O_3-induced mucosa-linked airway muscle hyperresponsiveness in the guinea pig

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MURLAS, CHRISTOPHER G., THOMAS P. MURPHY, AND VIDYASAGAR CHODIMELLA. *O_3-induced mucosa-linked airway muscle hyperresponsiveness in the guinea pig.* J. Appl. Physiol. 69(1): 7–13, 1990.—We investigated the effects of ozone exposure (3.0 ppm, 2 h) on the responsiveness of guinea pig airway muscle in vitro from animals developing bronchial hyperreactivity. Muscarinic reactivity in vivo was determined by measuring specific airway resistance (sRaw) in response to increasing concentrations of aerosolized acetylcholine (ACh) administered before and 30 min after exposure. Immediately after reactivity testing, multiple tracheal rings from ozone- and air-exposed animals were prepared and the contractile responses to increasing concentrations of substance P, ACh, or KCl were assessed in the presence of 10 μM indomethacin with or without 1 μM phosphoramidon, an inhibitor of neutral endopeptidase. Isometric force generation in vitro was measured on stimulation by cumulative concentrations of the agonists, and force generation (in g/cm^2) was calculated after determination of muscle cross-sectional area. The smooth muscle of mucosa-intact airways from guinea pigs with ozone-induced bronchial hyperreactivity proved to be hyperresponsive in vitro to substance P and ACh but not to KCl. Pretreatment with phosphoramidon abolished the increase in substance P responsiveness but had no effect on muscarinic hyperresponsiveness after ozone exposure. Furthermore, substance P responsiveness was not augmented in ozone-exposed airways in which the mucosa had been removed before testing in vitro. Likewise, muscarinic hyperresponsiveness was not present in ozone-exposed airways without mucosa. Our data indicate that airway smooth muscle responsiveness is increased in guinea pigs with ozone-induced bronchial hyperreactivity and suggest that this hyperresponsiveness may be linked to non-cyclooxygenase mucosa-derived factors.

acetylcholine; airway resistance; airway smooth muscle; bronchoconstriction; bronchial hyperreactivity; enkephalinase; metalloendopeptidase; muscarinic receptors; substance P; tachykinins; trachealis muscle

SEVERAL AIRWAY DISORDERS, including asthma and acute ozone-induced respiratory injury (1, 13, 33), are characterized by bronchial hyperreactivity. Both of these disorders are also characterized, in part, by pathological abnormalities of the respiratory mucosa (11, 19, 23, 24). In our own studies of ozone-induced bronchial hyperreactivity in the guinea pig (23, 24), we observed various signs of airway mucosal damage when hyperreactivity first manifested. This suggested to us that bronchial hyperreactivity may be linked to mucosal cell injury. From the work of many investigators, it appears that the airway mucosa in a variety of species may normally produce several factors that affect airway muscle tone (8–11, 20, 26, 31, 33). This conclusion derives from studies in which the physical removal of respiratory mucosae was shown to increase airway smooth muscle responsiveness in vitro to a variety of bronchoconstrictors. Whether and how other respiratory injuries unassociated with mucosal denudation, such as acute ozone-induced airway damage, produce airway muscle hyperresponsiveness are subjects of considerable interest.

Therefore we investigated whether smooth muscle from the airways of guinea pigs with ozone-induced hyperreactivity was hyperresponsive to agonist stimulation in vitro. Muscarinic reactivity, before and after ozone or air exposure, was determined by measuring specific airway resistance (sRaw) in unanesthetized spontaneously breathing animals to obviate the potential effects that endotracheal intubation and anesthesia could have had on our findings. Mucosa-intact tracheal tissue rings were obtained from ozone- and air-exposed animals and pretreated with indomethacin to inhibit effects on airway muscle over time of cyclooxygenase products generated in vitro (25, 34) that may be derived from the respiratory mucosa (4). We then assessed the responsiveness of those rings on stimulation by acetylcholine (ACh), KCl, or substance P. This was done in the presence or absence of phosphoramidon, an inhibitor of neutral endopeptidase (E.C. 3.4.24.11; also called enkephalinase), an enzyme that degrades substance P. Neutral endopeptidase appears to be present in certain respiratory mucosal cells (14, 15), and it has been shown to affect guinea pig airway response to substance P in vivo (32) and in vitro (30).

METHODS

Protocol

Thirty-six male Hartley strain guinea pigs (600–750 g body wt) were used in the study. sRaw (in ml·cmH_2O·ml^{-1}·s) and muscarinic reactivity to inhaled ACh were determined serially. All animals showed reproducible base-line sRaw values and response to serial muscarinic challenge testing. These animals were exposed to either 3.0 ppm of ozone (experimental) or to room air (control) for 2 h while awake and spontaneously breathing at rest. sRaw and bronchial reactivity to ACh were measured 30 min after exposure. Immediately thereafter, 24 of the guinea pigs were killed, and the tracheae were removed.

Ring segments of tracheal tissue from each animal were prepared and mounted in muscle chambers so that airway smooth muscle responsiveness to exogenous substance P, ACh, or KCl could be compared between air- and ozone-exposed animals with bronchial hyperreactivity. In some experiments, these airway rings were pretreated with 1 μM phosphoramidon, or the mucosa was removed before stimulation by these agonists.

Procedures

Airway studies in vivo. sRaw and muscarinic reactivity were measured as previously described in intact unanesthetized spontaneously breathing guinea pigs (22). 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 by using a pneumotachograph (no. 0, Fleish Instruments, Prés 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 with 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 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, West Germany) regulated by a variable voltage supply. The ozone level was monitored with an 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 as the result of endogenous β-adrenergic stimulation (5, 6). Reactivity to inhaled ACh was determined with 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 phosphate-buffered saline (PBS), 30 breaths of 0.1 mg/ml 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 base-line 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 (ED_{200}ACh in mg/ml) present just before reactivity testing was determined by interpolation. All ozone-exposed animals manifested at least a 50% decrease in ED_{200}ACh after ozone exposure and were considered hyperreactive. Mean values for the ED_{200}ACh were expressed as the geometric mean and geometric standard error. Changes in log values of ED_{200}ACh for each animal before and after ozone or air exposure were compared was assessed by using the paired t test. In all cases, differences were $P < 0.05$.

Airway smooth muscle studies in vitro. Tracheae were obtained from each guinea pig killed with pentobarbital sodium (Socumb-6 GR, Butler, Columbus, OH). Each trachea was cut transversely to yield multiple tracheal rings, each ~3 mm long. To minimize mucosal trauma, each preparation was mounted vertically on a 0.3-mm-diam stainless steel wire stirrup in a 10-ml muscle chamber and attached with 4.0 silk thread to a force-displacement transducer (model FT03, Grass Instrument, Quincy, MA) for measuring isometric tension. This tissue handling caused damage to the mucosal surface only at those points of contact. For experiments requiring airways without mucosa from ozone- and air-exposed animals, the luminal surface of each ring was gently rubbed with a dry wooden probe [mucosal removal was confirmed histologically (see below)]. Responses were displayed on a strip chart recorder (model 8000, Gould Instrument, Cleveland, OH). The tracheal rings were bathed in modified Krebs-buffered solution (maintained at 37°C and gassed continuously with 95% O_2-5% CO_2) of the following composition (in mM): 137.0 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$, 24.9 $NaHCO_3$, 1.2 NaH_2PO_4, 9.6 glucose; pH 7.40 at 37°C. A tension of 2 g was maintained on the tissues, and they were washed every 15 min during a 60-min equilibration period. After the last wash, 10 μM indomethacin were added to inhibit the possible effects of cyclooxygenase products generated in vitro on these experiments. Before starting the experiment, each ring was adjusted to optimal muscle fiber length by stretching it progressively until maximal contraction was elicited by standard submaximal current stimulus (20 V, 0.5 ms, 10 Hz). At this time, the rings were stimulated with 10 μM ACh followed by thorough washouts until a consistent response was established. During the entire experiment, optimum muscle length was maintained. Each preparation was exposed to substance P, ACh, or KCl added in cumulatively increasing concentrations to the muscle chambers. For the experiments, ring segments from the upper third of tracheae were used for substance P or KCl stimulation; from the middle third for KCl; or from the lower third for ACh or KCl. This comparison of agonist responses in like sections of guinea pig tracheae was done because previously published work in this tissue has shown that the proximal and distal ends differ in their responsiveness to ACh and to the electrical field stimulation (18). This differential muscarinic responsiveness may derive from cholinergic

innervation of the trachea. KCl and substance P responsiveness in tracheal muscle may, at least in part, similarily be determined (21, 31).

Responses to each agonist concentration were expressed in terms of g/cm^2 cross-sectional area as previously described (2, 21). Differences in the maximal contractile response to each agonist between groups of ozone- and air-exposed tracheal rings were compared by using the Mann-Whitney test with Bonferonni adjustment. Differences were considered significant for $P <$ 0.02.

Histological evaluation. Histological sections of the tracheal ring segments were obtained after the muscle studies in vitro had been completed. The 10-μm frozen sections were cut and stained with hematoxylin and eosin. They were each examined by light microscopy to confirm that the mucosa of tracheal rings were either intact or had been removed as intended for the experiments.

Drugs. ACh chloride (Sigma Chemical, St. Louis, MO) for reactivity testing in vivo was diluted in PBS of the following composition (in mM): 144 NaCl, 3.2 KCl, 6.5 Na_2HPO_4, 1.5 KH_2PO_4, 0.5 $CaCl_2 \cdot 6H_2O$; pH 7.40 at 37°C. For experiments in vitro, solutions of the following (in modified Krebs-buffered solution) were freshly made on the day of use: bestatin, indomethacin, pyrilamine, leupeptin, phosphoramidon, and substance P (all purchased from Sigma); phentolamine (Ciba-Geigy, Summit, NJ); and captopril (Squibb Pharmaceuticals).

RESULTS

In neither animal group was there a significant change in sRaw values after exposure. In those exposed to ozone, sRaw was 2.22 ± 0.09, and 2.35 ± 0.13 ml·cmH_2O·ml^{-1}·s before and after ozone, respectively. In those exposed to air, sRaw was 2.11 ± 0.07 and 2.26 ± 0.15 before and after air, respectively.

Muscarinic hyperreactivity was present in all animals exposed to ozone. Air-exposed animals show no change in reactivity over the same time. Mean changes in ED_{200} ACh for each group are shown in Fig. 1. The values for ED_{200}ACh before air or ozone exposure were not different.

Figure 2 shows responses (expressed as force generation) of airway muscle preparations from ozone- and air-exposed animals to cumulatively increasing concentrations of substance P in vitro. The responsiveness of mucosa-intact ozone-exposed airway tissue was greater at all concentrations of substance P tested (Fig. 2*A*). For the differences in maximal response to substance P between groups, $P < 0.01$.

There appeared to be no difference in substance P responsiveness between the ozone- and air-exposed groups of airways pretreated with 1 μM phosphoramidon (Fig. 2*B*). Phosphoramidon pretreatment abolished the increased responsiveness seen in ozone-exposed tissue. However, substance P-induced maximal force generation of both air- and ozone-exposed tissue was significantly increased by phosphoramidon pretreatment.

There was no difference seen in the responsiveness to substance P of ozone- and air-exposed airways devoid of mucosa (Fig. 2*C*). Mucosal denudation of air-exposed

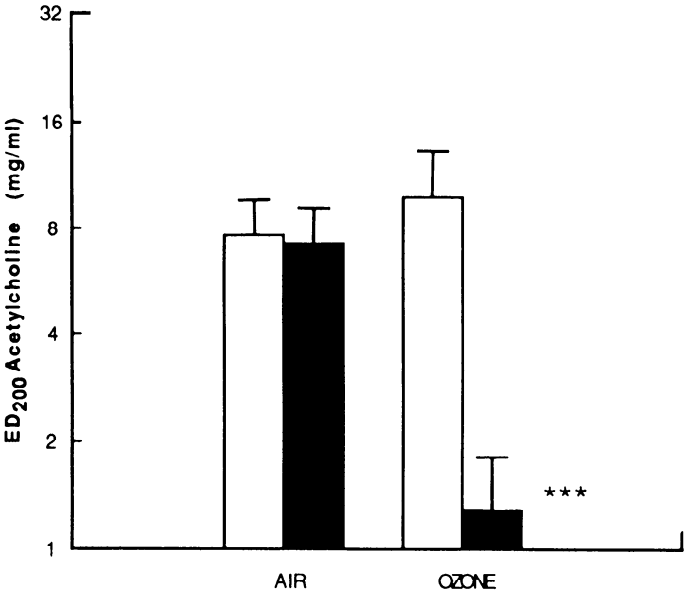

FIG. 1. Comparison of ED_{200}ACh before (open bars) and after (closed bars) exposure of guinea pigs to air or ozone. Values are means ± geometric SE for 18 pairs of animals. *** Postexposure value significantly lower than preexposure, $P < 0.005$.

tissue increased substance P responsiveness to a degree comparable with that caused by ozone exposure (Fig. 2, *A* and *C*). However, mucosal denuduation did not affect the substance P hyperresponsiveness of ozone-exposed tissues. The substance P responsiveness of ozone-exposed denuded rings was not different from that of ozone-exposed airways that were intact (Fig. 2, *A* and *C*). This contrasts with the increased responsiveness seen in the air-exposed airways devoid of mucosa compared with air-exposed tissue with intact mucosa (Fig. 2, *A* and *C*). Figure 2 also shows that phosphoramidon pretreatment increased the substance P responsiveness of intact airway tissues ($P < 0.02$). The phosphoramidon-induced increase of air-exposed tissue was greater than that caused by mucosal removal. Histological evaluation of tissue sections confirmed that the mucosa of tracheal rings was either intact or had been removed as intended for the experiments.

Antagonism of aminopeptidase, serine protease, or angiotensin-converting enzymes (with bestatin, leupeptin, or captopril, respectively) did not significantly affect responsiveness to 1 μM substance P of control, mucosa-intact airway preparations (Fig. 3). In addition, blockade of histaminic (H_1), α-, or β-adrenergic receptors (with pyrilamine, phentolamine, or propanolol, respectively) did not appear to affect the contractile response of guinea pig tracheal muscle to substance P. Values [as a percent of base-line response to substance P ($n = 4$)] were for pyrilamine, 95 ± 21 (SE), for phentolamine, 108 ± 13; and for propanolol, 112 ± 15.

Muscarinic responsiveness of airway preparations from the experimental and control groups are shown in Fig. 4. In the mucosa-intact preparations, force generation of ozone-exposed tissue was substantially greater at the highest ACh concentrations tested (Fig. 4*A*). This difference in responsiveness between the experimental and control groups was not present in rings without

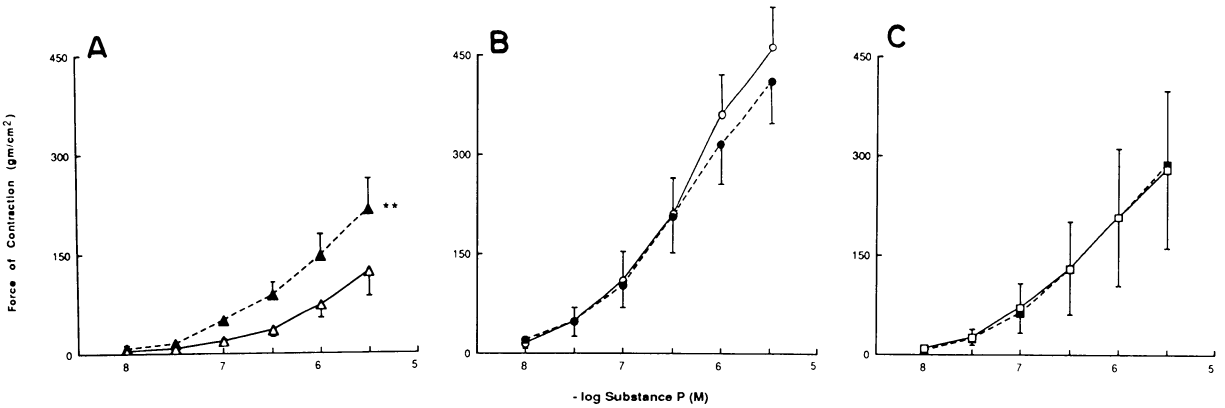

FIG. 2. Contractile response to cumulative concentrations of substance P of mucosa-intact (*A* and *B*) and mucosa-denuded tracheal ring segments (*C*) from animals exposed to ozone (closed symbols, dotted lines) or air (open symbols, solid lines). Responses of ozone- and air-exposed airway tissues pretreated with 1 μM phosphoramidon are shown in *B*. Responses are expressed in terms of force generation (in g) per cross-sectional area of airway smooth muscle (in cm^2). Values are means ± SE; $n = 6$ experiments in *A* and 4 in *B* and *C* (symbols without SE bars had SE less than symbol size). ** Value significantly different from control, $P < 0.01$. There was no difference in substance P responsiveness of ozone- and air-exposed airway tissue pretreated with phosphoramidon (*B*) or in preparations in which mucosa was removed after exposure and before testing in vitro (*C*). Mucosal denudation of air-exposed tissue increased substance P responsiveness to a degree comparable with that caused by ozone exposure (*A* and *C*). However, mucosal denudation did not affect substance P hyperresponsiveness of ozone-exposed tissues. Substance P responsiveness of ozone-exposed denuded rings was not different from that of intact ozone-exposed airways. Phosphoramidon pretreatment significantly increased substance P responsiveness of intact airway tissues ($P < 0.02$).

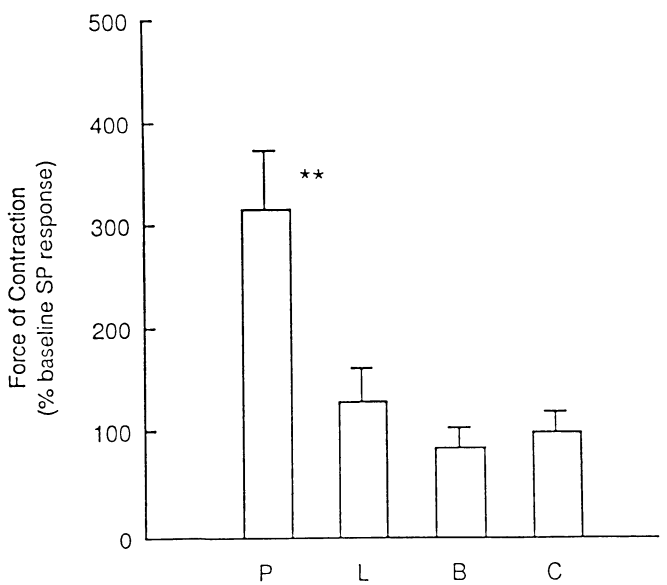

FIG. 3. Effects of enzyme antagonism by phosphoramidon (P, 1 μM), leupeptin (L, 10 μM), bestatin (B, 10 μM), or captopril (C, 10 μM) on contractile response of air-exposed mucosa-intact airway tissue to substance P (SP, 1 μM). Values are means ± SE of 5 cases for each antagonist, except for phosphoramidon cases, where $n = 6$. ** Significantly different from control, $P < 0.01$.

mucosa (Fig. 4*C*). However, within each group (Fig. 4, *A* and *C*), responses to ACh were significantly greater in preparations without mucosa ($P < 0.05$). Pretreatment of airway preparations with phosphoramidon did not affect muscarinic responsiveness of the airway muscle in either group (Fig. 4*B*).

Figure 5 shows the response to KCl of tracheal airway preparations from air- and ozone-exposed animals. No difference at any concentration of this agonist was seen.

DISCUSSION

We have found that smooth muscle of mucosa-intact airways from guinea pigs with ozone-induced bronchial hyperreactivity is hyperresponsive in vitro to substance P and ACh but not to KCl. Pretreatment with phosphoramidon, an antagonist of neutral endopeptidase, abolished the increased substance P responsiveness seen in the ozone- compared with the air-exposed group, but it had no effect on the difference in muscarinic responsiveness between these groups. Furthermore, we found that substance P responsiveness was not augmented in ozone-exposed airways in which the mucosa had been removed before testing in vitro (Fig. 2*C*). Likewise, muscarinic hyperresponsiveness was not present in ozone-exposed airways without mucosa. Our data indicate that smooth muscle responsiveness is increased in guinea pigs with ozone-induced bronchial hyperreactivity and suggest that this hyperresponsiveness may be linked to non-cyclooxygenase mucosa-derived factors.

To our knowledge, this is the first report of acute substance P hyperresponsiveness of airway muscle in vitro from animals with documented bronchial hyperreactivity. Subacute airway muscle hyperresponsiveness to substance P in airway disease was first described by Saban et al. (28) who studied bronchial muscle in vitro 4 days after guinea pigs had been innoculated with parainfluenza 3 virus. Both in the publication by Saban et al. and in a recent report (14) concerning ferret trachea evaluated 4 days after human influenza virus infection in vitro, the effect of viral airway infection on animal reactivity in vivo was not assessed. As others have found in ferret tissue (14), we found that guinea pig airway muscle substance P responsiveness in vitro was not indirectly influenced by histaminic (H_1), α-, or β-adrenergic receptor stimulation. In addition, the activity of a variety of enzymes other than neutral endopeptidase, including aminopeptidase, serine protease, and angiotensin-converting enzymes, did not appear to significantly affect substance P-induced airway muscle contraction in the guinea pig.

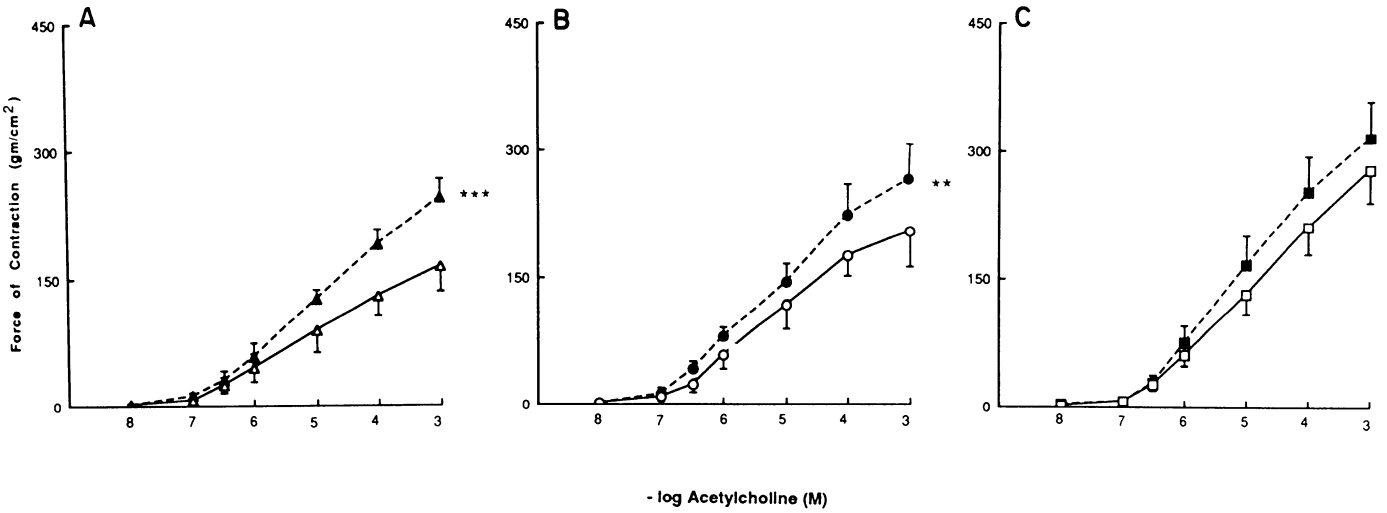

FIG. 4. Contractile responses to cumulative concentrations of ACh of mucosa-intact (*A* and *B*) and mucosa-denuded tracheal ring segments (*C*) from animals exposed to ozone (solid symbols, dotted lines) or air (open symbols, solid lines). Responses of ozone- and air-exposed airway tissues pretreated with 1 μM phosphoramidon are shown in *B*. Terms for responses are described in legend of Fig. 2. Values are means ± SE of 6 experiments, except in *B* where $n = 4$. At highest concentrations of ACh tested, ozone-exposed mucosa-intact airway tissue was substantially more responsive to stimulation in the absence (*A*) or presence (*B*) of phosphoramidon. There was no difference, however, in responsiveness of ozone- and air-exposed preparations in which mucosa was removed after exposure and before testing in vitro (*C*). Significantly different from control: ** $P < 0.01$, *** $P < 0.05$.

In conjunction with our earlier observations of striking histopathological injury, but not denudation, of guinea pig airway mucosa after ozone exposure (24), our present findings indicate that increased substance P responsiveness of guinea pig airway muscle occurs in airways with an intact, but functionally abnormal, mucosal cell layer. In contrast, substance P hyperresponsiveness in ferret airway tissue 4 days after viral infection occurred concomitant with extensive mucosal desquamation (14).

Our data also suggest that ozone-induced substance P hyperresponsiveness may be the result of decreased neutral endopeptidase activity of the respiratory mucosal cell layer. This conclusion is supported by the observations that *1*) denudation of air-exposed tissues caused these preparations to become as hyperresponsive to substance P as ozone-exposed intact tissues, and that *2*) mucosal denudation of ozone-exposed airway tissues did not further increase substance P responsiveness. In other words, removal of ozone-damaged mucosa, lacking neutral endopeptidase activity, had no effect on the underlying smooth muscle response to substance P, whereas removal of mucosa from air-exposed tissue with normal neutral endopeptidase activity increased substance P responsiveness. The fact that phosphoramidon pretreat-

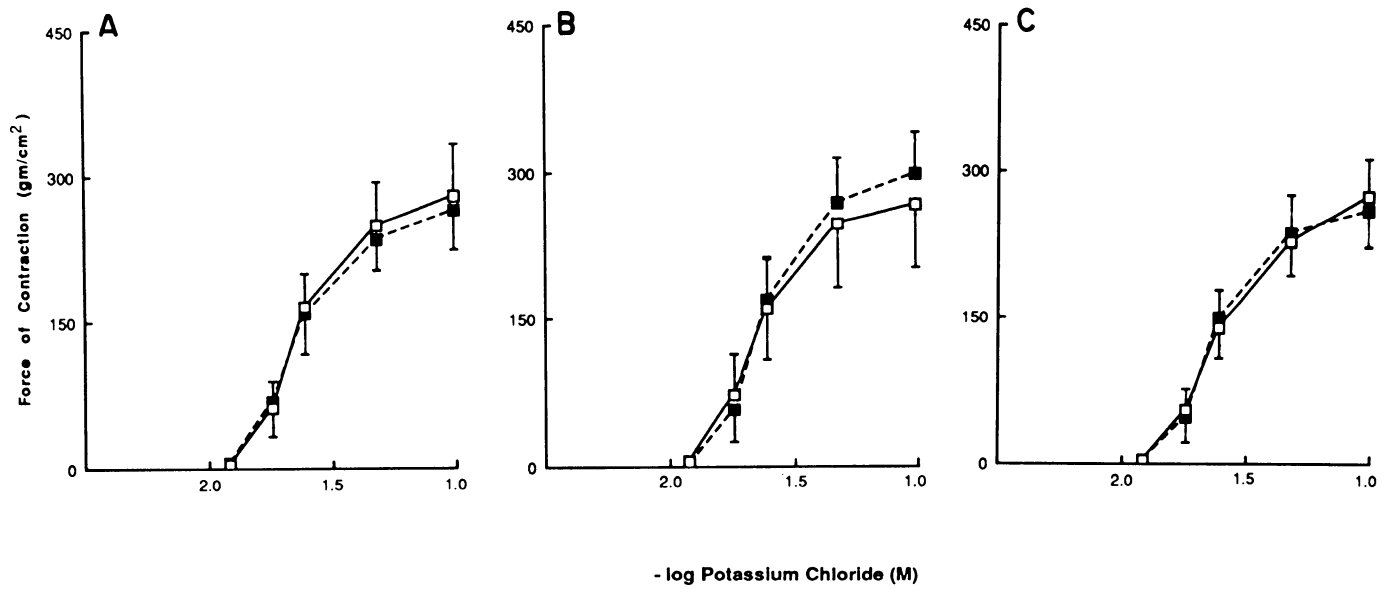

FIG. 5. Contractile responses to cumulative concentrations of KCl of mucosa-intact upper (*A*), middle (*B*), and lower (*C*) tracheal ring segments from animals exposed to ozone (solid symbols, dotted lines) or air (open symbols, solid lines). Terms for responses are described in legend of Fig. 2. Values are means ± SE of 6 experiments. There was no difference in responsiveness of ozone- and air-exposed airway tissue to KCl.

ment 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, suggests that the ozone exposure protocol we employed only partially inactivated airway neutral endopeptidase. In other words, phosphoramidon treatment inactivated all airway endopeptidase activity (both mucosal and nonmucosal), whereas ozone exposure inactivated only mucosal neutral endopeptidase. Consistent with this hypothesis are previous observations by other investigators that neutral endopeptidase activity is present on both mucosal and nonmucosal lung cells (15, 29).

The biochemical events occurring on oxidant injury of the airway mucosa are not well understood at present. Mucosal ozone exposure may generate oxygen-derived free radicals and oxidizing agents, like hydrogen peroxide and hypochlorous acid (HOCl), a product of chloride oxidation that may be catalyzed by myeloperoxidase, an enzyme abundant in some leukocytes such as eosinophils. Interestingly, we have recently found that exposure of guinea pig airway mucosa in vitro to 0.1 μM HOCl produced substance P hyperresponsiveness that was abolished by phosphoramidon pretreatment and that was not present in airways devoid of mucosa (22). This substance P hyperresponsiveness of HOCl-exposed mucosa-intact airways was associated with decreased neutral endopeptidase activity assayed by high-pressure liquid chromatography. These results and our current findings lead us to speculate that ozone-induced substance P hyperresponsiveness of airway muscle may be caused by the oxidation of airway mucosal neutral endopeptidase. On the surface of respiratory mucosal cell membranes (15, 29), this enzyme may be quite vulnerable to inhaled environmental pollutants (20a, 24a).

Other mechanisms that cannot be excluded include ozone-induced changes in airway muscle muscarinic receptors or receptor-linked events or the generation of a product that may antagonize neutral endopeptidase activity. The former seems unlikely based on our finding that maximal force generation was increased after ozone, suggesting nonreceptor mediation. The fact that *1*) ozone exposure did not increase the KCl responsiveness of intact or denuded airways and *2*) that the substance P and the ACh responsiveness of denuded airways was unaffected by ozone exposure suggests to us, but does not prove, that airway muscle hyperresponsiveness after ozone is not the result of changes in airway smooth muscle alone.

Our finding that muscarinic responsiveness was also increased in airway preparations with, but not without, mucosa suggests to us that ozone-induced airway muscle hyperresponsiveness is linked to more than one non-cyclooxygenase mucosa-derived factor. This conclusion is somewhat at variance with that made in a recent report in dogs by Jones et al. (16). They concluded that loss of an epithelial-derived relaxation factor was not responsible for the development of ozone-induced airway hyperreactivity in dogs, because they demonstrated increased muscarinic responsiveness in denuded tracheal preparations from both air- and ozone-exposed dogs. This evidence, in our opinion, does not mitigate against the possibility that ozone's effect on respiratory mucosal cells is partial rather than total (as is mucosal removal). It is clear from our data that mucosal removal causes a much greater change in airway muscarinic responsiveness than does mucosal ozone exposure alone. Thus, if assessed in combination (rather than individually where degrees of difference could be compared), the effect of the former (mucosal removal) may well obscure the effect of the latter (ozone exposure) on airway responsiveness.

Perhaps our difference of opinion also relates to an important difference in experimental design. We exposed animals to room air while awake and spontaneously breathing, rather than to "dry air" directly through an endotracheal tube while anesthetized as did Jones et al. (16). Barbet and colleagues (3) have recently demonstrated that breathing dry room air can cause significant tracheal mucosal damage. The potential effect of this type of respiratory mucosal damage on airway responsiveness in vivo and in vitro is of considerable interest. It is conceivable that dry air exposure through an endotracheal tube may damage the respiratory mucosa sufficiently to increase muscarinic responsiveness of airways in vitro. Thus these airways may not represent true control preparations. True control preparations may have otherwise shown a greater difference in response compared with intact ozone-exposed preparations than was the difference found by Jones and co-workers between denuded air- and denuded ozone-exposed airways. Employing a very similar protocol in the dog, Walters et al. (35) found no difference in muscarinic responsiveness of air- and ozone-exposed airways devoid of mucosa. Our findings in the guinea pig are similar to those of Walters et al.

The possibility that airway muscle hyperresponsiveness [to substance P (31) or to ACh] is the result of an ozone-induced decrease in airway acetylcholinesterase activity seems unlikely to us. This conclusion derives from our findings that ozone-exposed airways pretreated with phosphoramidon (compared with matched controls) were not hyperresponsive to substance P and that there was no difference in muscarinic responsiveness between matched pairs devoid of mucosa, as others have reported in the dog (35). It should be mentioned, however, that another group of investigators has found in mongrel dogs that some indexes of airway muscle muscarinic responsiveness (but not other indexes such as maximal response) after ozone were increased in preparations without mucosa (17). It is difficult for us to rationalize the differing results from these two studies in dogs in which very similar methods appear to have been employed.

From our work we conclude that airway muscle responsiveness in acute ozone-induced bronchial hyperreactivity is increased and that this hyperresponsiveness may be linked to non-cyclooxygenase mucosa-derived factors in the guinea pig. Our data indicate that neutral endopeptidase may be one of those factors that link ozone exposure to airway hyperresponsiveness. Furthermore, our data suggest that ozone partially inactivates airway mucosal neutral endopeptidase. Identification of other

factors and the cellular mechanisms by which smooth muscle contractility is augmented merit additional study.

ADDENDUM

Since the submission of this manuscript for publication, an article by Dusser et al. (7) has appeared, which is relevant to our own observations.

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