivity to substance P, we assessed the influence on airway substance P reactivity of phosphoramidon, an antagonist of NEP. Guinea pigs exposed to 3 ppm of ozone for 2 h were evaluated and compared with animals exposed to room air for the same time. To facilitate repeated testing over intervals >1 day as well as to obviate the possible effects that endotracheal intubation and anesthesia could have on airway neuromuscular tone, morphology, and substance P reactivity, animals were studied intact, unanesthetized, and spontaneously breathing. Because the difference in effects of phosphoramidon on substance P reactivity in the two groups suggested that ozone inactivated airway NEP, we measured in tracheal homogenates (made from each animal of other groups exposed to either ozone or air) NEP specific activity in the presence of 1 μM phosphoramidon and/or 100 μM amastatin, an antagonist of aminopeptidase activity. Furthermore, we assessed the effects of an aerosolized NEP preparation, partially purified from guinea pig kidney, on substance P reactivity of other guinea pigs exposed to ozone.

METHODS

Protocol

Thirty-two male Hartley strain guinea pigs (600-750 g body wt) were used in the study (Fig. 1). Baseline specific airway resistance (sRaw in ml · cmH_2O · ml^{-1} · s) and reactivity to intravenous substance P boluses were determined on at least two occasions before the time of experimentation to establish reproducibility of their responsiveness to intravenous substance P. Ten of these animals, chosen randomly, were exposed while awake, resting, and spontaneously breathing at rest to 3.0 ppm of ozone (experimental) for 2 h while the others were exposed to room air (control). sRaw and bronchial reactivities to substance P were remeasured 30 min after ex-

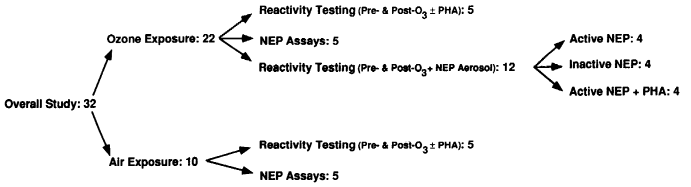

FIG. 1. Flow diagram of protocol for animals used in the study. Nos. indicate guinea pigs used in that subgroup. NEP, neutral endopeptidase; PHA, phosphoramidon treatment.

posure. Substance P was given at 2-min intervals in increasing doses until sRaw increased at least 200% above baseline values. Thirty minutes later phosphoramidon was administered as a single intravenous dose, and 5 min thereafter the dose-response curve to substance P was repeated. Because group differences in the effects of phosphoramidon were consistent with ozone inactivation of airway NEP, we measured NEP activity in tracheal homogenates made from 10 other guinea pigs exposed to either ozone or room air. Twelve other animals were used to evaluate the effect of an aerosolized NEP preparation, partially purified from guinea pig kidney, on substance P reactivity before and after ozone exposure. In eight of these animals, the NEP was either heat inactivated (as confirmed biochemically) before aerosol delivery and repeat substance P reactivity testing or followed thereafter by aerosolized phosphoramidon and retesting to further assess possible changes attributable to NEP inhalation.

Procedures

Airway studies in vivo. sRaw and substance P reactivities were measured as previously described in intact unanesthetized spontaneously breathing guinea pigs (16). 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 rotameter (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 inspiratory phase of the animal's breathing was measured, and sRaw was calculated from it.

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, 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 increasing doses of substance P delivered intravenously. 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 β-adrenergic stimulation (2, 3). Normal saline or substance P was delivered via a 0.61-mm-OD 0.28-mm-ID polyethylene cannula that had been previously placed in an external jugular vein. sRaw was measured continuously before and after bolus administration of 0.1–0.2 ml of substance P (Sigma Chemical, St. Louis, MO) dissolved in isotonic saline and 1% acetic acid. Substance P was given at 2-min intervals in increasing doses beginning with 0.025 μg until sRaw was at least 200% of the baseline value. Substance P reactivity was deemed reproducible if changes in the effective dose (in μg) of substance P that produced a dabling of sRaw ($ED_{200}SP$) were <50% of initial values. After each injection the jugular catheter (dead space ~0.08 ml) was flushed with 0.2 ml of isotonic heparinized saline (1,000 U heparin/10 ml). Thirty minutes later phosphoramidon was administered as a single dose (0.5 mg in 1 ml 0.5% albumin in isotonic saline). Five minutes after administration of phosphoramidon, the dose-response curve to substance P was performed again as described above. The dosages, routes, and timing of substance P and phosphoramidon administration were chosen on the basis of previously published work (17). Active or heat-inactivated guinea pig renal NEP or phosphoramidon, prepared as described below, was aerosolized 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). Substance P reactivity was again assessed 10 min thereafter.

Cumulative substance P dose-response curves were constructed by plotting, on semilogarithmic paper, baseline sRaw and the peak values of sRaw for each dose of substance P administered. $ED_{200}SP$ was determined by interpolation.

Histopathological evaluation. Histological sections of the right mainstem bronchus and lung were made for each case. Eight-micrometer-thick frozen sections were cut and stained with hematoxylin and eosin. They were each examined by light microscopy for histopathology. None of the cases studied had respiratory tract infections histopathologically.

Biochemical assays of NEP activity in tracheal tissue. Each guinea pig tracheal segment (200-250 mg in weight) was homogenized (Brinkman Instruments, Boston, MA) in 10 vol of ice-cold 50 mM tris(hydroxymethyl)aminomethane · (Tris) HCl buffer, pH 7.4. These whole tissue homogenates were sonicated twice at 75 W for 10 s and then centrifuged at 135,000 *g* for 1 h. The pellet was resuspended in 3 ml Tris · HCl buffer containing 0.1% Triton X-100. This was homogenized and placed in a shaker bath at 4°C for 2 h. The suspension was again centrifuged at 135,000 *g*. By employing a modification of meth-

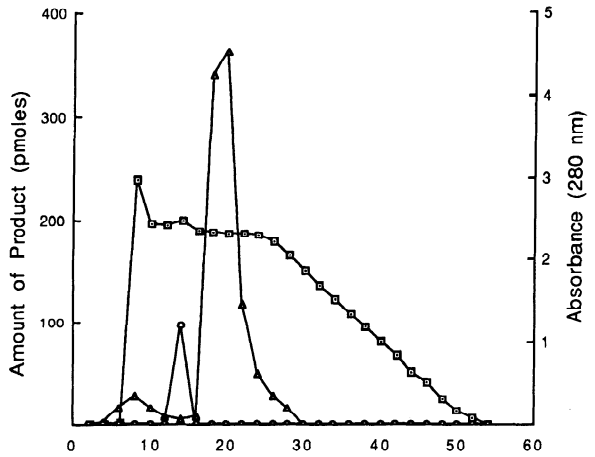

FIG. 2. DEAE-cellulose chromatographic fractionation of NEP and aminopeptidase activities of crude preparation derived from guinea pig kidney. For each fraction separated (as described in METHODS), amounts of products [alanine *p*-nitroaniline (triangles) and nitroaniline (circles)] and absorbance at 280 nm [index of protein concentration (squares)] are shown.

ods described by Kuwada and Katayama (11), the initial supernatant was assayed along with the secondary pellet and supernatant (the latter contained all of the activity in the tissue). Aliquots were incubated for 10 min with the chromogenic substrate succinyl (Ala)3-*p*-nitroaniline (SA_3NA) in the presence of 1 μM phosphoramidon and/or 100 μM amastatin, an antagonist of aminopeptidase activity. Chromophores were detected in the effluent fractions by absorbance measurement at 314 nm. The enzyme preparation was incubated with 0.5 mM SA_3NA, 50 mM Tris · HCl, and 0.1% Triton X-100, pH 7.4, in a final volume of 80 μl. When specific inhibitors were used, they were preincubated with enzyme for 15 min at 4°C. Reactions were run at 37°C for 10 min with gentle shaking and then stopped with 20 μl of 10% trichloroacetic acid (TCA). Samples were spun in a microcentrifuge (model 135A, Fisher) at 14,000 *g* for 10 min at 4°C.

Supernatants were directly analyzed using an high-performance liquid chromatography (HPLC) system (Waters Associates, Milford MA) to determine hydrolytic products of this substrate cleaved by the enzyme. They were applied to a Novapak C_{18} reverse-phase column (3.9 × 150-mm, Phenomenex, Rancho Palos Verdes, CA) and separated under isocratic conditions [40% methanol and 60% NaH_2PO_4 (10 mM), pH 3.0]. Products detected at 314 nm were identified by coelution with standards. Products were quantitated by comparing integrated peak areas to peak areas of known amounts of authentic standards. Authenticity was based on the sole production of alanine *p*-nitroaniline (ANA) from substrate and its inhibition by 1 μM phosphoramidon. Protein concentrations were measured using a microprotein assay (Bio-Rad Laboratories, Richmond, CA). Specific activity was expressed in moles of ANA produced per minute per milligram protein. Differences between groups were compared using independent *t* tests and were considered significant for $P < 0.05$.

Preparation and delivery of an aerosolized NEP preparation partially purified from guinea pig kidney. Guinea pig kidneys were removed from animals and homogenized with a Vertis microhomogenizer in 10 vol of 50 mM Tris · HCl buffer, pH 7.4. This was centrifuged at 1,000 *g* for 20 min at 4°C. The supernatant was decanted and centrifuged at 135,000 *g* for 1 h. The pellet was resuspended in 50 mM Tris · HCl buffer, pH 7.4, containing 0.1% Triton X-100. This was incubated at 37°C with gentle shaking for 2 h. The suspension was then centrifuged at 135,000 *g* for 1 h. The supernatant was decanted and made a 40% ammonium sulfate suspension. The suspension was stirred for 1 h at 4°C. This was centrifuged at 21,500 *g* for 20 min. The resulting pellet atop the solution was carefully removed and resuspended in 50 mM Tris · HCl buffer, with 0.1% Triton X-100, pH 7.4. This was dialyzed against 100 volumes of the same buffer. This solution was placed on a DEAE-52 column (4.8 × 10 cm) previously equilibrated with 50 mM Tris · HCl buffer containing 1 mM NaCl and 0.1% Triton X-100, pH 7.4. The column was washed with this same buffer until the OD of the eluent was at baseline. A gradient of NaCl from 1 to 600 mM was used to elute this crude preparation, and 15-ml fractions were collected and assayed for enzymatic activity on the substrate SA_3NA. There appeared to be a bimodal elution of NEP (Fig. 2) similar to that seen by previous authors employing ion-exchange chromatography on NEP isolated from human kidney tissue (7). Fractions 18–22, having high NEP activity, were pooled. These fractions demonstrated no aminopeptidase activity in that no nitroaniline (NA; retention time 9 min) was produced (11) from the substrate (Fig. 3). They were dialyzed against 10 mM sodium bicarbonate containing 0.1% Triton X-100. This dialysate was applied to a CM-52 column preequilibrated with the same buffer. The column was washed with this buffer until absorbance at 280 nm returned to baseline, and then a gradient of NaCl from 1 to 600 mM (total volume 575 ml) was begun. Fractions with high NEP activity were pooled, concentrated by lyophilization, and dialyzed. The dialysate was loaded onto a Sephadex G-200 column preequilibrated with 50 mM Tris · HCl, pH 7.4, containing 0.1% Triton X-100. Active fractions were pooled from the resulting eluate, lyophilized, and dialyzed for use in vivo. Dialysis was done against 100 vol of 10 mM sodium bicarbonate buffer, pH 7.4, overnight with one change of dialysate buffer.

Peptidase activity of this partially purified preparation (Fig. 4), assessed employing HPLC by measuring sub-

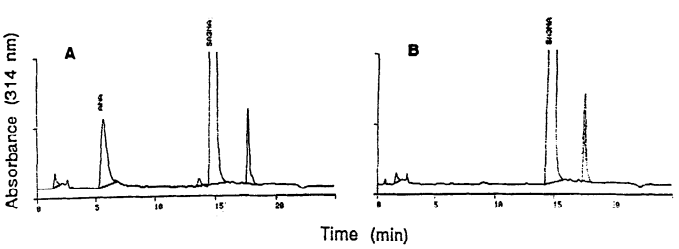

FIG. 3. High-pressure liquid chromatograms showing activity of NEP present in fractions of crude preparation derived from guinea pig kidney as described in METHODS. Production of alanine *p*-nitroaniline from substrate [succinyl $(Ala)_3$-*p*-nitroaniline; SA_3NA] in the presence of 100 μM amastatin characterized NEP activity (*A*) and was inhibited by 1 μM phosphoramidon; it was abolished in NEP aliquots that were heat inactivated (*B*).

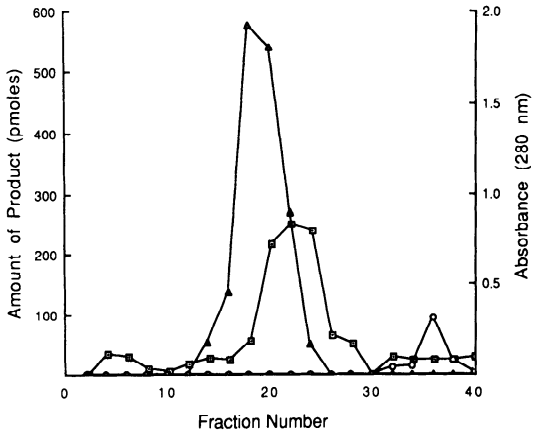

FIG. 4. Sephadex G-200 chromatographic fractionation of NEP and aminopeptidase activities of final partially purified guinea pig renal NEP preparation used in study. For each fraction separated (as described in METHODS), amounts of products [alanine *p*-nitroaniline (triangles) and nitroaniline (circles)] and absorbance at 280 nm [an index of protein concentration (squares)] are shown.

stance P degradation as previously described (14), was inhibited by phosphoramidon alone (Table 1). To measure substance P catabolism by this renal preparation, 10-μl aliquots of the preparation were incubated for 30 min with 0.1 mM substance P, with or without inhibitors, in a final volume of 100 ml (Table 1). Reactions were stopped by adding 50 ml of 5% TCA at 4°C and spun in a microcentrifuge at 15,000 *g* for 10 min at 4°C. Fifty-microliter aliquots of the reaction mixture were applied to a μBondapak C_{18} reverse-phase column (3.9 × 150 mm, Waters Associates, Milford MA). Substance P was resolved using a one-step linear gradient from 20 to 45% acetonitrile in 0.035% trifluoroacetic acid over 12 min. The column was eluted at 1 ml/min and monitored at 214 nm. Under these conditions, substance P eluted at ~11 min. Immediately thereafter, acetonitrile was increased to 70% within 2 min and maintained for the ensuing 5 min to wash off the column before subsequent use. Amounts of substance P degraded were quantified by comparing integrated peak areas to those established for known standards.

The specific activity of this partially purified NEP preparation was 180 nmol · min^{-1} · mg $protein^{-1}$. Then 0.5 ml of this preparation diluted in 2.5 ml of normal saline was used for aerosolization. It contained 15 μg of protein, ~40% of which was aerosolized during a 90-breath inhalation. A like dilution of heat-inactivated enzyme (confirmed by the HPLC assay as described above) was prepared for experimental use by heating the enzyme solution at 100°C for 5 min. For animals tested only after active or heat-inactivated NEP inhibition, both enzyme solutions contained 100 μM amastatin to inhibit possible aminopeptidase activity of the aerosols delivered that may not have been detectable by our HPLC microassay. In preliminary studies, inhalation of aerosol generated from 100 μM amastatin alone was shown to have no effect on substance P reactivity in guinea pigs. For animals retested both after NEP and after phosphoramidon inhalation after reactivity assessment postozone, the aerosol contained no amastatin. Reactivity postphosphoramidon (10 μM; 5 min) was assessed immediately after this treatment.

Drugs. For the experiments in vivo, solutions of the following were freshly made on the day of use: amastatin, phosphoramidon, propranolol, and substance P (all purchased from Sigma); and captopril (a gift from Squibb Pharmaceuticals, New Brunswick, NJ).

Statistical analysis. Values (means ± SE) for each animal subgroup (Fig. 1) of the change in log ED_{200}SP after air or ozone exposure (before or after phosphoramidon aerosol inhalation) were compared with values of the change in log ED_{200}SP before exposure using the Student's *t* test. Values (means ± SE) of NEP activity from tracheal homogenates of air- and ozone-exposed animals were similarly compared. Ratios of the log ED_{200}SP after air or ozone exposure (compared with the log ED_{200}SP before exposure), and after active or inactive NEP or phosphoramidon (compared with the log ED_{200}SP before NEP), were compared with a value of 1.0 by employing the *t* test. Differences were considered significant for $P < 0.05$.

RESULTS

Ozone exposure caused a substantial increase in airway reactivity to substance P. Mean values of the changes in log ED_{200}SP were 0.27 ± 0.07 (SE) for the ozone-exposed group and 0.08 ± 0.04 for the air-exposed group (Figs. 5–7). Figures 5 and 6 show the effect of phosphoramidon treatment on substance P reactivity after either ozone or air exposure. In contrast to animals exposed to ozone, phosphoramidon caused a substantial increase in the substance P reactivity of air-exposed guinea pigs.

Mean values for the ratio of the change in ED_{200}SP after phosphoramidon compared with the change in ED_{200}SP before phosphoramidon (both determined after ozone or air exposure) are shown in Fig. 8. The effect of phosphoramidon was significant in the air-exposed group only ($P < 0.01$).

Figure 9 illustrates NEP activities of tracheal homogenates from animals of both groups. Mean values were 18.1 ± 1.9 nmol · min^{-1} · mg $protein^{-1}$ for the ozone-exposed group and 25.1 ± 2.4 nmol · min^{-1} · mg $protein^{-1}$ for air-exposed animals ($P < 0.05$).

The effects of active and heat-inactivated NEP aerosols on guinea pig substance P reactivity after ozone are shown in Fig. 10. Active NEP reduced substance P reactivity after ozone exposure to preozone levels in all animals tested. Aerosolized phosphoramidon treatment

TABLE 1. *Degradation of substance P by preparation partially purified from guinea pig kidney*

Inhibitor Added to Assay	%Disappearance of Substance P
No inhibitor (Control)	23.5±1.1
Amastatin (10 μM)	25.9±1.6
Captopril (200 μM)	24.5±0.4
Phosphoramidon (1 μM)	0±0.4*

Values are means ± SE for experiments. Degradation of substance P, expressed as percent disappearance relative to baseline, was measured by high-performance liquid chromatography as described in METHODS. Peptidase activity present in this preparation was inhibited by phosphoramidon alone. * Significantly different from control value ($P < 0.01$).

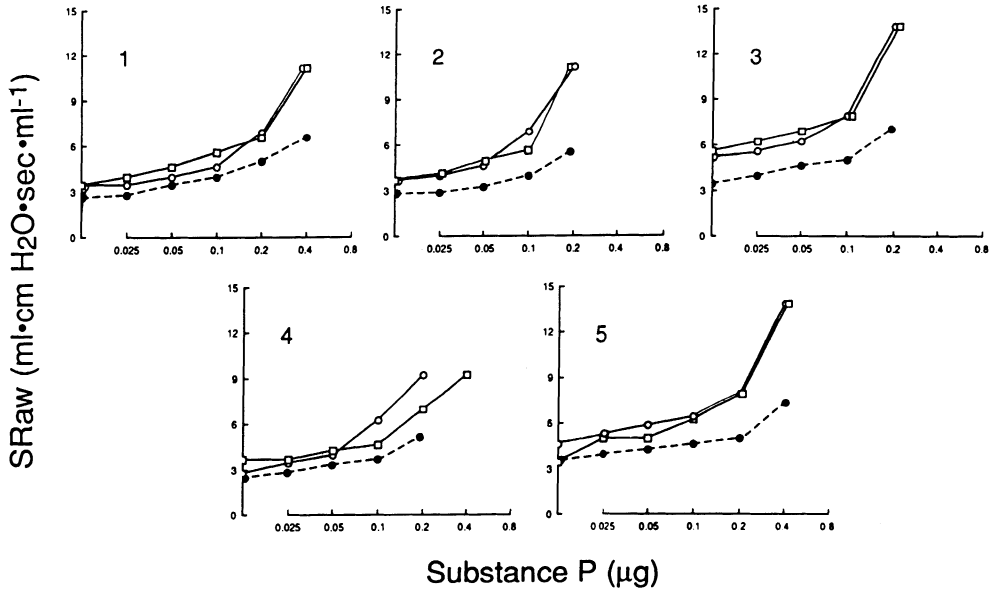

FIG. 5. Individual dose-response curves demonstrating effect of ozone exposure on airway reactivity to increasing doses of intravenous substance P administered before (open circles) and after (open squares) intravenous phosphoramidon. Substance P reactivity before ozone exposure is indicated by closed circles and dashed lines. sRaw, specific airway resistance.

post-NEP reversed this effect (Fig. 11). In comparison, heat-inactivated NEP inhalation had no significant influence on substance P reactivity postozone. Comparisons of the changes in log ED_{200}SP before and after active or inactive NEP inhalation are shown in Fig. 12. The decrease in substance P reactivity produced by active NEP inhalation postozone was considerable ($P < 0.01$). Mean ratios of the change in log ED_{200} substance P after ozone, NEP, and phosphoramidon inhalation are shown in Fig. 13.

DISCUSSION

In contrast to the substantial increase in substance P reactivity that phosphoramidon produced in control animals, we found that phosphoramidon treatment had no significant effect on substance P reactivity in ozone-exposed animals. These findings were consistent with the possibility that ozone-induced airway injury was associated with decreased airway NEP. This possibility was corroborated by our evidence that NEP activity, measured by HPLC, was significantly decreased in ozone-exposed airways. Furthermore, we found that inhalation of an aerosolized NEP preparation, freshly isolated and partially purified from guinea pig kidney tissue, reversed the acute increase in airway substance P reactivity caused by ozone in guinea pigs. Inhalation of phosphoramidon post-NEP inhibited this effect. Heat-inactivated NEP had no influence on ozone-induced hyperreactivity.

To our knowledge, this is the first study to demonstrate that an airway disorder associated with the loss of NEP activity can be reversed by inhalation of an aerosol containing that enzyme. Normally, this enzyme is present in certain respiratory mucosal cells (5, 9, 14) and

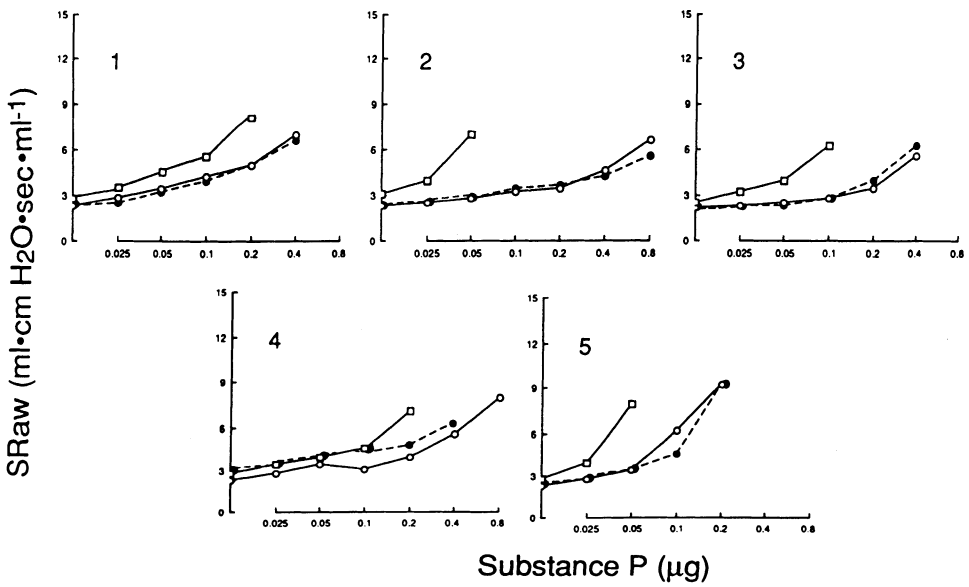

FIG. 6. Individual dose-response curves demonstrating effect of air exposure on airway reactivity to increasing doses of intravenous substance P administered before (open circles) and after (open squares) intravenous phosphoramidon. Substance P reactivity before air exposure is indicated by closed circles and dashed lines. In contrast to ozone-exposed animals, phosphoramidon administration increased reactivity in air-exposed guinea pigs.

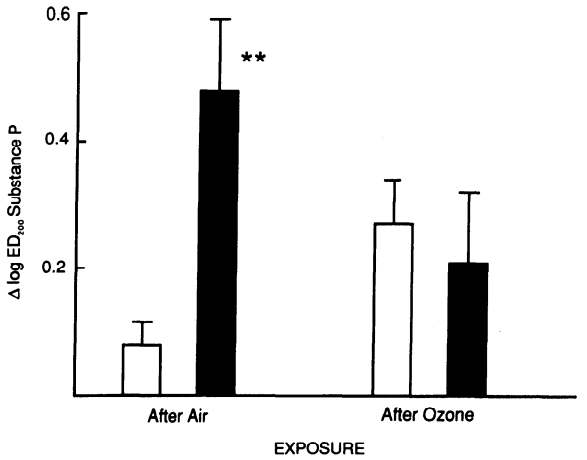

FIG. 7. Comparison of values (means ± SE) for air- and ozone-exposed groups of changes in effective dose of substance P that produced a doubling of baseline specific airway resistance (log ED_{200} substance P) after exposure and before (open bars) or after phosphoramidon administration (closed bars) as described in METHODS. In contrast to ozone-exposed subgroups, mean value for air-exposed animals after phosphoramidon (**) was significantly greater than that for air-exposed guinea pigs before phosphoramidon ($P < 0.01$).

appears to affect guinea pig airway response to substance P both in vivo (20) and in vitro (19). Other investigators have found that inhaled recombinant NEP, originally derived from a human cultured cell line, also has pharmacological activity in the guinea pig on substance P-induced coughing (10).

Previous work has indicated that there are striking early pathological signs of ozone-induced airway mucosal injury in the guinea pig when airway hyperreactivity first manifests (15). Although loss or matting of mucosal epithelial cell cilia is common shortly after high-level ozone exposure, frank mucosal denudation is not present. This contrasts with the extensive mucosal damage shown in 4-day-old virus-infected ferret airway tissue hyperresponsive to substance P (9). It has been shown by many investigators that physical removal of the airway mucosa per se may increase responsiveness of airway muscle in

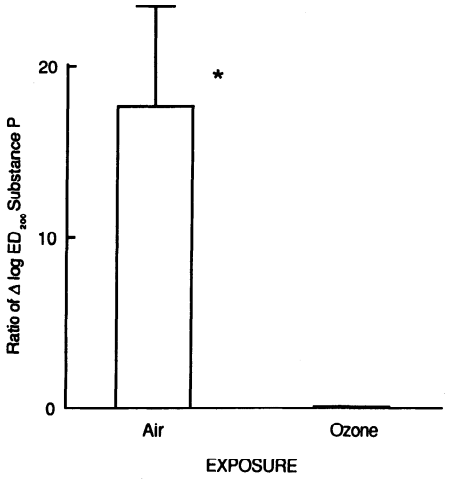

FIG. 8. Values (means ± SE) of ratio of change in log ED_{200} substance P after phosphoramidon administration to change in log ED_{200} substance P before phosphoramidon in both air- and ozone-exposed animals. *Value significantly different from 1.0 ($P < 0.01$).

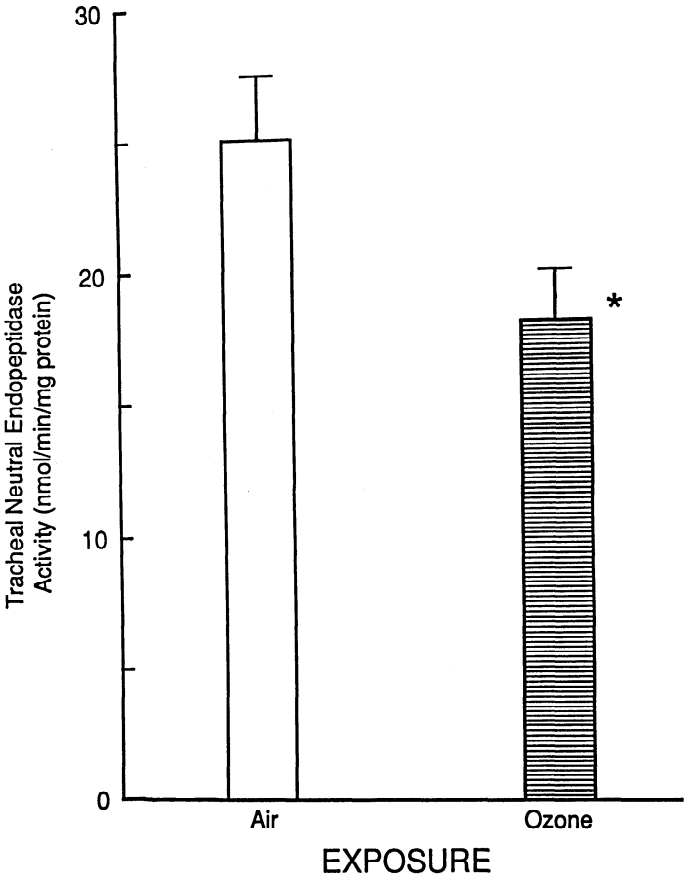

FIG. 9. Effect of ozone exposure on airway NEP activity. Values shown for control (air-exposed) and ozone-exposed groups are means ± SE of 5 cases each. *Ozone value significantly less than control value ($P < 0.05$).

vitro to a variety of bronchoconstrictors. The results of our studies, including those reported here, suggest to us that ozone-induced airway hyperreactivity in the guinea pig is linked to a morphologically intact but functionally abnormal respiratory mucosal cell layer. The precise biochemical events during airway oxidant injury that lead to this dysfunction are not well understood at present. Several events are likely, including the generation of oxygen-derived free radicals and oxidizing agents, such as hydrogen peroxide and hypochlorous acid (HOCl), a product of chloride oxidation catalyzed by myeloperoxidase. HOCl appears to have a direct effect on the NEP of airway epithelial cells (14, 22).

It is clear from the work of several groups of investigators that NEP may be important in the modulation of airway muscle tone in vivo by tachykinins. NEP is a transmembrane enzyme inserted into the microvillar plasma membrane bilayers of certain cell types. In the airways of species studied thus far, NEP appears to be present in gland and smooth muscle cells as well as in airway mucosal cells (5). Shore and colleagues (18) have shown in guinea pigs that intravenous thiorphan, another NEP antagonist, enhanced intravenous substance P-induced bronchoconstriction. In the same species, Dusser and co-workers (4) subsequently demonstrated that inhaled phosphoramidon potentiated substance P aerosol-induced bronchoconstriction. These investiga-

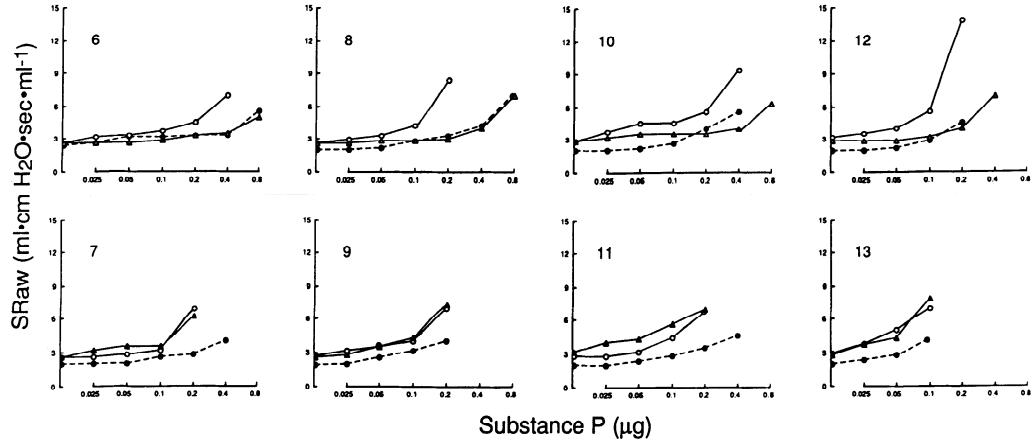

FIG. 10. Individual dose-response curves demonstrating effect of active (*top panels*, open triangles) and heat-inactivated NEP inhalation (*bottom panels*, closed triangles) on increase in substance P reactivity produced by ozone (open circles). Substance P reactivity before ozone exposure is indicated by closed circles and dashed lines. Inhalation of aerosolized NEP reversed ozone-induced increase in substance P reactivity. Heat-inactivated NEP aerosol had no such effect.

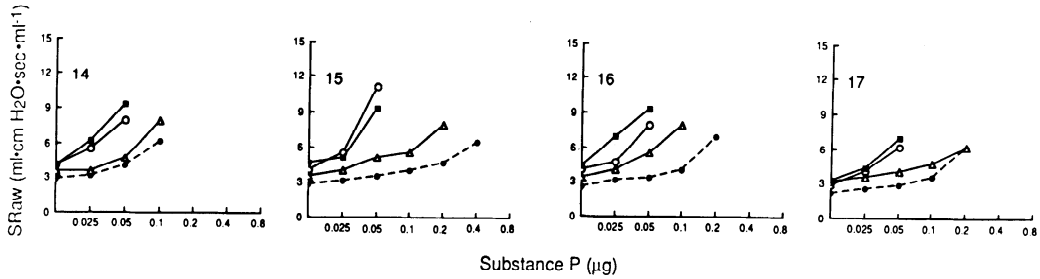

FIG. 11. Individual dose-response curves demonstrating effect of active NEP inhalation (open triangles) and phosphoramidon inhalation post-NEP (closed squares) on increase in substance P reactivity produced by ozone (open circles). Substance P reactivity before ozone exposure is indicated by closed circles and dashed lines. Inhalation of aerosolized NEP reversed ozone-induced increase in substance P reactivity. This effect was inhibited by phosphoramidon inhalation.

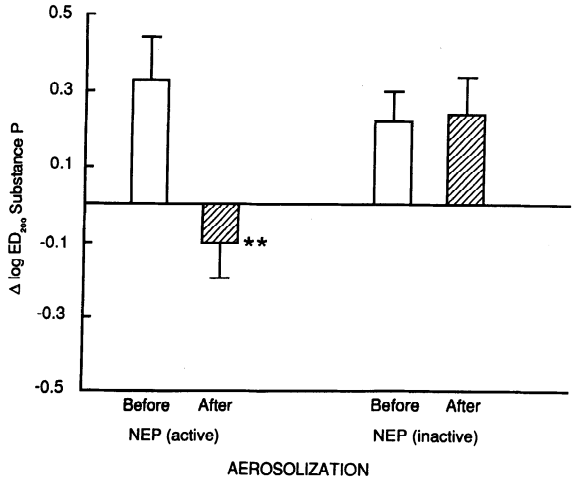

FIG. 12. Comparison of values (means ± SE) for changes in log ED_{200} substance P of ozone-exposed animals before and after inhalation of either active (*left*) or heat-inactivated (*right*) NEP aerosol. **Value of ozone-exposed group after active NEP inhalation was significantly greater than value for group before inhalation ($P < 0.01$).

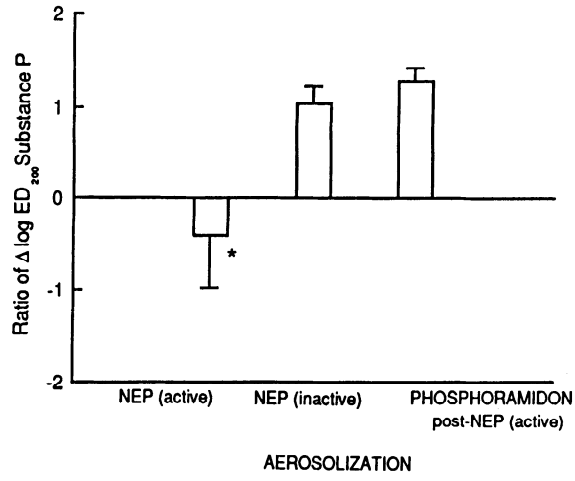

FIG. 13. Values (means ± SE) of ratio of change in log ED_{200} substance P after active or inactive NEP or post-NEP phsophoramidon aerosol inhalation by ozone-exposed animals. *Value significantly different from 1.0 ($P < 0.05$).

tors also showed that atropine-resistant responses to vagal nerve stimulation were potentiated by aerosolized phosphoramidon, suggesting that NEP-like activity may influence the effects of endogenous trachykinins released by pulmonary vagal nerves in vivo.

Our data lead us to speculate that oxidant-induced increases in airway reactivity to substance P may be caused by inactivation of airway mucosal NEP, although there are alternative explanations. This speculation derives from other recently reported work as well (3, 13, 14, 17). Our finding in the present study that ozone-induced hy-

perreactivity to intravenous substance P was reversed by aerosolized NEP and that this hyperreactivity was associated with decreased airway tissue NEP has relevance to our past work (13) in which we found that *1*) mucosal denudation of air-exposed airway tissues caused these preparations to become as hyperresponsive to substance P as ozone-exposed intact tissue and *2*) mucosal denudation of ozone-exposed airway tissues did not further increase substance P responsiveness. In other words, removal of ozone-damaged mucosa, possibly lacking NEP activity had no effect on the underlying smooth muscle response to substance P, whereas removal of mucosa from air-exposed tissue, with presumably normal NEP activity, increased substance P responsiveness. The fact that phosphoramidon treatment of either air- or ozone-exposed mucosa-intact tissues caused greater maximal force generation to substance P than was observed in either ozone-exposed intact or mucosa-denuded tissues (13) suggests to us that our ozone exposure protocol only partially inactivated airway NEP. In other words, while phosphoramidon treatment inactivates all airway endopeptidase activity (both mucosal and nonmucosal), ozone exposure may inactivate only mucosal endopeptidase. Concerning the present study, this may explain why the small (although statistically significant) decrease in airway NEP activity after ozone exposure was not larger. The nonmucosal component of total tracheal activity, which is substantial, may obscure the ozone-induced changes in mucosal NEP.

Nonetheless, there may be alternative explanations for the data reported here. Although airway NEP hypoactivity appears to be linked to ozone-induced hyperreactivity and aerosolized NEP can reverse it, our study does not prove that oxidation of respiratory mucosal NEP is a cause of this disorder. Because the major site of degradation of intravenous substance P is the vascular endothelium, it is possible that ozone-induced hyperreactivity derives from damage at this level rather than at the level of the airway luminal surface. Because ozone is a potent and highly reactive airway oxidant, either itself (if toxic ozone levels occur within lung vessels) or secondary mediators it generates upon airway mucosal injury may increase vascular permeability and/or alter perfusion of the vascular bed, thereby allowing more intravenous substance P to reach the airways. Although these possibilities require future investigation, the fact that guinea pig airway tissue behaves pharmacologically in vitro, as if its mucosal endopeptidase activity has been reduced by ozone (13), favors the explanation provided in the preceding paragraph.

On the external surface of respiratory mucosal cell membranes, NEP may be quite vulnerable to inhaled environmental pollutants as are the cilia of mucosal epithelial cells. In addition to ozone, other inhaled toxicants also appear to produce increases in airway reactivity to substance P and are associated with decreased airway NEP activity (3, 17). In such conditions, the cells of the respiratory mucosa (and, possibly, of other airway cells) that may be affected by air pollutant injury have not been identified, nor have the mechanisms by which NEP is inactivated and can be reconstituted. The relationship of these issues to naturally occurring human airway diseases and their management merit additional study.

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