HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hypoactivity

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MURLAS, CHRISTOPHER G., THOMAS P. MURPHY, AND ZHIHUI LANG. *HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hypoactivity.* Am. J. Physiol. 258 (Lung Cell. Mol. Physiol. 2): L361–L368, 1990.—We investigated whether exposure of guinea pig tracheal tissue to hypochlorous acid (HOCl) or hydrogen peroxide (H_2O_2) by perfusion through the airway lumen affected the responsiveness of airway muscle to ACh, KCl, or substance P in the presence or absence of 1 μM phosphoramidon, an inhibitor of neutral endopeptidase (NEP). Pairs of tracheal segments were immersed in a Krebs solution (pH 7.40 at 37°C) and connected to perfusion circuits so that the lumen of one segment of each pair could be perfused with Krebs solution while the other was perfused for the same time (10 min) with either 0.1 μM HOCl or 10 mM H_2O_2. Segments after perfusion were cut into rings of similar size and placed in muscle chambers so that airway muscle force generation in vitro could be measured on stimulation by cumulative agonist doses. In addition, cell homogenates were made from other, similarly perfused tracheal segments to assess NEP activity using reverse-phase, high-pressure liquid chromatography (HPLC). We found that smooth muscle of mucosa-intact guinea pig airways perfused with HOCl, but not H_2O_2, was hyperresponsive to substance P but not to ACh or KCl. HOCl-perfused rings were not different from Krebs solution-exposed rings pretreated with phosphoramidon. There was no increase in substance P responsiveness of HOCl-exposed airways in which the mucosa had been removed before testing in vitro. The substance P hyperresponsiveness of HOCl-exposed, mucosa-intact airways was associated with decreased NEP activity. To more directly inspect the possible effect of HOCl or phosphoramidon exposure on airway substance P catabolism, disappearance of this tachykinin from perfusates circulated through additional tracheal segments (utilizing a closed circuit system) was also assessed by HPLC. It was found that HOCl or phosphoramidon exposure decreased substance P disappearance from these perfusates. Our results suggest that the smooth muscle hyperresponsiveness to substance P caused by airway perfusion with HOCl is produced by HOCl inactivation of mucosal NEP.

airway hyperreactivity; asthma; bronchoconstriction; bronchial reactivity; enkephalinase; guinea pig; hypohalous acids; metalloendopeptidase; muscarinic receptors; oxidant injury; tachykinins; trachealis muscle

OXIDANT AIRWAY INJURY in various species produces bronchial hyperreactivity (1, 5, 7, 15). At least some of these disorders may be linked to the generation of H_2O_2 and hypohalous acids, including HOCl, a product of eosinophil peroxidase- and myeloperoxidase-catalyzed oxidation of chloride (29, 32). During airway injury caused by some inhaled irritants, these two oxidants may occur at high concentrations within or on the luminal surface of the airway mucosa. The effects of mucosal oxidant injury on airway smooth muscle function has been the subject of considerable speculation. It has been shown by many investigators (4, 6, 13, 18, 27) that airway mucosa cells produce factors that affect airway muscle tone in a variety of species. This conclusion derives almost solely from studies in vitro in which physical removal of respiratory mucosa has been shown to influence airway smooth muscle responsiveness to various agonists. Whether other airway injuries, such as oxidant exposure (16), that do not appear to be associated with acute mucosal denudation in vivo also affect airway muscle responsiveness is uncertain.

To address this issue, we utilized airways in which liquid perfusion of an airway segment could assure an easily quantifiable and preferential exposure of an intact, normal airway mucosa to agents of either of two important oxidants, HOCl or H_2O_2. We assessed whether such perfusion of guinea pig airways augmented airway muscle responsiveness to subsequent stimulation by substance P, acetylcholine (ACh), or KCl in vitro. Mucosa-intact tissue rings were cut from tracheal segments that had been perfused with either HOCl, H_2O_2, or a Krebs-buffered solution. Before testing in vitro, these tissues were pretreated with indomethacin to inhibit time-dependent effects on airway muscle of cyclooxygenase products generated in vitro (17, 31), which may be derived from the respiratory mucosa (4). The responsiveness of these airway rings to exogenous substance P, ACh, or KCl was tested in the presence or absence of phosphoramidon, an inhibitor of neutral endopeptidase (NEP; EC 3.4.24.11; also called enkephalinase), an enzyme that affects guinea pig airway response to substance P in vivo (30) and in vitro (26) that appears to be present on certain respiratory mucosal cells (11). To substantiate evidence suggesting that observed effects on contractility were associated with changes in neutral endopeptidase activity, we also measured substance P disappearance or NEP activity in other guinea pig tracheae perfused in vitro.

METHODS

Protocol

Tracheae from male Hartley-strain guinea pigs (550–700 g body wt) were used in pairs throughout the study:

tracheal segments pretreated with 10 μM indomethacin were mounted in individual chambers so that the lumen of one segment of each pair could be perfused with a Krebs-buffered solution (pH 7.40 at 37°C) while the other was perfused for the same time (10 min) with either 0.1 μM HOCl or 10 mM H_2O_2. The serosal surfaces of these segments remained immersed in Krebs solution during the perfusion period. Immediately thereafter, the segments were cut into rings of similar size and placed in muscle chambers so that airway muscle force generation in vitro could be measured on stimulation by cumulative agonist doses. Airway smooth muscle responsiveness to exogenous substance P, ACh, or KCl was compared between the HOCl-, H_2O_2-, and Krebs solution-perfused groups. In some experiments, these airway rings were pretreated with 1 μm phosphoramidon, or the mucosa was removed, before stimulation by the agonists. We also measured NEP activity in tracheal homogenates made from other HOCl- and Krebs solution-exposed airways. Because these experiments suggested that HOCl exposure inactivated airway NEP, additional experiments were done to assess whether substance P disappearance within a closed perfusion circuit (containing an airway segment) was decreased by HOCl or phosphoramidon exposure as would be expected if HOCl was acting by decreasing NEP catabolism of this tachykinin.

Procedures

Airway contraction studies in vitro. Tracheae were obtained from guinea pigs killed with 60 mg/kg ip pentobarbital sodium (Butler, Columbus, OH). The tracheae were quickly excised and gently cleaned of debris, including blood. Perfusion of the tracheal segments was accomplished by securing the ends with suture to lipped adaptors that were part of twin perfusion circuits fed by gravity (at 1 ml/min) from glass reservoirs. Each pair of tracheae was placed in these adjacent perfusion circuits so that the external surface could be immersed horizontally in chambers containing a Krebs-buffered solution of the following composition (pH 7.40 at 37°C and gassed continuously with 95% O_2-5% CO_2 (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, and 9.6 glucose. One trachea of each pair was perfused with Krebs solution while the other was perfused for the same time with either 10 mM H_2O_2 (a concentration that, by itself, had no effect on airway muscle tone) or 0.1 μm HOCl [a concentration that may substantially decrease bovine tracheal mucosal NEP (11)]. In all experiments, the perfusion protocol (which followed a 2-min washout with Krebs solution of the entire circuit) was 14 min in duration, and the flow rate was 1 ml/min. The control trachea of each pair was perfused with Krebs solution for this entire period while the experimental trachea was perfused for the first 2 min with Krebs solution, for the ensuing 10 min with either HOCl or H_2O_2, and for the last 2 min with Krebs solution.

For studies concerning potential substance P catabolism by tracheal segments, the perfusion circuit was closed so that a 2-ml volume of 5 μM substance P could be recirculated for 10 min before and after the 2-min Krebs solution perfusion periods (which were separated by the 10-min HOCl, H_2O_2, phosphoramidon, or control perfusion period). In those experiments employing phosphoramidon, the perfusate also contained 1 μM phosphoramidon during the second substance P period. At the beginning and end of the two substance P perfusion periods, a 100 ml aliquot of perfusate was removed from the circuit to measure substance P (see below). Disappearance of substance P (in %) was expressed by 100 minus the ratio (multiplied by 100) of the amount measured at the end of each substance P period compared with the amount present at the beginning. The effect of HOCl or phosphoramidon was assessed by comparing the percent disappearance after exposure with 0.1 μM HOCl or 1 μM phosphoramidon to the percentage before that exposure. Mean values of the percent disappearance before and after control perfusion periods were not different.

For the airway contraction studies, the tracheae were removed from the perfusion apparatus and the terminal 2–3 mm of each end were removed. Preliminary experiments and histological examination had indicated that the histology and responsiveness of the remaining segments were not affected by the perfusion protocol. The remaining segments were then carefully cut transversely into ~3-mm-long rings. To minimize mucosal trauma and optimize mechanical function, each ring was tied with 4.0 silk thread at the cartilaginous attachment of one end of the airway muscle fibers, hung vertically from a force-displacement transducer (model FT03; Grass Instrument, Quincy, MA), and attached at the other end of the fibers to a 0.3-mm diameter stainless steel wire stirrup at the base of a 10-ml muscle chamber filled with Krebs solution. As documented histologically, this tissue handling caused damage to the mucosal surface only at those points of contract. For experiments requiring airway rings after perfusion that were devoid of mucosa, 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). An initial 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 was added to inhibit the possible effects of cyclooxygenase products generated in vitro. Before starting the experiment, each ring was adjusted to a muscle fiber length that produced maximal contraction to a submaximal current stimulus (20 V, 0.5 ms, 10 Hz) by stretching it progressively until that response was achieved (14, 22). During the entire experiment, this muscle length was maintained. The rings were stimulated with 10 μM ACh followed by thorough washouts until a consistent response was established. Thereafter, each preparation was exposed to either substance P, ACh, or KCl added in increasing concentrations and cumulatively to the muscle chambers. For each experiment and animal pair, rings from the upper third of tracheae were used for stimulation by substance P, KCl, or ACh. This comparison of agonist responses in like sections of guinea pig tracheae was done because previously published work (9) in this

tissue has shown that the upper and lower ends differ in their responsiveness to ACh and to electrical field stimulation. 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, be similarly determined (14, 28). Responses to each agonist concentration were expressed in terms of grams per square centimeters cross-sectional area (CSA) where CSA (2) was calculated as follows (14)

$$\text{CSA (cm}^2\text{)} = \frac{\text{blotted wet wt of dissected trachealis muscle (g)}}{\text{ring posterior wall height (cm)}} \times \text{tissue density } (\sim 1.06\ \text{g/cm}^3)$$

Differences in the maximal contractile response to each agonist between groups of ozone- and air-exposed tracheal rings were compared using independent t tests and were considered significant for values of $P < 0.02$.

Histological evaluation. Histological sections of the tracheal ring segments were made after the muscle studies in vitro had been completed. Eight-micrometer thick frozen sections were cut and stained with hematoxylin and eosin. They were each examined by light microscopy to identify whether the tracheal mucosa was denuded or intact and if intact whether pathological signs were present.

Biochemical assays of neutral endopeptidase activity in tracheal tissue. Each guinea pig tracheal segment (200–250 mg in wt) was homogenized in 3 ml of ice-cold 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.20. 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 with a tissue homogenizer (Brinkman Instruments) and placed in a shaker bath at 37°C for 2 h. The suspension was again centrifuged at 135,000 g. The resulting supernatant was assayed for NEP activity using a modification of methods previously described by Kuwada and Kotayama (10). Aliquots of this solution were incubated with the chromogenic substrate succinyl $(Ala)_3$-p-nitroaniline (SA_3NA) in the presence of 1 μM phosphoramidon and/or 100 μM amastatin for 30 min. Chromophores were detected in the effluent fractions by absorbance measured at 314 nm. The enzyme preparation was incubated with SA_3NA (0.5 mM), 50 mM Tris-HCl, and 0.1% Triton X-100, pH 7.2 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 60% trichloroacetic acid. Samples were centrifuged (Fisher, model 135A) at 14,000 g for 10 min at 4°C.

The incubation mixture was directly analyzed using a high-performance liquid chromatography (HPLC) system (Waters Associates, Milford, MA) to determine the hydrolytic products of this substrate cleaved by the enzyme. The substrate and hydrolytic products were applied to a Novapak C-18 reverse-phase column (3.9 × 150 mm, Phenomenex, Rancho Palos Verdes, CA) and separated with a linear gradient of increasing concentrations of HPLC grade methanol (*solvent A*) mixed into 10 mM NaH_2PO_4, pH 2.5 (*solvent A*) at a flow rate of 1 ml/min. The solvent mixture was initially 20% *solvent A*-80% *solvent B*. *Solvent A* was increased to 48% in 17 min for analysis of the products of SA_3NA hydrolysis. Products detected at 314 nm were identified by coelution with standards. Products were quantitated by comparing integrated peak areas with 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 values of $P < 0.05$.

Biochemical measurement of substance P catabolism by perfused trachea. Aliquots (100 ml) were injected on a Novapak C-18 reverse-phase column. Substance P was resolved with a one-step linear gradient from 10 to 42% acetonitrile in 0.1% 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 ~12 min (Fig. 1). Immediately thereafter, acetonitrile was increased to 70% within 2 min and maintained for the ensuing 5 min to wash off the column before subsequent

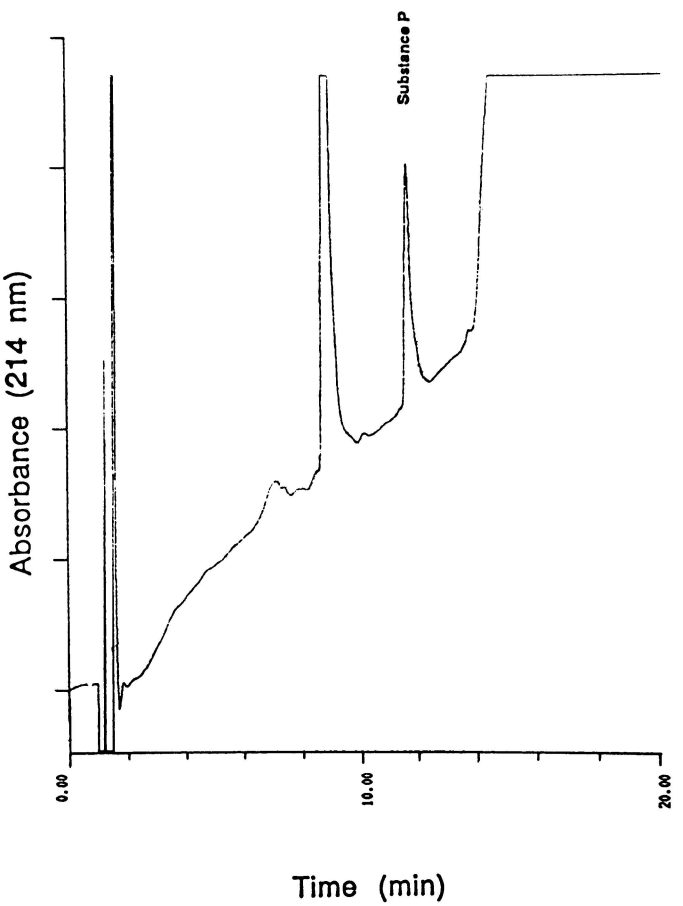

FIG. 1. Reverse-phase, high-pressure liquid chromatography profile of substance P recovered from perfusates containing 1 μM phosphoramidon (large peak immediately to *left* of substance P peak) recirculated through closed-circuit system described in METHODS.

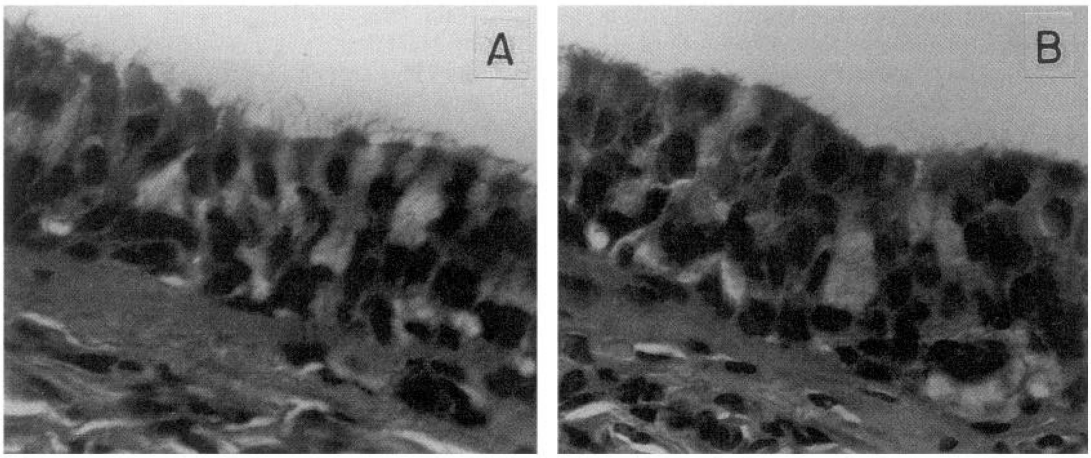

FIG. 2. *A*: light micrograph of tracheal mucosa from a Krebs-buffered solution-perfused airway segment: pseudostratified columnar epithelium included ciliated columnar cells and goblet cells (hematoxylin-eosin stain; original magnification: ×500). *B*: micrograph of tracheal mucosa from airway segment exposed to HOCl: striking, focal matting or loss of epithelial cilia was observed (hematoxylin-eosin stain; original magnification ×500).

use. Amounts of substance P degraded were quantified by comparing integrated peak areas to those established for known standards.

Drugs. Solutions of the following (in modified Krebs-buffered solution) were freshly made on the day of use: bestatin, indomethacin, leupeptin, propranolol, phosphoramidon, and pyrilamine (all purchased from Sigma); phentolamine (Ciba-Geigy, Summit, NJ); and enalaprilat (gift of Merck, Rahway, NJ).

RESULTS

Krebs solution-perfused tracheal segments demonstrated normal airway mucosal histology (Fig. 2*A*). Although the mucosa from HOCl-perfused preparations was not denuded, it was clearly abnormal by comparison. The most striking finding by light microscopy was matting or loss of epithelial cilia (Fig. 2*B*). The submucosal and muscle layers of the tracheal wall were similar to those in the sham-exposed cases.

Figure 3 shows responses (in g/cm^2) of airway preparations from HOCl- and Krebs solution-exposed tracheal segments to cumulatively increasing concentrations of substance P in vitro. The increase in responsiveness of the HOCl-exposed airway tissue was substantial (Fig. 3*A*). There appeared to be no difference in substance P responsiveness between the HOCl and control groups of airways pretreated with 1 μM phosphoramidon (Fig. 3*B*). The substance P responsiveness of phosphoramidon pretreated, Krebs solution-exposed tissues (Fig. 3*B*, solid lines) and HOCl-perfused tissue (Fig. 3*A*, dotted lines) were not different, and each was substantially greater than the control preparations exposed to Krebs solution alone (Fig. 3*A*, solid lines). There was no difference in

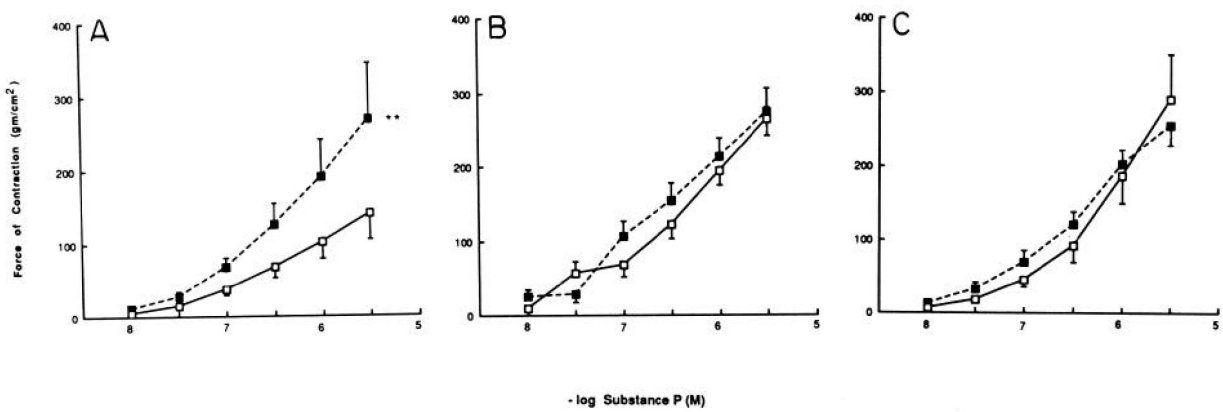

FIG. 3. Contractile responses to cumulative concentrations of substance P of mucosa-intact (*A* and *B*) and mucosa-denuded segments (*C*) tracheal rings from segments exposed in pairs to either HOCl (solid symbols, dotted lines) or Krebs solution (open symbols, solid lines). Responses of HOCl and air-exposed airway tissues pretreated with 1 μM phosphoramidon are shown (*B*). Responses are expressed in terms of force generation (in g) per cross-sectional area of airway smooth muscle (in cm^2). Each symbol represents mean ± SE of 5 experiments, except in *B* where 8 experiments were used (symbols without SE bars had SE less than symbol size). ** Experimental value significantly different from control value ($P < 0.01$). HOCl-exposed, mucosa-intact airway tissue was substantially more responsive to substance P stimulation than was control tissue (*A*). There was no difference in the substance P responsiveness of HOCl- and Krebs solution-exposed airway tissue pretreated with phosphoramidon (*B*) or in preparations in which mucosa was removed after exposure and before testing in vitro (*C*).

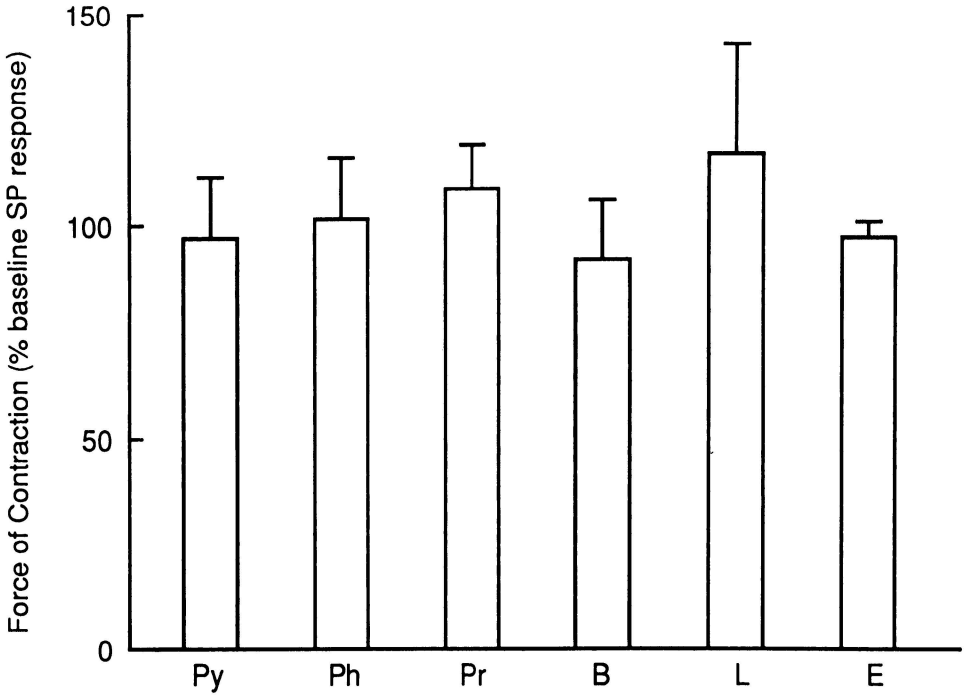

FIG. 4. Effects of pyrilamine (Py, 10 μM), phentolamine (Ph, 10 μM), propranolol (Pr, 10 μM), bestatin (B, 10 μM), leupeptin (L, 10 μM), or enalaprilat (E, 10 μM) on contractile response to substance P (SP, 1 μM). Values are means ± SE of 7 cases for each antagonist, except for phentolamine and propranolol where 6 were used for each.

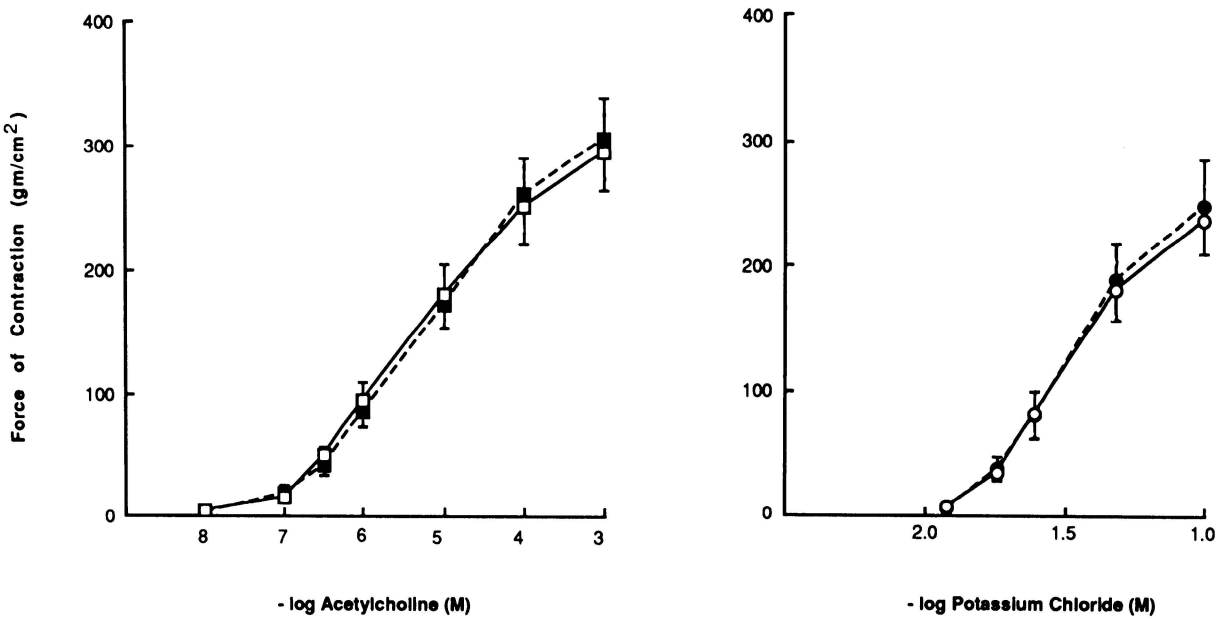

FIG. 5. Contractile responses to cumulative concentrations of ACh or KCl of mucosa-intact tracheal rings from segments exposed in pairs to either HOCl (solid symbols, dotted lines) or Krebs solution (open symbols, solid lines). Terms for responses and asterisks are described in Fig. 1. For ACh experiments, each symbol represents mean ± SE of 6 experiments; for KCl ones, 6 were used. At all concentrations of ACh or KCl tested, there was no difference in responsiveness of the HOCl- and Krebs solution-exposed airway tissue.

responsiveness to substance P between HOCl and control airways devoid of mucosa (Fig. 3*C*). The substance P responsiveness of HOCl-exposed, denuded rings appeared quite similar to that of the HOCl-exposed airways that were intact (Figs. 3, *A*, dotted lines, and *C*, dotted lines). In contrast, the responsiveness seen in the Krebs-exposed airways devoid of mucosa was increased compared with Krebs solution-exposed tissue in which the mucosa was left intact (Fig. 3, *A*, solid lines, and *C*, solid lines). Figure 3 also shows that the increased substance P responsiveness produced by phosphoramidon pretreatment was not different from that produced by HOCl perfusion.

Blockade of histaminic (H_1), α-adrenergic, or β-adrenergic receptors (with pyrilamine, phentolamine, or propranolol, respectively) did not appear to affect the contractile response of guinea pig tracheal muscle to substance P (Fig. 4). In addition, antagonism of aminopeptidase, serine protease, or angiotensin-converting enzymes (with bestatin, leupeptin, or enalaprilat, respectively) did not affect substance P responsiveness.

Muscarinic responsiveness of airway preparations

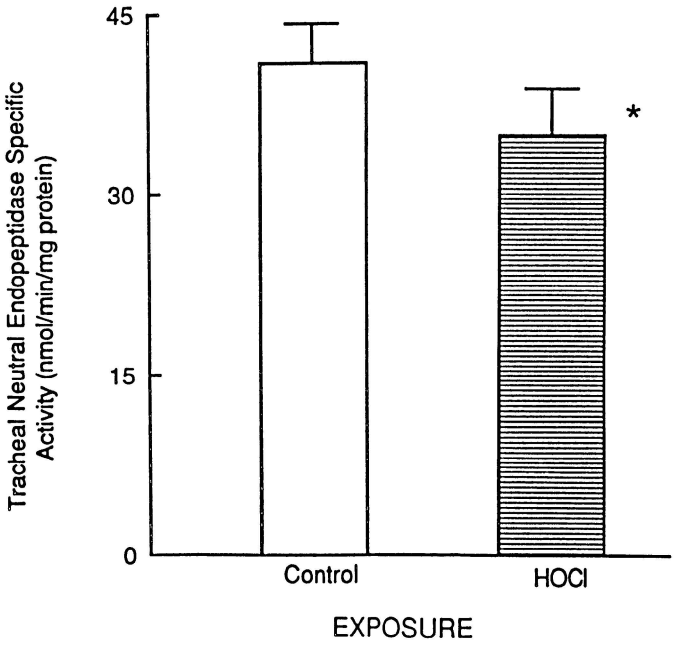

FIG. 6. Effect of HOCl exposure on airway NEP activity. Values shown for control (Krebs solution)- and HOCl-exposed groups are means ± SE of 5 cases each. * Significantly different from control group, $P < 0.05$.

TABLE 1. *Disappearance of substance P from guinea pig tracheal segment perfusates*

Condition	%Disappearance
Control (no inhibitor present)	89.2±0.7
HOCl (0.1 μM)	49.2±8.2*
Phosphoramidon (1 μM)	66.9±9.0*

Values are means ± SE of 4 experiments. Aliquots (100 ml) from guinea pig tracheal segment perfusates containing 5 μM substance P (in 2 ml Krebs solution) were analyzed. Disappearance of intact substance P was quantitated as described in METHODS. * Significantly different from control value ($P < 0.05$).

from the experimental and control groups is shown in Fig. 5*A*. No differences between groups in response to ACh were seen. Figure 5*B* shows data comparing the KCl responsiveness of both groups. No differences were noted between groups in response to this agonist.

Measurements of NEP activity of whole tracheal homogenates made from both HOCl- and Krebs solution-perfused tracheal segments are shown in Fig. 6. This activity was abolished by 1 μM phosphoramidon. The mean value of NEP activity from the experimental cases was 35.0 ± 3.9 nmol·min^{-1}·mg protein^{-1} compared with 41.1 ± 3.3 for the control group ($P < 0.05$, $n = 5$).

Data concerning the disappearance of substance P from closed-circuit perfusates are shown in Table 1. Compared with control cases, 1 μM phosphoramidon exposure led to a significant decrease in the disappearance of substance P from perfusates (Table 1). This decrease would be expected if substance P catabolism by the perfused tracheal tissue was reduced. Likewise, HOCl exposure also produced a significant decrease in substance P disappearance from perfusates. The degree of difference produced by HOCl and phosphoramidon (compared with controls) was not statistically different from each other.

Figure 7 shows responses of airway preparations from H_2O_2- and Krebs solution-exposed tracheae to cumulatively increasing concentrations of substance P, ACh, or KCl. Exposure to H_2O_2 did not significantly affect responsiveness to any of these agonists.

DISCUSSION

We have found that smooth muscle of mucosa-intact guinea pig airways perfused with HOCl is hyperresponsive to substance P but not to ACh or KCl. Phosphoramidon pretreatment or mucosal removal produced a similar pattern of results. The substance P hyperresponsiveness produced by HOCl perfusion was associated with decreased airway neutral endopeptidase activity and decreased disappearance of substance P from tracheal segment perfusates. Likewise, phosphoramidon exposure decreased substance P disappearance suggesting inhibition of substance P catabolism by tracheal tissue. Our results suggest that the increased smooth muscle responsiveness to substance P produced by luminal exposure of airway tissue to HOCl is produced by HOCl inactivation of airway mucosal NEP.

To our knowledge, this is the first demonstration that

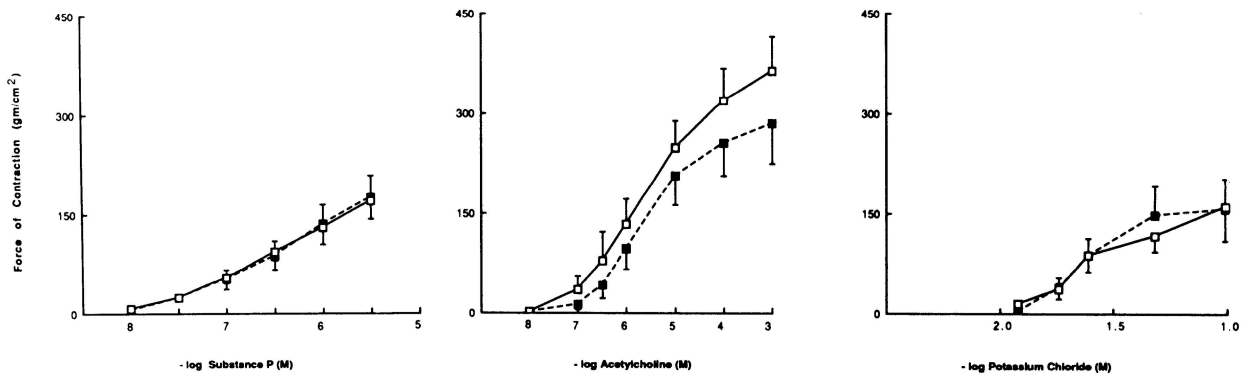

FIG. 7. Contractile responses to cumulative concentrations of substance P, ACh, or KCl of mucosa-intact tracheal rings from segments exposed in pairs to either H_2O_2 (solid symbols, dotted lines) or Krebs solution (open symbols, solid lines). Terms for responses are described in Fig. 3. Each symbol represents mean ± SE of 4 experiments. At all concentrations of substance P, ACh, or KCl tested, there was no difference in responsiveness of the H_2O_2- and Krebs solution-exposed airway tissue.

exposure to an oxidizing agent like HOCl may selectively augment the smooth muscle responsiveness of mucosa-intact airways to certain bronchoconstrictors like substance P while the responsiveness to other agonists remains unaltered. Subacute airway muscle hyperresponsiveness to substance P in airway disease was first described by Saban et al. (23), who studied tracheal muscle 4 days after guinea pigs were infected with parainfluenza 3 virus. Substance P hyperresponsiveness of ferret tracheae evaluated 4 days after influenza infection in vitro has also been recently reported (8). As other investigators (8) have found in ferret tissue, we found that guinea pig airway muscle substance P responsiveness in vitro was not indirectly influenced by histaminic, α-adrenergic, or β-adrenergic receptor stimulation. In addition, the activity of a variety of enzymes other than NEP, including aminopeptidase, serine protease, and angiotensin-converting enzymes, did not appear to significantly affect substance P-induced, guinea pig airway muscle contraction in vitro.

Our experience suggests that the increased substance P responsiveness of guinea pig airway muscle that can be produced by HOCl airway injury is linked to an intact but functionally abnormal respiratory mucosal cell layer (15, 16). The substance P responsiveness of airways devoid of mucosa that were exposed to HOCl was not different from that of Krebs solution-exposed airways without mucosa. Although there were changes in the guinea pig airway mucosa after HOCl injury, we found that mucosal denudation did not occur. Therefore, this type of injury contrasts with the extensive subacute mucosal desquamation that has been shown in virus-infected ferret airway tissue hyperresponsive to substance P (8). It has been shown by many investigators (4, 6, 13, 18, 27) that physical removal of the airway mucosa per se may increase responsiveness of airway muscle in vitro to a variety of other bronchoconstrictors.

Although the mucosa of airway preparations studied was intact after HOCl perfusion, our data indicate that the substance P hyperresponsiveness present was associated with a decrease in NEP activity of whole tracheal homogenates. The significance of this decrease is corroborated by the observed decrease in disappearance of perfusate substance P from tracheal segments exposed to HOCl. The evidence from this study also suggests that NEP activity of airway cells other than the respiratory mucosa is substantial and consequently that whole tracheal NEP activity may obscure the effect of HOCl on mucosal NEP activity. We have recently reported additional information suggesting that HOCl has a direct effect on NEP airway mucosal cells (11). Enzymatic activity of cytosolic and membrane fractions from bovine tracheal mucosa homogenates was assessed by HPLC assay of products cleaved from succinyl-$(Ala)_3$-*p*-nitroalanine (10) in the presence of amastatin, an aminopeptidase antagonist. The high specific enzymatic activity that was identified and inhibited by 1 μM phosphoramidon, was significantly reduced by a 10-min exposure to 0.1 nM HOCl. These results and our current findings lead us to speculate that HOCl-induced substance P hyperresponsiveness of airway muscle is caused by oxidation of airway mucosal NEP. Other possibilities that cannot be dismissed at this time include HOCl-induced changes in airway smooth muscle substance P receptors or receptor-linked events and the generation of product(s) that may antagonize NEP and/or substance P activity.

The finding that muscarinic responsiveness was not increased in HOCl-perfused airways suggests that the hyperresponsiveness to substance P observed is not due to a HOCl-induced decrease in airway acetylcholinesterase activity. If this were so, responses to ACh in the experimental preparations probably would have been increased as well. In addition, the fact that KCl and ACh responsiveness in vitro was not increased suggests that HOCl-induced airway muscle hyperresponsiveness to substance P is not simply due to a decrease in the diffusion barrier imposed by the mucosa (13) or to HOCl-induced changes in airway smooth muscle itself.

In this study, we found that exposure of guinea pig airway preparations to 10 mM H_2O_2 for as long as 10 min did not significantly change airway muscle responsiveness to substance P, ACh, or KCl. The effect of H_2O_2 perfusion on airway smooth muscle responsiveness to bronchoconstrictors has not been previously studied to our knowledge, although the direct effect of immersion in H_2O_2 on airway muscle contractility has been investigated (25). In that study, it was found that H_2O_2 immersion contracted bovine tracheal muscle, and this effect was enhanced under hypoxic conditions and blocked by cyclooxygenase inhibitors. In two recent studies (3, 19), the effect of tissue immersion in (rather than perfusion with) H_2O_2 on pulmonary arterial smooth muscle tone has also been evaluated. The reported results seem somewhat contradictory. Exposure of bovine pulmonary artery preparations to H_2O_2 produced a concentration-dependent relaxation of precontracted rings by a mechanism that appeared to be independent of the endothelium or prostaglandin mediators (3). In comparison, exposure of rat pulmonary artery rings to free radicals generated in vitro by xanthine-xanthine oxidase resulted in contractions that were endothelium cyclooxygenase product independent but that were completely blocked by catalase, suggesting that H_2O_2 was the major reactive species (19). These results conflict with studies concerning canine coronary smooth muscle in which H_2O_2 directly depressed smooth muscle contractions and also initiated the release of endothelium-derived relaxing factor(s) (21). Comparing these studies with our own, it would appear that the effects of oxidant tissue injury on smooth muscle responsiveness are dependent on a variety of factors, including the particular oxidant(s) generated, the route and degree of exposure, and the species and organ tissue studied, especially as these relate to products of archidonic acid mutation that may be elaborated.

The results of this investigation may be of some relevance to the pathophysiology of airway hyperreactivity in respiratory disorders that may be linked to damage of the respiratory mucosa (16a) and the generation of oxidants like HOCl. The production of such hypohalous acids into the airway microenvironment may augment smooth muscle responsiveness to endogenous neuro-

transmitters like substance P (14a). The present work identifies this possibility and indicates a mechanism by which respiratory mucosal oxidant injury may lead to this hyperresponsiveness.

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