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Hyaluronan blocks porcine pancreatic elastase-induced mucociliary dysfunction in allergic sheep

Mario Scuri, Juan R. Sabater, and William M. Abraham

Division of Pulmonary and Critical Care Medicine, Miller School of Medicine,
University of Miami at Mount Sinai Medical Center, Miami Beach Florida

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Scuri M, Sabater JR, Abraham WM. Hyaluronan blocks porcine pancreatic elastase-induced mucociliary dysfunction in allergic sheep. *J Appl Physiol* 102: 2324–2331, 2007. First published March 29, 2007; doi:10.1152/jappphysiol.00568.2006.—Neutrophil elastase is a mediator common to asthma, chronic obstructive pulmonary disease, and cystic fibrosis and thought to contribute to the pathophysiology of these diseases. Previously, we found that inhaled hyaluronan blocked elastase-induced bronchoconstriction in allergic sheep through its control of tissue kallikrein. Here, we extend those studies by determining if inhaled hyaluronan can protect against the elastase-induced depression in tracheal mucus velocity, a surrogate marker of whole lung mucociliary clearance. We measured tracheal mucus velocity in allergic sheep before, and sequentially for 6 h after, aerosol challenge with porcine pancreatic elastase alone and after pretreatment with 1.5 or 6 mg aerosolized hyaluronan. Elastase (2.55 U) decreased tracheal mucus velocity. Pretreatment with 6 mg, but not 1.5 mg, hyaluronan inhibited the elastase-induced decrease in tracheal mucus velocity. Hyaluronan (6 mg) given 1 h after elastase challenge was ineffective, suggesting the involvement of secondary mediators. The elastase-induced depression in mucus transport appeared to be mediated, in part, by reactive oxygen species and bradykinin because pretreatment with either aerosolized catalase (38 mg/3 ml) or the bradykinin B₂-receptor antagonist HOE140 (400 nM/kg) was also effective in blocking the response. These latter two findings are consistent with oxygen radical-induced degradation of hyaluronan with concomitant loss of its regulatory effect on tissue kallikrein, resulting in kinin generation. This hypothesis is supported by the demonstration that hyaluronan failed to block the oxygen radical-induced fall in tracheal mucus velocity resulting from xanthine-xanthine oxidase challenge and that inhaled bradykinin itself can slow mucociliary transport.

polysaccharides; asthma; chronic obstructive pulmonary disease; serine proteases; cystic fibrosis

THE SLOWING of mucociliary clearance is a common pathophysiological characteristic of asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis and contributes to the morbidity of these diseases (48). Therefore, uncovering potential factors that contribute to this reduced mucus clearance is important. Neutrophil elastase (NE) is one mediator whose concentrations are increased in airway samples obtained from patients with these diseases (9–11, 21, 22, 49). The putative contribution of increased NE to the reduced mucus clearance is based on experimental evidence showing that NE can affect individual components of the mucociliary apparatus as well as direct evidence that NE can slow mucus clearance in vivo. Specifically, NE is a known mucus secretagogue (23, 29, 47), has cilio-inhibitory properties (3, 45), can stimulate the pro-

duction of reactive oxygen species (ROS) (12), and can stimulate epithelial sodium channels, which reduces the periciliary fluid layer contributing to mucus stasis (5). Consistent with these effects on the various individual components of the mucociliary apparatus, our in vivo studies in sheep showed that aerosol challenge with either human NE (HNE) (35), or elastase obtained from degranulated sheep neutrophils (32) slowed tracheal mucus velocity (TMV), a marker of whole lung mucociliary clearance. Furthermore, O’Riordan and colleagues (32) showed that the reduction in TMV that occurs after antigen challenge in allergic sheep was associated with an increase in neutrophils and free elastase activity in bronchoalveolar lavage (BAL) fluid and that this effect could be prevented and/or reversed with natural and synthetic elastase inhibitors including α 1-protease inhibitor and a synthetic HNE inhibitor, ICI-200,355 (32). It is important to note that the effects on TMV in these studies were independent of the bronchoconstrictor responses seen with antigen challenge because the agents did not protect against the antigen-induced airway constriction. Collectively, these studies suggest that clearance of mucus from the airways is slowed in the presence of increased airway elastase activity.

Hyaluronan (HA) is a linear polymer formed by a repeating disaccharide structure of glucuronic acid and *N*-acetylglucosamine with molecular weights ranging from $\sim 2 \times 10^5$ to $\sim 10 \times 10^6$. HA is abundant in biological fluids and plays a significant role in several biological processes, including cell-cell and cell-matrix signaling and regulation of cell migration and proliferation (17). Many of these biological functions are mediated by cell surface receptors CD44 and RHAMM (receptor for hyaluronic acid-mediated motility), or CD168 (8, 20). In the lung, HA can be found as a soluble polymer or complexed with other proteoglycans, and its homeostasis has been shown to be deranged in a variety of pathological conditions, including acute and chronic lung diseases (31). This latter point is of interest because of previous studies showing that under normal conditions, HA interacts with RHAMM on ciliated airway epithelial cells and that functionally the binding of HA to RHAMM stimulates ciliary beating (16, 28). Furthermore, HA was shown to bind and inactivate tissue kallikrein (TK), the enzyme responsible for the generation of kinins (7, 14, 16), pluripotent inflammatory mediators that contribute to bronchoconstriction and airway hyperresponsiveness (2, 13). Alternatively, during inflammatory conditions, degradation of HA by hyaluronidase or ROS results in a loss of the HA-protective functions. This was demonstrated experimentally by treating

Address for reprint requests and other correspondence: W. M. Abraham, Dept. of Research, Mount Sinai Medical Center, 4300 Alton Rd., Miami Beach, FL 33140 (e-mail: abraham@msmc.com).

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submucosal gland cells with xanthine-xanthine oxidase (X/XO) to generate ROS, resulting in HA depolymerization and subsequent TK activation (7, 16). The degradation and TK activation could be blocked by pretreating cells with ROS scavengers or adding exogenous HA (7, 16).

These in vitro studies provided mechanistic support for our previous work demonstrating the in vivo interactions of elastase, HA, and TK. We first showed that inhaled porcine pancreatic elastase (PPE) caused bronchoconstriction, that this constrictor response was associated with increased BAL TK activity, and that kinin levels increased in BAL fluid. Consistent with the role of TK and kinins in this PPE-mediated response was the finding that the bronchoconstriction could be blocked by a bradykinin (BK) B₂-receptor antagonist, NPC-567 (39, 42). Subsequently we showed that pretreatment with inhaled HA blocked the PPE-induced increase in BAL TK activity and the bronchoconstriction, as well. The protective effects seen with HA were both dose and molecular weight dependent (39). Finally, to ensure that the actions of HA were not specific to PPE, we showed that pretreatment with inhaled HA blocked HNE-induced bronchoconstriction (38).

Although these studies indicated that HA can block the constrictor effects resulting from aerosol challenge with either PPE or HNE, the effects of HA on elastase-induced slowing of mucus clearance have not been determined. Therefore, in this study, we tested the hypothesis that HA would block elastase-induced reduction in TMV. We used PPE for these studies because of our extensive previous work with this elastase and because of the similarities between PPE and HNE in their constrictor responses when given alone or the lack thereof when given in the presence of HA. On the basis of our previous studies showing that natural and synthetic elastase inhibitors could reverse elastase-mediated decreases in TMV (32), we also determined if HA could reverse the PPE-induced reduction in TMV. Not surprisingly, we found that pretreatment with HA inhibited the PPE-induced decrease in TMV, but that giving HA after PPE was ineffective. To determine if the failure of HA to reverse the PPE-induced reduction in TMV could be related to the release of ROS, we examined the effect of the radical scavenger catalase (Cat) on the PPE-induced TMV response and determined if HA could block the decrease in TMV caused by ROS. Furthermore, since TK activation leads to kinin generation, we determined if the PPE-induced response could be blocked with a BK B₂-receptor antagonist and if BK, itself, slowed TMV. Finally, we provide in vivo evidence that HA may have a direct effect on mucus clearance.

METHODS

Adult ewes (mean weight 40.8 kg) allergic to *Ascaris Suum* antigen were used for this study. The allergic designation indicates that the animals demonstrate a bronchoconstrictor response to inhaled *Ascaris suum* and that they have heightened responses to inhaled BK compared with nonallergic animals (1). These animals, however, were not exposed to antigen during the course of these studies. For all studies described below, repeat experiments in any one animal were separated by at least 72 h. The study was conducted under the approval of the Mount Sinai Medical Center Animal Research Committee.

Measurement of TMV

TMV, a marker of mucociliary clearance, was measured with roentgenographic technique as previously described (32, 36). The

animals were restrained in a shopping cart, in an upright position with their heads immobilized. They were intubated with a cuffed endotracheal tube (7.5 cm ID; Mallinckrodt Medical, St. Louis, MO) introduced through the left nostril, using a flexible fiber-optic bronchoscope after topical anesthesia with 2% lidocaine solution (Hospira, Lake Forest, IL). The inspired air was warmed and humidified with a Bennett humidifier (Puritan-Bennett; Lenexa, KS). To minimize possible impairment of TMV caused by an inflated cuff, the endotracheal tube cuff remained deflated throughout the study, except for the short periods of aerosol administration. The animals were also given water (60 ml) every other hour through a nasogastric tube to minimize possible changes in TMV caused by dehydration.

To measure TMV, we insufflated 5–10 radiopaque Teflon particles onto the tracheal mucosa. The particles were ~1 mm in diameter and 0.8 mm thick and weighed between 1.5 and 2 mg. The particles were punched out of a strip of Teflon impregnated tape (Saint-Gobain Performance Plastic Bristol, RI). A modified suction catheter connected to a source of compressed air (50 psi) at a flow rate of 3 to 4 l/min was used to introduce the particles via the endotracheal tube. The catheter remained within the endotracheal tube only during actual insufflation. No contact with the tracheal surface was made. The movement of the particles was then measured over a 1-min period, using videotaped fluoroscopy.

Aerosols

All aerosols were generated using a disposable medical nebulizer (Raindrop, Nelcor-Puritan Bennett, Carlsbad, CA). The output from the nebulizer generated an aerosol with mass median aerodynamic diameter of 3.2 μ m (geometric SD 1.9) as determined by an Andersen cascade impactor (25). The output of the nebulizer was directed into a plastic T-piece, which was interconnected to the inspiratory port of a Harvard piston ventilator (Harvard Apparatus, Natick, MA) with the animal's tracheal tube. To control aerosol delivery, a dosimeter system consisting of a solenoid valve and a source of compressed air (20 psi) was used. The solenoid valve was activated for 1 s at the beginning of the inspiratory cycle of the ventilator. Aerosols were delivered at a tidal volume of 500 ml and a rate of 20 breaths/min as previously described (2).

Agents

PPE, BK, X, XO from buttermilk, and Cat were purchased from Sigma Aldrich (St. Louis, MO). PPE was dissolved in PBS to a stock concentration of 5 mg/ml. Aliquots of 2.55 and 1.275 U were kept at -20°C, dissolved in 3 ml PBS the day of the experiment, and delivered as aerosols until completion. HA from pig skin (average molecular mass ~70 kDa) was purchased from Seikagaku (Tokyo, Japan). Aliquots of 6 and 1.5 mg were diluted in 3 ml PBS the day of the experiment and delivered as aerosols until completion. BK was dissolved in PBS to a concentration of 5 mg/ml and delivered as an aerosol (20 breaths). The bradykinin B₂ antagonist HOE140 was purchased from Clinalfa (Oakland, CA). A final solution of 400 nM/kg was prepared in 3 ml PBS the day of the experiment and delivered as an aerosol until completion. For the generation of ROS, X (0.1% in PBS) was delivered by aerosol over 4 min followed by XO (4.1 U/2 ml) aerosolized to completion as previously described by us (26). Cat (38 mg/3 ml H₂O) was delivered as aerosol until completion as previously described by us (26).

Protocol

Study 1: Effect of PPE on TMV. TMV was measured and then the sheep inhaled 3 ml PBS as a control for subsequent pharmacological interventions (see below). Thirty minutes later, TMV was remeasured followed by inhalation challenge with either 2.55 or 1.275 U of PPE. TMVs were then measured at 0.5 and 1 h and then hourly up to 6 h after challenge. The units of PPE used for this study were those used in our previous studies (39, 42).

To control for changes of TMV over time, animals were given PBS alone. TMV was measured before and for 6 h after PBS challenge.

Study 2: Effect of HA on PPE-induced changes in TMV. STUDY 2A. TMV was measured, and then the sheep were given either 1.5 or 6 mg HA by inhalation. TMV was remeasured 30 min after treatment, and then the sheep were challenged with PPE. Post-PPE TMVs were measured as described above.

To control for changes in TMV due to HA treatment alone, TMV was measured, and then the animals were given 6 mg HA. TMV was followed for 6 h.

STUDY 2B. To determine if HA could reverse the PPE-induced fall in TMV, animals were treated with 6 mg inhaled HA, 1 h after challenge with PPE, and measurements of TMV were carried out through 6 h.

Study 3: Contribution of ROS to the PPE-induced reduction in TMV. STUDY 3A. Our findings that HA could not reverse the PPE-induced reduction in TMV suggested to us that PPE could stimulate the release of secondary mediators that could contribute to the reduction in TMV (43). We have previously shown that ROS can slow TMV (43). Because elastase can stimulate ROS in the airways (12) and HA is degraded by ROS (7), we tested the possibility that ROS might be a factor in the PPE-induced fall in TMV. In the first series of experiments, sheep were pretreated with aerosolized Cat (38 mg/3 ml PBS), immediately before PPE challenge. TMV was measured before and after Cat treatment and then serially after PPE challenge as described above. The dose of Cat used in these studies was previously shown by us to block ROS-induced bronchoconstriction (26).

STUDY 3B. The results of the Cat pretreatment experiments suggested a role for ROS. To test this directly, we challenged sheep with aerosolized X/XO, as previously published by our group (26). TMV was measured before and serially for 6 h after X/XO challenge. To determine if HA could modify the effects of X/XO, we repeated these studies, but 30 min before X/XO challenge the sheep were administered 6 mg HA. TMV measurements were made as described in study 2A.

STUDY 3C. We then determined if Cat could reverse the PPE-induced decrease in TMV by giving Cat 1 h after PPE challenge. TMV was measured before and after PPE challenge as described above.

Study 4: Effect of BK on TMV. STUDY 4A. Our previous work showed that PPE causes an increase in BAL fluid TK activity and

kinin levels. The effect of these kinins on TMV, however, has not been reported. Therefore, we determined if BK affects TMV and if the effect was mediated by BK B₂ receptors. TMV was measured before and after PBS and then for up to 6 h after inhalation challenge with BK (5 mg/ml; 20 breaths). The dose of BK used for this study was the same as we have used previously to induce bronchoconstriction in allergic sheep (1). The same protocol was repeated except that the BK B₂ antagonist HOE140 (400 nM/kg) was given by aerosol 30 min before BK challenge.

STUDY 4B. Finally, to show that the BK pathway contributes to the PPE-induced slowing of TMV, we pretreated sheep with HOE140 30 min before PPE challenge. TMV was measured for 6 h as described above.

Statistics

All data were analyzed using a multivariate ANOVA for repeated measures. If a significant effect was found, a post hoc *t*-test with Bonferroni correction was used to identify significant pairs. Individual comparisons were made using paired and unpaired *t*-test when appropriate (Sigmastat 2.0 for Windows, SPSS, Chicago, IL). Values in the text and Figs. 1–8 are presented as means $\pm$ SE; *P* < 0.05 was considered significant using a two-tailed test.

RESULTS

Table 1 provides the raw values that equate to the initial TMV values (100%) and the percent changes in TMV at 1 and 6 h after PPE challenge as illustrated in Figs. 1–8. The initial values represent the TMV values measured 30 min after treatment with either PBS (control) or the described pharmacological agent immediately before PPE challenge. There were no statistical differences between these TMV values and the baseline TMV value (obtained before treatment with placebo or drug) for any study (data not shown). Also shown in Table 1 for reference are the numbers of sheep used in each specific protocol.

Study 1: Effect of PPE on TMV. Figure 1 shows the effects of two doses of PPE compared with PBS on TMV. Both doses of PPE significantly reduced TMV compared with PBS with

Table 1. Initial TMV values (100%) and percent changes in TMV 1 and 6 h after PPE challenge in studies 1–4

Study Challenge	Initial TMV Values, mm/min	1 h Postchallenge	6 h Postchallenge
<i>Study 1: PPE-induced changes</i>			
PPE (2.55 U) alone (<i>n</i> = 11)	11.5 $\pm$ 0.6	48 $\pm$ 7%	57 $\pm$ 4%
PPE (1.75 U) alone (<i>n</i> = 4)	10.1 $\pm$ 0.5	76 $\pm$ 3%	78 $\pm$ 5%
PBS alone (<i>n</i> = 6)	9.3 $\pm$ 0.4	98 $\pm$ 2%	80 $\pm$ 1%
<i>Study 2: HA and PPE-induced changes</i>			
HA (6 mg) + PPE (<i>n</i> = 6)	10.1 $\pm$ 0.4	91 $\pm$ 4%	88 $\pm$ 6%
HA (1.5 mg) + PPE (<i>n</i> = 6)	11.1 $\pm$ 0.2	55 $\pm$ 4%	60 $\pm$ 3%
HA (6 mg) posttreatment (<i>n</i> = 5)	11.4 $\pm$ 0.8	54 $\pm$ 4%	63 $\pm$ 6%
HA alone (<i>n</i> = 4)	10.4 $\pm$ 0.9	101 $\pm$ 2%	93 $\pm$ 2%
<i>Study 3: ROS and PPE-induced changes</i>			
Cat pretreatment + PPE (<i>n</i> = 5)	9.6 $\pm$ 0.4	91 $\pm$ 4%	87 $\pm$ 3%
Cat posttreatment (<i>n</i> = 5)	10.6 $\pm$ 0.9	59 $\pm$ 1%	70 $\pm$ 4%
X/XO (<i>n</i> = 4)	12.3 $\pm$ 1.6	52 $\pm$ 5%	49 $\pm$ 5%
HA (6 mg) pretreatment + X/XO (<i>n</i> = 3)	11.1 $\pm$ 1.3	64 $\pm$ 10%	63 $\pm$ 9%
<i>Study 4: BK-induced changes</i>			
BK alone (<i>n</i> = 5)	12.5 $\pm$ 1.0	69 $\pm$ 4%	71 $\pm$ 4%
HOE140 pretreatment + BK (<i>n</i> = 4)	9.4 $\pm$ 0.5	94 $\pm$ 4%	86 $\pm$ 4%
HOE140 pretreatment + PPE (<i>n</i> = 3)	14.9 $\pm$ 2.4	85 $\pm$ 4%	75 $\pm$ 8%

Values are means $\pm$ SE; *n* = no. of animals. TMV, tracheal mucus velocity; PPE, porcine pancreatic elastase; HA, hyaluronan; ROS, reactive oxygen species; Cat, catalase; X/XO, xanthine-xanthine oxidase; BK, bradykinin. Statistical analysis is in text and figures.

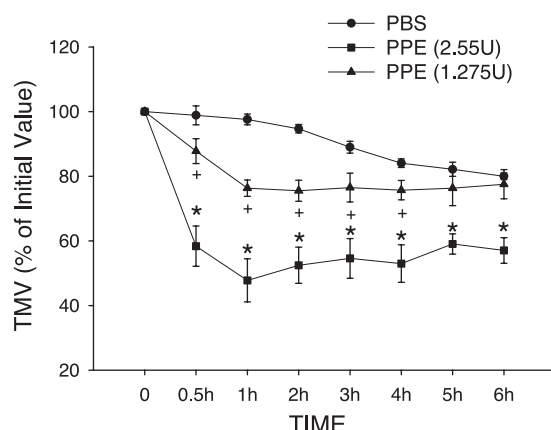


Fig. 1. Dose-related effects of inhaled porcine pancreatic elastase (PPE) on tracheal mucus velocity (TMV). PPE at the 2.55-U dose decreased TMV to $48 \pm 7\%$ of initial value 1 h after challenge, whereas the 1.275-U dose caused TMV to decrease only to $76 \pm 3\%$ of initial value 1 h after challenge (see Table 1). In all subsequent studies, the 2.55-U dose of PPE was used. Values are expressed as means $\pm$ SE for 4–11 sheep. * $P < 0.05$ vs. PBS and PPE (1.275 U); + $P < 0.05$ vs. PBS.

the maximum response seen 1 h after challenge (Table 1). The higher dose of PPE (2.55 U) gave the most robust response, and so this dose was used in the subsequent studies. Also seen in Fig. 1 was the slight decrease in TMV over the 6-h time course in the vehicle (PBS) treatment arm. This has been observed in our previous studies and likely reflects a combination of drying and irritation of the airways despite our attempts to humidify the inspired air (35–37, 51). This decrease was, however, minor compared with that seen with PPE.

Study 2: Effect of HA on PPE-induced reduction in TMV. Figure 2 shows the effect of pretreating sheep with two different doses of HA on the PPE response. Inhaled HA at 1.5 mg provided no protection against the PPE-induced decrease in TMV. The high dose of HA (6 mg), however, blocked the PPE-induced decrease in TMV over the entire measurement period ($P < 0.05$ vs. HA 1.5 mg + PPE and PPE alone; Fig. 2). Interestingly, the low dose of HA was able to block the fall

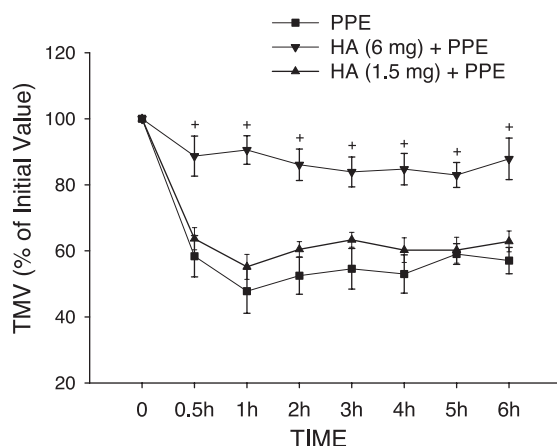


Fig. 2. Dose-dependent effects of inhaled hyaluronan (HA) on PPE-induced decrease in TMV. Pretreatment with inhaled HA (6 mg) 30 min before PPE challenge completely blocked the PPE-induced decrease in TMV, whereas inhaled HA (1.5 mg) provided no protection. Values are expressed as means $\pm$ SE for 6–11 sheep. + $P < 0.05$ vs. HA (1.5 mg) + PPE and PPE alone.

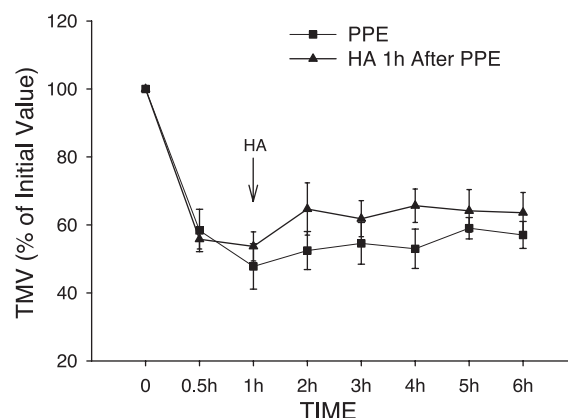


Fig. 3. Posttreatment with HA does not reverse the PPE-induced fall in TMV. Treatment with HA (6 mg) 1 h after PPE challenge failed to restore the PPE-induced decrease in TMV. Values are expressed as means $\pm$ SE for 5–11 sheep.

in TMV produced by the low dose (1.275 U) of PPE (data not shown).

Whereas pretreatment with 6 mg HA effectively blocked the PPE-induced slowing of TMV, giving HA 1 h after airway challenge with PPE failed to reverse the PPE-induced decrease in TMV (Fig. 3).

Figure 4 shows that HA alone had no acute effect on TMV but prevented the late (i.e., >3 h) decrease in TMV seen after PBS (Table 1).

Study 3: Contribution of ROS to the PPE-induced reduction in TMV. The failure of HA to reverse the PPE-induced reduction in TMV suggested that PPE could stimulate the release of secondary mediators, not affected by HA, and that these mediators could contribute to the reduction in TMV. ROS is one such candidate (7, 43). Figure 5 shows that inhaled Cat given immediately before PPE completely blocked the PPE-induced fall in TMV throughout the entire 6 h ($P < 0.05$ vs. PPE alone and Cat posttreatment; Fig. 5). As was seen with HA, when Cat was given 1 h after PPE challenge, it failed to reverse the PPE-induced decrease in TMV (Fig. 5), which suggests that the ROS-mediated effects occurred within the first hour after PPE challenge.

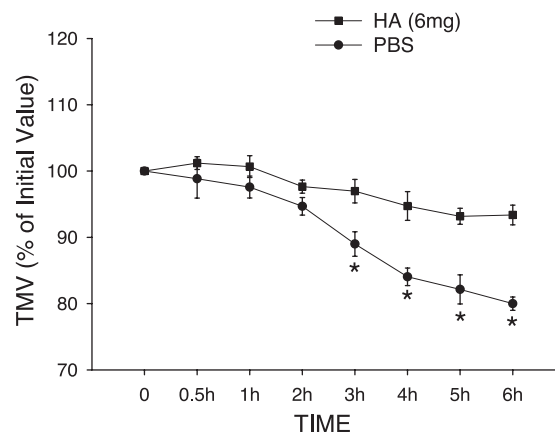


Fig. 4. HA prevents time-related decrease in TMV. HA alone (6 mg) prevented the late physiological depression in TMV seen with PBS, suggesting a possible stimulatory effect. Values are expressed as means $\pm$ SE for 4–6 sheep. * $P < 0.05$ vs. HA.

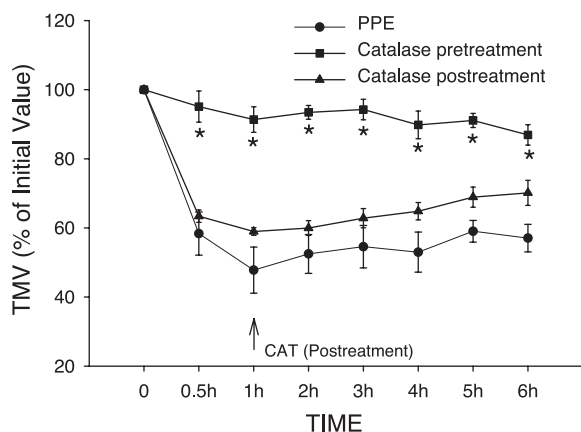


Fig. 5. Effect of catalase (Cat) on PPE-induced fall in TMV. Pretreatment with inhaled Cat completely blocked the PPE-induced fall in TMV throughout the entire measurement period, whereas inhaled Cat given 1 h after PPE challenge failed to restore the PPE-induced decrease in TMV. Values are expressed as means $\pm$ SE for 5–11 sheep. * $P < 0.05$ vs. PPE alone and Cat posttreatment.

The protective effects of Cat pretreatment on the PPE-induced fall in TMV indicated that ROS contributed to this response. To further support this argument, we challenged sheep with X/XO to provide a direct ROS challenge to the airways (26, 41). Figure 6 shows that airway challenge with X/XO caused a decrease in TMV similar in magnitude and time course to that seen with PPE. We then tested the effects of HA pretreatment on this X/XO-induced response. Pretreatment with HA failed to affect the X/XO-induced decrease in TMV, a result consistent with *in vitro* studies showing that ROS can degrade HA.

Study 4: Effect of BK on TMV. The studies showing that Cat pretreatment, but not posttreatment, affected the PPE-induced response suggested a mediator downstream of ROS might also be involved in the PPE response. BK was a likely candidate because PPE increases TK activity and BK levels in BAL. Figure 7 shows that inhaled BK can decrease TMV ($P < 0.05$ vs. PBS). Consistent with previous *in vitro* studies examining the role of BK on airway mucus function, we found that the BK-induced fall in TMV was mediated by BK B_2 receptors as

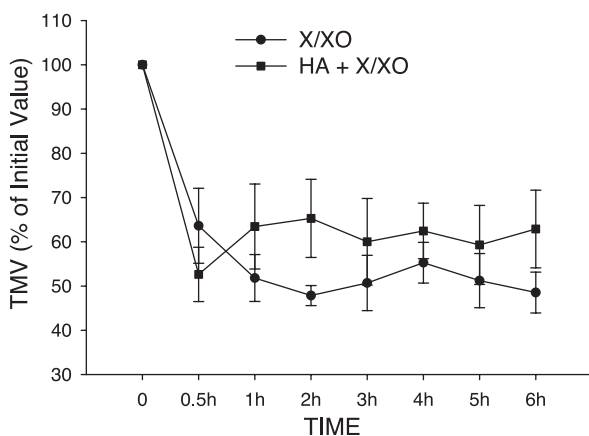


Fig. 6. Effect of HA on reactive oxygen species (ROS)-induced fall in TMV. Generation of ROS by xanthine-xanthine oxidase (X/XO) challenge resulted in a decrease in TMV similar to that observed with PPE. Pretreatment with inhaled HA (6 mg) failed to block the ROS-induced decrease in TMV. Values are expressed as means $\pm$ SE for 3–4 sheep.

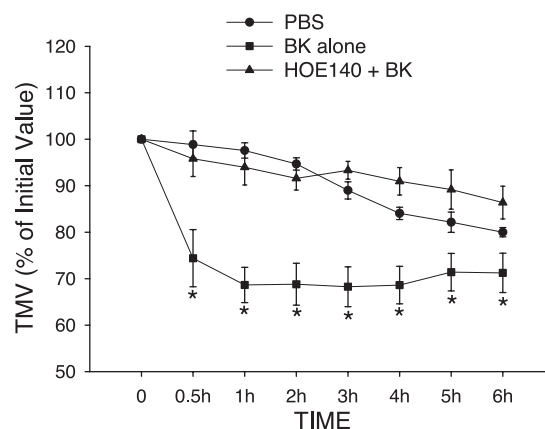


Fig. 7. Effect of bradykinin (BK) on TMV. Inhaled BK caused TMV to decrease to $69 \pm 4\%$ of initial value 1 h after challenge and remained depressed throughout the entire measurement period (6 h). This effect was completely blocked by pretreatment with the BK B_2 -receptor antagonist HOE140. Values are expressed as means $\pm$ SE for 4–6 sheep. * $P < 0.05$ vs. PBS and HOE140 + BK.

the effect could be blocked by pretreatment with the BK B_2 -receptor antagonist HOE140. The demonstration that BK can reduce TMV combined with the failure of Cat and/or HA given 1 h after challenge to reverse the PPE-induced fall in TMV would suggest that the release of BK occurred within this 1 h window. This conclusion is consistent with the appearance of BK in BAL fluid 5 min after PPE challenge (39) and the results shown in Fig. 8, indicating that the BK B_2 -receptor antagonist HOE140 blocks the PPE-induced fall in TMV. Interestingly, even though HOE140 protected against the fall in TMV over the 6-h time course, the most pronounced effect was seen over the first 2 h after PPE challenge.

DISCUSSION

The results of this study show that pretreatment with inhaled HA blocks the PPE-induced decrease in TMV. The extent of the protective effect of HA was related to the amount of exogenous HA given, as lowering the dose resulted in only a partial inhibitory effect. HA given after PPE challenge was not able to reverse the PPE-induced depression in TMV, a finding

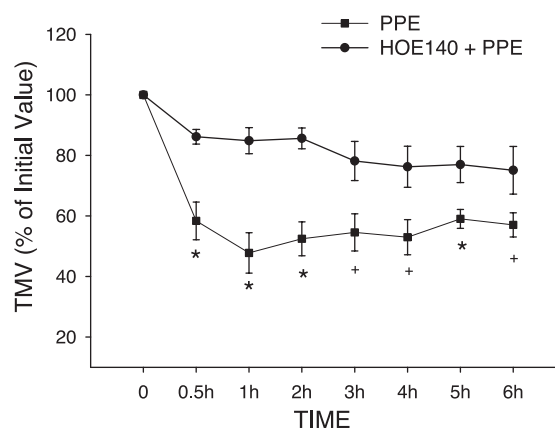


Fig. 8. Effect of HOE140 on PPE-induced changes in TMV. Pretreatment with inhaled HOE140 blocked the PPE-induced slowing of TMV. Values are expressed as means $\pm$ SE for 3–11 sheep. * $P < 0.05$ vs. HOE140 + PPE; + $P < 0.10$ vs. HOE140 + PPE.

consistent with *in vitro* results indicating that ROS can depolymerize HA, causing it to lose effectiveness. The observations that Cat pretreatment blocked the PPE-induced decrement in TMV, ROS reduced TMV in a fashion similar to that seen with PPE, and HA was ineffective in blocking the ROS-mediated response support a role for ROS in the PPE-induced fall in TMV. An unexpected finding, however, was that posttreatment with Cat, like HA, could not reverse the PPE-induced fall in TMV. While the failure of HA to reverse the PPE-induced response could be explained because of ROS-induced HA degradation, the failure of Cat to reverse the effect suggested the contribution of a mediator not responsive to Cat that could also contribute to the decline in TMV. Since we have previously shown that PPE releases BK and that BK is not affected by Cat (15), our finding that BK can slow TMV is consistent with this. Finally, our demonstration that the PPE-induced fall in TMV could be blocked by the BK B₂-receptor antagonist HOE140 confirms a role for kinins in the TMV response seen in this study.

We used PPE in these studies rather than HNE because our previous work with PPE provided a mechanistic basis for interpreting the results of these experiments (39, 42). We realize that PPE has only 43% homology with HNE and that this difference can affect the responses seen with the two elastases (44). However, *in vivo* we have not been able to distinguish between the two elastases in terms of the bronchoconstrictor responses they elicit or the blockade of this constriction by HA (38, 39, 42). The results of this study show further similarities in that PPE, as we had observed with HNE previously, slows TMV (35). In further support of the *in vivo* similarities, we also confirmed in one animal that the HNE-induced decrease in TMV could be blocked by pretreatment with the 6-mg dose of HA used in these studies (data not shown). Nevertheless, despite the apparent similarities in responses to PPE and HNE in our hands, the interpretation of the results obtained with PPE must be considered speculative for HNE.

The present study complements and extends findings from a series of *in vivo* and *in vitro* studies designed to understand the effects of elastase on airway function. Initially we showed that inhaled PPE induced bronchoconstriction in our allergic animals. As expected, this constrictor effect was blocked by natural and synthetic HNE inhibitors α 1-protease inhibitor and ICI-200,355, respectively. Surprisingly, though, the constrictor effect was also blocked by the BK B₂-receptor antagonist NPC-567, suggesting that PPE released kinins in the airways. Consistent with the pharmacological protection with NPC-567 was the finding that PPE caused a significant increase in BAL TK activity, the enzyme responsible for kinin generation, and the demonstration that BK levels increased in BAL fluid within 5 min after PPE challenge (39, 42). Subsequent *in vitro* studies by Forteza and coworkers (14) showed that HA binds and inactivates TK. These *in vitro* observations were substantiated *in vivo* by showing that HA could block the PPE-induced bronchoconstrictor effect and the increase in TK activity in BAL (39). The HA protective effects were not specific for PPE because we showed that HA blocked HNE-induced bronchoconstriction as well (38). Collectively, then, these studies indicated that pretreatment with inhaled HA could block the constrictor effects of PPE and/or HNE and that the purported mechanism of action was by modulation of airway TK activity

and the subsequent release of kinins. Our current observations using TMV as the end point, rather than bronchoconstriction, are consistent with these earlier studies.

In our study we used HA with an average molecular mass of 70 kDa, similar to that used by Cantor et al. (6) in their hamster model of elastase-induced emphysema. This molecular weight HA did not induce an inflammatory response in their studies, nor has HA of similar molecular weights been shown to cause any abnormal reaction when given to humans (18, 19, 24). Cantor et al. (6) also showed that there is no direct interaction between HA and elastase, which supports the concept that the protective effects of HA occur through its control of TK.

As seen in our previous studies, the degree of protection obtained with the 70-kDa HA used here was dependent on the amount given, with the 6-mg dose (0.2%) providing complete protection whereas the 1.5-mg dose (0.05%) failed to inhibit the fall in TMV (39). The present results also reinforce the important factor for HA-induced protection is molecular weight and not the source from which HA is derived, as the present study was done with HA obtained from pig skin compared with the previous study where HA was obtained from pig trachea (39). Other studies confirm that the important factor is the molecular weight of the HA and not the source (7, 14, 16).

Whereas pretreatment with HA was effective in preventing the PPE-induced decrease in TMV, posttreatment failed. *In vitro* studies indicated that TK binding to HA, which results in TK inactivation, can be disrupted by factors that affect HA turnover. Such factors include hyaluronidase (14) and ROS (7). Because elastase can release ROS, it is conceivable that ROS generated by PPE challenge could effectively depolymerize endogenous HA, such that the addition of exogenous HA was insufficient to control TK activity when given after the PPE. To test this, we used a pharmacological approach that we and others have used previously to demonstrate ROS involvement rather than attempting to measure ROS directly. We first determined if Cat pretreatment would protect against PPE-induced depression in TMV. The concentration of Cat used in these studies was the same as that used to block the generation of ROS in the airways using X/XO challenge in a previous study (26). Our finding that Cat blocked the PPE-induced decrease in TMV supported a role for ROS in the PPE-induced TMV response. We then confirmed that ROS generation with X/XO could itself reduce TMV. These findings are consistent with and support the previous *in vitro* studies showing that ROS depolymerizes HA, causing it to lose its protective effect (i.e., binding TK) (7). Finally, we determined if the dose of HA used in these studies could block the X/XO-induced TMV response. HA was unable to block the X/XO response, suggesting that the 6-mg dose of 70-kDa HA used in this study was insufficient to counteract the ROS produced by X/XO. This rationale can also explain the failure of HA treatment given 1 h after PPE challenge to reverse the fall in TMV, i.e., because by 1 h sufficient degradation of endogenous HA would be expected such that additional administration of this dose of HA was insufficient to affect the response.

We found that Cat given 1 h after the PPE challenge was also unable to reverse the TMV response. This suggested the actions of an additional mediator released downstream of PPE-ROS, insensitive to Cat, but yet able to slow TMV. BK

was a logical choice because 1) it is generated by TK; 2) its levels increase in BAL within 5 min of PPE challenge (39); and 3) the dose of Cat used in this study does not block its airway effects (15), and the BK airway constrictor effects are blocked by the 6-mg dose of HA used in this study (40). These results suggested that BK was the appropriate mediator except that BK had not been shown to reduce TMV. Therefore, it was important to demonstrate this effect and to show that the effect of BK was mediated by BK B₂ receptors, as has previously been described for this mediator (4, 27, 30, 46). Our results confirmed that BK does slow TMV and that the effect could be blocked by the BK B₂-receptor antagonist HOE140. Our findings that the PPE-induced slowing of TMV was blocked by the BK B₂-receptor antagonist confirms a role for BK in the PPE-induced TMV response. While these results are consistent with reports that BK stimulates glycoconjugate secretion in a number of mammalian tissues including human submucosal glands and nasal mucosa via BK B₂ receptors (4, 27, 30, 46), they are somewhat at odds with other reports of the effects of BK on mucociliary function. The fact that BK reduced TMV in our studies is in contrast to reports that BK stimulated mucus clearance in normal human subjects and stimulated ciliary beat frequency in dogs (34, 50). However, in asthmatic patients, BK was associated with mucociliary impairment (33). This dichotomy in the mucociliary response is similar to that seen with constrictor responses to BK in normal subjects and asthmatics. Since the animals used in this study are allergic, our results appear consistent with the response of BK in asthmatics (33).

It is of interest that after HA treatment, TMV remained within 10% of baseline throughout the 6-h time course, effectively inhibiting the small decline in TMV that occurs over the 6-h period after PBS inhalation. As indicated above, this decline in TMV has been seen previously by us and has been attributed to a combination of drying and irritation of the airway despite our attempts to humidify the inspired air (35). The differences between the HA and the PBS alone were small but significant but could represent the first in vivo support for the in vitro observation of a potential stimulatory effect of HA on ciliary beat frequency (28). However, this conclusion is speculative as ciliary beat frequency is only one factor that contributes to overall mucus clearance.

In conclusion, our findings suggest that HA could be important in controlling the mucociliary dysfunction caused by the release of elastase in the airways and so provides new information on the effects of glycosaminoglycans in inflammatory airway diseases.

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Present address for M. Scuri: Dept. of Pediatrics, West Virginia University School of Medicine, 2267 Health Sciences South, PO Box 9104, Morgantown, WV 26506.

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