Inhaled porcine pancreatic elastase causes bronchoconstriction via a bradykinin-mediated mechanism

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Scuri, M., R. Forteza, I. Lauredo, J. R. Sabater, Y. Botvinnikova, L. Allegra, and W. M. Abraham. Inhaled porcine pancreatic elastase causes bronchoconstriction via a bradykinin-mediated mechanism. J Appl Physiol 89: 1397–1402, 2000.—Neutrophil elastase has been linked to inflammatory lung diseases such as chronic obstructive pulmonary disease, adult respiratory distress syndrome, emphysema, and cystic fibrosis. In guinea pigs, aerosol challenge with human neutrophil elastase causes bronchoconstriction, but the mechanism by which this occurs is not completely understood. Our laboratory previously showed that human neutrophil elastase releases tissue kallikrein (TK) from cultured tracheal gland cells. TK has been identified as the major kininogenase of the airway and cleaves both high- and low-molecular weight kinogen to yield lysyl-bradykinin. Because inhaled bradykinin causes bronchoconstriction and airway hyperresponsiveness in asthmatic patients and allergic sheep, we hypothesized that elastase-induced bronchoconstriction could be mediated by bradykinin. To test this hypothesis, we measured lung resistance (RL) in sheep before and after inhalation of porcine pancreatic elastase (PPE) alone and after pretreatment with a bradykinin B2 antagonist (NPC-567), the specific human elastase inhibitor ICI 200,355, the histamine H1-antagonist diphenhydramine hydrochloride, the cysteiny1 leukotriene 1 receptor antagonist montelukast, or the cyclooxygenase inhibitor indomethacin. Inhaled PPE (125–1,000 μg) caused a dose-dependent increase in RL. Aerosol challenge with a single 500 μg dose of PPE increased RL by 132 ± 8% over baseline. This response was blocked by pretreatment with NPC-567 and ICI-200,355 (n = 6; P < 0.001), whereas treatment with diphenhydramine hydrochloride, montelukast, or indomethacin failed to block the PPE-induced bronchoconstriction. Consistent with pharmacological data, TK activity in bronchial lavage fluid increased 134 ± 57% over baseline (n = 5; P < 0.02). We conclude that, in sheep, PPE-induced bronchoconstriction is in part mediated by the generation of bradykinin. Our findings suggest that elastase-kinin interactions may contribute to changes in bronchial tone during inflammatory diseases of the airways.

asthma; inflammation; tissue kallikrein; sheep

ELASTASE IS A PROTEOLYTIC enzyme contained in the azurophicly granules of polymorphonuclear leukocytes and has been reported to cause epithelial damage (6, 32), vascular hyperpermeability (21), mucus hypersecretion (13, 25), mucus gland metaplasia (30), and a reduction in mucociliary clearance (29, 34). Recent studies show that aerosol challenge with human neutrophil elastase causes bronchoconstriction and airway hyperresponsiveness (AHR) in guinea pigs and that these responses could be blocked by a recombinant half-length secretory leukocyte protease inhibitor (r1/2SLPI) that contains the elastase inhibitory site of natural SLPI (33). Although these findings suggest that elastase-induced bronchoconstriction is dependent on elastase proteolytic activity, the mechanism responsible for the constrictor effect remains unknown.

Our laboratory showed that, in vitro, human neutrophil elastase causes the release of tissue kallikrein (TK) from primary cultures of ovine tracheal gland cells (17). TK is the major kininogenase in the airways (8) and cleaves both high and low molecular weight kinogen, yielding lysyl-bradykinin (kallidin). Kallidin is a potent vasoactive peptide that causes vasodilation, vascular permeability, and bronchoconstriction, all of which are important features in the pathophysiology of asthma. Inhaled bradykinin causes bronchoconstriction and AHR in asthmatic patients and allergic sheep (2, 20, 22) and TK-like activity has been shown to increase in nasal washings and bronchialveolar lavage fluid (BALF) from human subjects and sheep after allergen challenge (7–9). Our laboratory also showed that, in addition to antigen challenge, TK-like activity is increased in BALF of sheep challenged with a variety of inflammatory stimuli, such as ozone, bacterial supernatants, and metabisulfite, that cause bronchoconstriction and/or AHR. Furthermore, these airway responses could be blocked by treating the animals with bradykinin B2 receptor antagonists (16, 18, 24). Collectively, these findings suggest that stimuli that increase lung TK activity appear to cause airway abnormalities via the generation of kinins. Therefore, we hypothesized that elastase-induced bronchoconstriction could be mediated by kinins. To

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test this hypothesis, we determined whether inhaled porcine pancreatic elastase (PPE) would cause bronchoconstriction in conscious sheep and whether this effect could be blocked by the bradykinin B₂ receptor antagonist NPC-567. To provide further support for this mechanism, we also determined whether aerosol PPE challenge caused an increase in the BALF TK activity.

METHODS

A total of 18 sheep (mean weight: 30.5 kg) were used for this study. With the exception of one animal, all sheep had a history of airway sensitivity to inhalation of Ascaris suum antigen. The one nonallergic animal was used to determine whether the response was limited to allergic airways. The study was conducted at Mount Sinai Medical Center, under the approval of Mount Sinai Medical Center Animal Research Committee.

Airway mechanics. To study the PPE-induced changes in airway mechanics, the animals were restrained in an upright position in a cart, with their heads immobilized. A balloon catheter was advanced through one nostril into the lower esophagus, after topical anesthesia with 2% lidocaine solution. The animals were intubated with a cuffed endotracheal tube, through the other nostril, using a flexible fiberoptic bronchoscope. Pleural pressure was measured via an esophageal catheter (filled with 1 ml of air) that was positioned 5 to 10 cm from the gastroesophageal junction. In this position, the end-expiratory pleural pressure ranged between −2 and −5 cm H₂O. Lateral pressure in the trachea was measured with a sidehole catheter (inner dimension, 2.5 mm) that was advanced through and positioned distal to the tip of the endotracheal tube. Transpulmonary pressure, the difference between tracheal and pleural pressure, was measured with a differential pressure transducer catheter system. For the measurement of pulmonary resistance (RL), the proximal end of the endotracheal tube was connected to a pneumotachograph (Fleisch, Dyna Sciences, Blue Bell, PA). The signals of flow and transpulmonary pressure were recorded on an oscilloscope recorder linked to a computer, which calculated RL online. Respiratory volume was obtained by digital integration of the flow signal and was used, together with transpulmonary pressure and flow, at isovolumetric points to derive RL. (35), as previously described (15). Analysis of 5–10 breaths was used for the determination of RL.

Aerosols. A disposable medical nebulizer (Raindrop, Puri- tan Bennett, Lenexa, KS) was used to generate all aerosols. The output from the nebulizer generated an aerosol with mass median aerodynamic diameter of 3.2 μm (geometric SD 1.9), as determined by an Andersen cascade impactor. 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) in 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 (one-half total lung capacity) and a rate of 20 breaths/min.

Agents. PPE and indomethacin were purchased from Sigma Chemical (St. Louis, MO). PPE was dissolved in 3 ml of phosphate buffered saline (PBS; pH 7.4), and indometha- cin was dissolved in sodium bicarbonate and given intravenously (2 mg/kg body wt), as described previously (5, 11, 28). The elastase inhibitor ICI-200,355 (10 mg) was dissolved in PBS (2 mg/ml) and delivered as an aerosol, as previously described (27). The bradykinin B₂ antagonist NPC-567 (o-Arg-[Hyp, D-Phe²]-bradykinin) was a gift from Nova Pharmaceutica (Baltimore, MD) and was dissolved in PBS (5 mg/ml), and 20 breaths of this solution were delivered as aerosol, as previously described (18). The histamine H₁-antagonist diphenhydramine hydrochloride was purchased from Elkins-Sinn (Cherry Hill, NJ) and was given intravenously (1 mg/kg body wt). Montelukast, a cysteinyl leukotriene 1 receptor antagonist, was a gift from Merck and was administered intravenously (0.15 mg/kg). The doses of the pharmacological agents used in this study were chosen based on previous experience in the model. ICI-200,355 was shown to have an effect similar to human α₁-protease inhibitor against sheep neutrophil elastase in vitro (27) and the elastase-mediated effects resulting from antigen challenge in vivo (27). NPC-567 was shown to block bradykinin-induced bronchoconstriction (2) but not carbachol-induced bronchoconstriction (31). Likewise, indomethacin (5, 11), diphenhydramine hydrochloride (1), and montelukast (23) were shown to block responses mediated by the prostanoids histamine and leukotriene D₄, respectively, in sheep. All treatments were given 30 min before elastase challenge.

Bronchoalveolar lavage for TK analysis. The distal tip of a specially designed 80-cm fiberoptic bronchoscope was wedged into a randomly selected subsegmental bronchus. Lung lavage was performed by slow infusion and gentle aspiration of 30 ml of PBS (pH 7.4) at 37°C, using a 30-ml syringe attached to the working channel of the instrument. The effluent was filtered through a double layer of gauze; 10 ml were placed in a tube containing 100 μl EDTA (Sigma Chemical) and 100 μl soybean trypsin inhibitor (40 mg/ml; Sigma), and the remaining BALF was placed in a tube with no additives. All tubes were immediately placed on ice and then centrifuged at 250 g at 4°C for 15 min. The supernatant was recentrifuged at 3,000 g at 4°C for 15 min, saved, and frozen at −80°C for subsequent analysis.

Analysis of BALF. Before mediator analysis, BALF supernatant was thawed and recentrifuged at 12,500 g at 4°C for 15 min. BALF was then analyzed for TK activity. TK-like activity in unconcentrated BALF supernatants was measured by cleavage of dl-Val-Leu-Arg pNA, as described by us previously (15) and was expressed as arbitrary units (1 unit = change in optical density at 405 nm in 24 h).

PROTOCOLS

Effects of PPE on lung resistance. In a separate series of experiments, the dose-response relationship between inhaled PPE and RL was determined. On individual experiment days, six sheep were challenged with one dose of PPE ranging from 125 to 1,000 μg. Challenges were at least 72 h apart. On the day of the experiment, baseline RL was measured, the animals were given PBS, and then a second measurement of RL was obtained. Animals were then challenged with aerosolized PPE. Measurements of RL were made immediately (0–2 min) and 5, 10, 15, and 30 min after challenge.

Pharmacology of PPE-induced bronchoconstriction. In the first series of experiments, sheep (n = 6) were challenged with 500-μg aerosols of PPE 30 min after pretreatment with 3 ml PBS alone, ICI-200,355 (10 mg), NPC-567 (20 breaths of 5 mg/ml solution), or diphenhydramine hydrochloride (1 mg/kg iv). In a second series of experiments, a separate group of sheep (n = 3–6) was challenged with 500-μg aerosols of PPE 30 min after pretreatment with 3 ml PBS alone, montelukast (0.15 mg/kg iv), or indomethacin (2 mg/kg iv). ICI-200,355 and NPC-567 were given as aerosols, and diphenhydramine...
hydrochloride, montelukast, and indomethacin were given intravenously through the external jugular vein. R_l was then measured again, before and 30 min after each treatment, to ensure that the compounds did not cause a change in bronchial tone. R_l was measured immediately (0–2 min) after challenge with 500 μg PPE and 5, 10, 15, and 30 min after, as described above. All experiments were separated by at least 72 h and were done in random order.

Effect of PPE on TK activity in bronchial lavage. In five sheep, TK-like activity in BALF was measured at baseline and 30 min after aerosol challenge with 500 μg PPE. Bronchial lavage was performed according to previously described procedures. There was a 30-min interval between the baseline lavage and PPE challenge.

Statistics. Changes in R_l for unpaired groups were first analyzed using one-way ANOVA. Post hoc comparisons were made with an unpaired t-test using the Bonferroni correction. Changes in R_l for paired groups were analyzed with a multivariate ANOVA for repeated measures, followed by paired t-test with Bonferroni correction (SIGMASTAT 2.0 for Window, SPSS, Chicago, IL). Change in TK-like activity were log10-transformed and then analyzed using a paired t-test. Values are presented as means ± SE. *P < 0.05 was considered significant.

RESULTS

Effects of PPE on R_l. Doses of inhaled PPE between 125 and 1,000 μg caused a linear increase in R_l (Fig. 1). The 500-μg dose of PPE produced a 151 ± 16% increase in R_l, which was significantly different (P < 0.05) from baseline and the 125- and 250-μg doses. On the basis of previous studies with inhaled peptides (2) and other provocative mediators (4), the increase in R_l caused by the 500-μg dose of PPE was determined to be severe enough to allow for pharmacological assessment of the mediators involved. Furthermore, the concentration of PPE (500 μg/3 ml) is in the range of those measured in sputum samples obtained from asthmatic patients and patients with cystic fibrosis (12).

The bronchoconstrictor response to inhaled PPE was short-lived. The peak increase in R_l was observed immediately (within 2 min) after challenge. The increase in R_l then began to decline rapidly, returning to baseline values by 30 min after challenge (Fig. 2). The response did not appear to be limited to allergic sheep, because 500 μg of PPE increased R_l to 2.5 cmH_2O·l^{-1}·s^{-1} from a post-PBS value of 0.95 cmH_2O·l^{-1}·s^{-1} in one animal that was not Ascaris suum sensitive.

Pharmacology of PPE-induced bronchoconstriction. PPE-induced bronchoconstriction was completely blocked by pretreatment with NPC-567 and ICI-200,355 (P < 0.001; n = 6 for both) but not by diphenhydramine hydrochloride (Fig. 2). In a separate series of experiments, we found that neither the peak nor the time course of the PPE-induced bronchoconstriction was inhibited by the montelukast or indomethacin (Fig. 3).

BALF TK activity. Consistent with the pharmacological data, PPE challenge caused BALF TK activity to increase by 134 ± 57% (P < 0.02; Fig. 4)

DISCUSSION

The results of this study indicate that inhaled PPE causes bronchoconstriction in conscious sheep via a kinin-mediated mechanism. This conclusion is supported by the novel observations that 1) PPE-induced bronchoconstriction has a time course and pharmacology similar to that previously seen with inhaled bradykinin in sheep (2) and 2) inhaled PPE caused an increase in BALF TK activity, which has been previously associated with increased kinin levels in the airways (16, 18, 24). The finding that PPE-induced bronchoconstriction was blocked by the specific elastase inhibitor ICI-200,355 indicates that PPE-induced bronchoconstriction requires PPE to have an active proteolytic site.
It may have been optimal to use human neutrophil elastase in the present studies, but cost factors and similarities in secretory responses obtained with PPE and human elastase made PPE an attractive alternative. Additional support for this choice is based on the known inhibitory activity of ICI-200,355 against PPE (37) and the observations that ICI-200,355 and a1 proteinase inhibitor (a1-PI), a natural elastase inhibitor, blocked both the in vitro and in vivo effects of elastase obtained from stimulated ovine neutrophils (27).

Our results extend previous findings reported in guinea pigs, in which aerosols of human neutrophil elastase were used to induce bronchoconstriction and AHR. These elastase-induced effects were blocked by r1/2SLPI, which has anti-elastase activity (33). Further characterization of the mechanisms involved, however, was not undertaken. The hypothesis for our study was based on our laboratory’s previous observations that, in vitro, human neutrophil elastase caused the release of TK from primary cultures of ovine tracheal gland cells (17) and that a variety of irritant stimuli that cause bronchoconstriction and AHR were associated with increased BALF TK activity/kinin levels (16, 18, 24). Additionally, these airway responses were blocked by NPC-567 (2).

The finding that ICI-200,355 was effective in blocking PPE-mediated responses in sheep suggests that an active molecule is required to initiate these events. Previous studies show that ICI-200,355 did not block the bronchoconstriction to inhaled kininogen, which is a substrate for TK (15). These results are consistent with and support our present findings, i.e., in the presence of baseline TK activity, one would not expect an elastase inhibitor to protect against kinin-mediated bronchoconstriction if the substrate for kinin generation is introduced into the airways. In the absence of an increased substrate load, however, ICI-200,355 should block the PPE-mediated responses, given that PPE challenge is associated with an increase in BALF TK activity. With this line of reasoning, it is not surprising that both the kininogen- and PPE-induced bronchoconstriction are blocked by the bradykinin B2 antagonist NPC-567.

Our present findings highlight a number of similarities between the responses to inhaled bradykinin and PPE. The time course of the PPE-induced bronchial response was similar to that previously seen in our laboratory with inhaled bradykinin (2). Both agents induce a rapid, although short lived (~30 min), response, and the bronchoconstriction produced by both bradykinin and PPE was blocked by NPC-567. Likewise, the constrictor responses to bradykinin and PPE were not affected by histamine H1-antagonism. Collectively, therefore, these data support our hypothesis that the PPE-induced bronchial response in sheep is mediated via kinin generation.

Although the majority of the sheep used in this study demonstrated airway hypersensitivity to inhaled As-

![Fig. 3. The effect of montelukast, a cysteinyl leukotriene 1 receptor antagonist, and the cyclooxygenase inhibitor indomethacin on PPE-induced bronchospasm. Neither agent affected the response to PPE (500 µg) challenge. Values are expressed as means ± SE for 3–6 sheep (see Table 1).](image)

**Table 1. Effect of inhaled PPE on lung resistance**

<table>
<thead>
<tr>
<th>Series</th>
<th>n</th>
<th>Prechallenge</th>
<th>Peak Change</th>
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<tbody>
<tr>
<td><strong>Series 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPE alone, control</td>
<td>6</td>
<td>1.04 ± 0.04</td>
<td>2.39 ± 0.03</td>
</tr>
<tr>
<td>NPC-567 + PPE</td>
<td>6</td>
<td>0.98 ± 0.03</td>
<td>1.04 ± 0.04*</td>
</tr>
<tr>
<td>ICI-200,355 + PPE</td>
<td>6</td>
<td>1.04 ± 0.02</td>
<td>1.00 ± 0.02*</td>
</tr>
<tr>
<td>Diphenhydramine + PPE</td>
<td>6</td>
<td>1.05 ± 0.02</td>
<td>2.59 ± 0.12</td>
</tr>
<tr>
<td><strong>Series 2</strong></td>
<td></td>
<td></td>
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<tr>
<td>PPE alone, control</td>
<td>6</td>
<td>1.05 ± 0.02</td>
<td>2.50 ± 0.12</td>
</tr>
<tr>
<td>Montelukast + PPE</td>
<td>3</td>
<td>1.00 ± 0.03</td>
<td>2.28 ± 0.08</td>
</tr>
<tr>
<td>Indomethacin + PPE</td>
<td>3</td>
<td>1.01 ± 0.04</td>
<td>2.35 ± 0.14</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for lung resistance (Rl; in cmH2O/L/sec). Peak change in Rl was the value obtained immediately after (0–2 min) porcine pancreatic elastase (PPE) challenge (see Fig 2). NPC-567, a bradykinin B2 antagonist; ICI-200,355, a specific human elastase inhibitor; montelukast, a cysteinyl leukotriene 1 receptor antagonist. *P < 0.05 vs. PPE alone and diphenhydramine + PPE.

![Fig. 4. Effect of PPE challenge (500 µg) on tissue kallikrein (TK)-like activity in sheep bronchoalveolar lavage fluid. Statistical analysis was performed as log10-transformed values. Values are means ± SE for 5 sheep, *P < 0.05 vs. baseline.](image)
caris suum antigen, we did have the opportunity to study one nonallergic sheep. In this animal, PPE also caused bronchoconstriction. This would indicate that the PPE-induced response is not limited to allergic airways. Our results are consistent with those obtained with human neutrophil elastase in unsensitized guinea pigs (33).

Our data do not support the concept that aerosol challenge with PPE stimulates mast cell degranulation. If PPE-induced bronchoconstriction caused mast cell degranulation, then one would have expected some protection from the histamine H1 antagonist; however, this was not the case. Likewise, neither montelukast nor indomethacin showed any protective effect. Given these data, it is unlikely that mast cells played a role in this response.

Elastase has been implicated in chronic obstructive pulmonary disease and emphysema, but recent studies have suggested it may also contribute to the pathophysiologic process in asthma. Neutrophil elastase has been found in the secretions of patients during exacerbations of asthma (12, 26, 36). In our own laboratory, studies found increased levels of free elastase after antigen challenge in sheep (27). The increased free elastase was associated with a lung neutrophilia and contributed to the antigen-induced mucociliary dysfunction in these animals, because mucociliary impairment was blocked with exogenous α1-PI and ICI-200,355.

Although elastase appears to contribute to the pathophysiologic responses (mucociliary dysfunction) resulting from antigen challenge, it is unlikely that elastase plays a major direct role in the constrictor responses that follow antigen challenge, because α1-PI, at a dose that blocked the changes of mucociliary clearance, had no effect on the antigen-induced bronchoconstriction. This conclusion differs from the conclusion of Fujimoto and colleagues (19), who showed that pretreatment with the elastase inhibitors ONO-5046 and FR-13403 partially inhibited both the early and late antigen-induced responses in sheep. The mechanism by which these agents work to inhibit the early bronchoconstrictor response to antigen is unclear, because neutrophil (and, hence, elastase) have not been implicated in this event. One possible explanation may be related to the specificity of the compound used (19). The fact that these agents did affect the early bronchoconstrictor response suggests that they may not be as specific as reported, especially at the doses used. In vitro, ONO-5046 can inhibit trypsin (27), and the profile of action of ONO-5046 in sheep is consistent with such a mechanism (i.e., trypsin inhibition) (10). The effects of another natural elastase inhibitor, SLPI, on antigen- and trypsin-induced bronchial responses confirms this thinking. Thus antigen- and trypsinase-induced bronchoconstriction are blocked by SLPI and the trypsinase inhibitor APC-366 but not by α1-PI (3, 14). These data, collectively, do not support the argument that elastase-mediated bronchoconstriction involves mast cell degranulation.

In conclusion, our data suggest a novel regulatory pathway by which elastase contributes to the inflammatory process in the airways. Further elucidation of the elastase/kinin system interaction may have potential therapeutic implications.

REFERENCES


