KINETICS OF IN VITRO BRONCHOCONSTRICTION IN AN ELASTOLYTIC MOUSE MODEL OF EMPHYSEMA

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Running title: cholinergic responses in murine COPD model

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Summary:

We used thin slice videomicroscopy to examine the kinetics of constriction in small airways in situ.

Balb/C mice inhaled elastase (0-20 IU), then were left to recover for 14 days before euthanization and lung removal. Cholinergic responsiveness was assessed in thin lung slices. Magnitude and velocity of narrowing in response to $10^{-5}$ M ACh, as well as the full concentration-response relationship for ACh ($10^{-8}$ - $10^{-5}$ M) were assessed.

In vivo exposure to elastase was accompanied by statistically significantly decreased magnitudes and velocities of contraction, but no change in the Ach concentration-response relationship. On the other hand, overnight in vitro exposure of slices from control animals to elastase (2.5 µg/ml) resulted in increased magnitudes and velocities of airway narrowing, with impaired relaxation, as well as marked tearing of the airways from the surrounding parenchyma: these changes are characteristic of decreased tethering forces on the airway wall.

Thus, the lung slice technique coupled with videomicroscopic analysis of airway contraction velocities provides a powerful tool to studying airway-parenchymal interactions. The elastolytic model of emphysema, which manifests with airspace enlargement and loss of parenchymal attachments, is accompanied by decreased airway contraction kinetics. The mechanism(s) underlying this loss of function remain to be elucidated.

Keywords: airway smooth muscle; cholinergic; collagen; connective tissue; contraction; elastin;
Introduction:

Chronic obstructive pulmonary (COPD) --- characterized by airways inflammation, bronchitis and lung remodeling (both emphysematous and fibrotic changes) (1-3) --- is already one of the major causes of mortality and morbidity across the world, and is increasing in prevalence (4; 5). The major triggering factor is cigarette smoke, which accounts for 80-90% of COPD cases. However, in the population of smokers, only 15% of the subjects develop chronic airflow limitations (6). Several serine proteases and matrix metalloproteases are expressed in association with COPD in humans (7). Also, many different experimental approaches (intranasal instillation of elastase; gene-targeted up-regulation of elastase expression or down-regulation of alpha-antitrypsin; use of various protease inhibitors) have highlighted important roles for endogenous proteases in airspace enlargement, and have identified many interactions between these proteolytic systems (7). In addition to playing a key role in destruction of the extracellular matrix, particularly elastin, proteases may also be important in regulating inflammation through the generation of chemokines and cytokines, and by blazing trails for cells through tissue barriers (7).

Awareness of this central or even causal role of proteases has led to the development of several rodent models of COPD (reviewed by Fehrenbach (8)), including one in which the animals are made to inhale porcine pancreatic elastase intranasally (9; 10). This challenge results in an inflammatory response which resolves within days, as well as structural changes which persist for at least 100 days. Stone et al. (11) have previously reported the dose-dependent effects of elastase in hamsters: even doses as low as 6 units of elastase produced mild emphysema, as detected by pulmonary function testing. Likewise, Gray and Mitchell (12) described the effects of intramural injection of elastase in isolated bronchial segments of pigs: this led to uncoupling of the airway wall from the parenchyma, resulting in increasing responsiveness in vitro. Qian and Mitzner (13) reported that emphysematous
parenchyma showed greater sensitivity to Ach compared with control parenchyma and significantly greater maximal contractility to Ach in the elastase-induced hamster model of emphysema.

Using this approach, it has been possible to examine and quantify various histological and immune/inflammatory indices associated with experimentally-induced emphysema. However, COPD is also characterized by partially reversible airflow obstruction, atelectasis and gas trapping, and airway hyperresponsiveness, all contributing to compromised gas exchange in the lung (14; 15). These aspects of COPD and emphysema have proven harder to plumb; changes in airway function per se are particularly difficult to examine. Although overall airflow and resistance can be measured in vivo or in excised lungs, it is not easy to attribute changes to specific segments of the airways: vis-B-vis, the larger airways versus the various bronchiolar vessels (conducting, terminal or respiratory). Imaging techniques such as positron emission tomography or computerized tomography have allowed for discrimination of some of the larger of these airways in living animals, but these approaches are limited by temporal and spatial resolution, and require costly and sophisticated equipment. The recent introduction of the thin lung slice technique may overcome some of these barriers. Using this latter approach, the lungs are inflated with a gel-like substance, then cut into slices as thin as 50 µm which can then be studied using standard videomicrometric techniques and the responsiveness of even terminal bronchioles monitored at video rates while still situated in their native environment (i.e., with parenchymal attachments largely intact). This approach has been used to examine airway wall – parenchyma interactions in a rat model of allergen-induced airway hyperresponsiveness (16; 17), but not the elastolytic model of COPD described above.

We combined this latter approach with a murine model of emphysema in order to explore the effects of parenchymal destruction on airway smooth muscle contraction kinetics. Balb/C mice were used, since these show a robust reproducible response to cholinergic stimulation when studied using the thin lung slice technique (18). We have added velocity measurements in Ach-induced contraction
to understand better the effect of elastase-treatment on the interactions of the airway with the parenchyma and the effects of changes in tethering imposed by surrounding parenchyma. We hypothesized that decreased tethering might manifest as greater magnitude of narrowing, faster velocity of contraction and slower velocity of relaxation (the latter being particularly evident in taking the ratio of the two velocities).
Materials and Methods

Animal handling and in vivo delivery of elastase

All experiments were approved by the Animal Research Ethics Board of McMaster University and carried out according to the guidelines of the Canadian Council on Animal Care.

Female Balb/c mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, PQ, Canada) and maintained under specific pathogen-free conditions in an access-restricted area, on a 12-h light-dark cycle, with food and water provided *ad libitum*. One group of animals were anesthetized with isofluorane according to institutional guidelines, and 0-20 IU of elastase/100g body weight administered intranasally (15 µl to each nostril) before returning them to the facility for recovery for fourteen days. Otherwise, elastase-exposed and naïve animals were sacrificed by CO₂ inhalation in a closed chamber for 1 minute and lungs removed for use in histological or physiological studies as described below.

Lung slicing

Lungs were sliced using the protocol first described by Sanderson *et al.* (19) and adapted within our laboratory. Briefly, following euthanization, the chest wall was removed, the trachea cannulated using an intravenous catheter (20G Intima, Becton Dickinson, Sandy UT), and the lungs inflated with ~1.2 ml of agarose (type VII-A low gelling temperature; Sigma Aldrich, St. Louis MO, USA) warmed to 37°C; unless indicated otherwise, the latter was made up to a concentration of 2% in Hanks’ buffered saline solution (HBSS). Approximately 0.1-0.2 ml of air was subsequently injected in order to flush the agarose-HBSS out of the airways of interest (those which would be visualized) so that they would be free to contract and not subject to intraluminal resistance. The agarose was gelled by cooling the lung preparation to 4°C for 5-10 min, after which the left lobe was removed and sliced (120µm thickness) using an EMS-4000 tissue slicer (Electron Microscope Sciences, Fort Washington,
PA) at 4°C. The slices were washed in HBSS and inspected under a phase contrast microscope: those slices which were not torn or otherwise damaged were then incubated overnight in DMEM supplemented with antibiotics and antimycotics at 37°C and 5% CO₂.

In vitro measurement of airway contraction

Lung slices were taken out after overnight incubation and transferred to fresh HBSS for 30 minutes. We selected those which possessed airways that nearly filled the CCD camera imaging area (approximately 125 µm outer diameter) and which displayed an intact epithelium and parenchyma, as well as active epithelial ciliary beating, and which were not plugged with agarose. Slices which met these criteria were mounted on a glass cover slip (45x50 mm; Fisher Scientific, Suwanee, GA), and held in position by a piece of nylon mesh (250 µm mesh; CMN-0250D, Small Parts, Miami Lakes, FL) as well as a second glass cover slip (22x40 mm); this did not appear to offer any frictional opposition to movement of the airway (it appeared unconstrained to move over the glass surface, although we did not specifically measure this). Dulbecco’s modified Eagle’s medium (DMEM) was superfused between the two cover slips and over the tissue slice at a rate of approximately 4.5 ml/min. Ach was added/removed via this perfusate and had excellent access to the airway of interest, as indicated by a latency for onset of the cholinergic response of less than 5 seconds. Phase contrast images were acquired with a high resolution CCD solid state video camera (CV-252 Nikon, Japan), and recorded in time lapse (3-ms exposure, 0.2 frames/sec) using image acquisition software (Video Savant, IO Industries, London, ON, Canada). Video images were transformed to recordings of airway area versus time using Scion image analysis software (Scion, Frederick, MD), which converted the 10-bit video images to binary and then measured cross-sectional area of the airway in each frame by pixel summing. Finally, the recordings were analyzed and statistical comparisons made using SigmaPlot and Minitab software.
**Histology**

The trachea of each excised lung was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA) and inflated with 10% formalin at a constant pressure of 20 cm H$_2$O. Lungs were fixed for 24 hours and embedded in paraffin. 3 µm-thick sections were stained with haematoxylin and eosin. Mean chord length was assessed as described previously (20). Five random fields per lung were digitized using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). Sections were analyzed using Leica Qwin Image Processing Software (Leica Imaging Systems, Cambridge, U.K.). Large airways and bronchi were excluded from the analysis. Data are shown as mean chord length of the airspace, a measure independent of the thickness of alveolar septa.

**In vitro exposure to elastase**

Where specifically indicated, slices from naïve animals (those which did not inhale elastase *in vivo*) were incubated overnight with porcine pancreatic elastase (Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 2.5 µg/ml in DMEM supplemented with antibiotics and antimycotics at 37°C and 5% CO$_2$. After overnight incubation, slices were analysed under a phase contrast microscope and airways with minimal visible damage were selected for assessment of cholinergic responsiveness.

**Materials**

Cell culture reagents were obtained from Invitrogen Life Technologies – GIBCO (Carlsbad, CA). DMEM for slice incubation was supplemented with PennStrep (penicillin 10,000 units/ml, streptomycin 10,000 µg/ml), amphotericin B (125 µg), L-ascorbic acid 35 µg/ml, transferrin 5 µg/ml, selenium 3.25 ng/ml and insulin 2.85 µg/ml. HBSS in most cases was supplemented with HEPES buffer (0.2 M pH 7.4). All other reagents were obtained from Sigma Chemical (St Louis, MO) unless
specified otherwise. Porcine pancreatic elastase (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% sodium chloride at a concentration of 1 mg/ml, divided into 250 µl aliquots and stored at -20°C.

Statistics

Airway areas were standardized as a per cent of lumen area measured at the beginning of the experiment (immediately prior to challenge with the first bolus dose of Ach). The first derivative of such recordings of airway area was taken to obtain the velocities of narrowing or of dilation. We also obtained the velocities of smooth muscle shortening by using area measurements to estimate the radius of the airway (assuming the latter to be circular; area = π x r²), then using radius to calculate circumference as the product of π and diameter: in this way, we were able to estimate the changes in length on a frame-by-frame basis, and the first derivative of this as the velocity of shortening. All data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between different groups were carried out using ANOVA (unpaired); Students’ t-test was used for comparing velocities of lumen narrowing in the same tissue. Correlation analysis was done using Pearson’s test. p < 0.05 was considered statistically significant.
Results

In vivo elastase-treatment causes airspace enlargement

In an initial set of experiments, we quantified the degree of airspace enlargement induced by various concentrations of elastase given intranasally (from 0.02 to 20 IU elastase per 100g body weight) 2 weeks prior to removal of the lungs. Figure 1 shows that 20 and 10 IU per 100 g body weight induced widespread emphysematous lesions. Only minimal and focal airspace enlargement was observed at 5 IU per 100 g body weight. No tissue damage was observed at concentrations <2 IU per 100 g body weight (data not shown). Quantification of airspace enlargement (Fig. 1E) showed statistically significantly increased mean chord length at 20 and 10 IU per 100 g body weight (p<0.05).

Cholinergic responses in control tissues

The experimental protocol which we used to assess airway responsiveness to cholinergic stimulation is illustrated in Figure 2. Tissues were first challenged with $10^{-5}$ M Ach to evoke a maximal constrictor response: in naïve tissues, this elicited a rapid and dramatic decrease in airway diameter which reached a peak within 5 minutes. Ach was then washed from the tissues by superfusing with regular HBSS for 5-10 minutes, during which time the airway relaxed back toward its baseline diameter. Finally, the full cholinergic concentration-response relationship was explored by challenging the tissues with Ach at concentrations ranging from $10^{-8}$ to $10^{-5}$ M.

Figure 2C summarizes the velocities of narrowing/dilation, derived on a frame-by-frame basis from the first derivative of the lumen area recording, in the same representative lung slice: the velocity of narrowing (an active process) in this trace was more than twice as fast as that for relaxation (a passive process). The ability of this approach to discriminate different contraction velocities is also highlighted in the second portion of the trace in which the responses to increasing concentrations of Ach are shown: in particular, both $10^{-7}$ M Ach and $10^{-6}$ M Ach evoked an approximately 20% decrease
in airway area, but the latter did so with a velocity almost 7 times greater than that of the former. Alternatively, it is possible to estimate velocities of smooth muscle shortening per se, by converting changes in lumen area to changes in lumen circumference (see Methods). We found the velocities of shortening were nearly identical to those of lumen narrowing, differing only slightly at extremely small airway diameters (e.g., see Figure 2C and 6B, thick grey lines), indicating that monitoring changes in airway area is a useful surrogate for following changes in smooth muscle length. That notwithstanding, given that the airways were not perfectly circular, we used velocities of lumen narrowing in our statistical comparisons.

On average, $10^{-5}$ M Ach evoked a mean decrease in initial airway area of $53.6 \pm 3.12\%$ (n=5; Fig. 3B), and the velocity of contraction (Fig. 3C) was statistically significantly greater than that of relaxation (Fig. 3D; p<0.05); the mean ratio of these two velocities was $4.14 \pm 0.69$ (Fig. 3E).

The mean cumulative Ach concentration-response relationship in these control tissues is given in Fig. 4 (●).

**Validation of the experimental approach**

Before employing this novel approach to examine the effects of altered tethering forces on airway function, we assessed the impact of several different experimental parameters on the measurements made.

Bai et al. (21) have shown that the magnitudes of constrictor responses in lung slices vary over a very wide range of airway sizes: however, they did not include contraction/relaxation velocities in those comparisons. In the present study, in which a much smaller range of airway sizes was examined, we found that neither the magnitudes nor the velocities of contraction correlated in a statistically significant manner with airway size (Fig. 5; p>0.05, Pearson’s correlation test).
To address whether the agarose used to inflate the lungs might affect the mechanical responses being measured (16), we halved the concentration of agarose, finding this to have no statistically significant effect on either the magnitudes nor velocities of contraction or relaxation (Table 1).

Lung compliance measured in whole animal studies varies with extent of lung inflation. Using the thin lung slice technique, the lungs are inflated to full physiological lung capacity with approximately 1.2 ml of agarose, depending on the relative size of the animal. We evaluated the dependence of our measurements on the degree of lung inflation. Even when the volume of agarose injected was decreased by a third, there was no statistically significant effect on either the magnitude nor velocities of the measured responses (Table 1; p>0.05, t-test).

**Effect of in vivo elastase treatment on cholinergic responsiveness**

When tissues from animals which had inhaled elastase *in vivo* were examined using the experimental protocol described above, we found that elastase treatment had a consistent, profound and statistically significant effect on the responses to cholinergic stimulation, to a degree that was dependent upon the concentration of elastase used. In particular, the mean magnitude of the response to the first bolus challenge with Ach was decreased even in those tissues which had received 5 IU elastase *in vivo*, and were nearly abolished in those treated *in vivo* with 15 or 20 IU elastase (Fig. 3B; p<0.05, ANOVA). Not surprisingly, the mean cholinergic response in tissues pretreated *in vivo* with 10 IU elastase was intermediate between these two extremes: however, we noted with interest that the responses in individual tissues ranged from being apparently normal (similar to those in naïve tissues) to being as severely affected as the 15 and 20 IU elastase-treated tissues.

We also found the velocities of contraction and relaxation to be significantly decreased (p<0.05, ANOVA) compared to naïve animals in tissues from animals that had inhaled elastase *in vivo*, and that the degree of inhibition was [elastase]-dependent (Fig. 3B-3E).
Finally, the Ach concentration-response relationship was examined in tissues pre-exposed in vivo to 5 or 10 IU elastase (the marked inhibitory effect of 15 and 20 IU elastase on the responses to maximal cholinergic stimulation precluded investigations of sub-maximal cholinergic responses). The mean magnitudes of the responses to $10^{-8}$ to $10^{-5}$ M Ach are given in Fig. 4: there was no statistically significant effect of elastase in vivo (5 or 10 IU) on the cholinergic concentration-response relationship (ANOVA).

**Effect of in-vitro elastase treatment on cholinergic responses**

In order to address the effects of simple destruction of elastic tissues without the confounding influences of inflammation, fibrosis, and repair, lung slices from another group of naïve animals were incubated overnight with varying concentrations of elastase added to the culturing medium, then examined the next day using the experimental protocol described above. Slices pretreated overnight in vitro with 100 µg/ml were very soft and fragile, with patchy parenchyma, epithelial degeneration, and noticeably less epithelial ciliary beating activity. Those treated with 50 µg/ml elastase had active cilia, but the parenchyma was also patchy and fragile. Slices pretreated overnight with 5 and 2.5 µg/ml elastase had normal appearing epithelium and less parenchymal damage (e.g., see Figure 6A): we elected to use 2.5 µg/ml elastase in studying the effects of overnight in vitro elastase treatment on cholinergic responsiveness.

Slices treated in this way exhibited a larger and much more brisk contractile response to Ach and a considerably more sluggish relaxation upon wash-out of Ach (e.g., see representative tracings in Fig. 8B and 8C); in fact, the airway often did not return to its baseline diameter, perhaps because the airways had torn away from their parenchymal attachments during the constrictor response. The mean magnitudes and velocities of this response are given in Fig. 3: one-way ANOVA revealed these to be
statistically increased following in vitro elastase treatment, relative to control tissues. The velocity of relaxation, on the other hand, was significantly suppressed (p<0.05).

The Ach concentration-response relationship was also altered by this direct overnight elastase treatment: although 10^-8 M Ach was sub-threshold in the tissues described above, it now elicited a profound, albeit transient, contractile response (Fig. 6). Likewise, responses to 10^-7 M Ach were significantly augmented. The mean concentration-response relationship is given in Fig. 4.
Discussion

Videomicrometry of lung slices has been used extensively to study a wide variety of mechanical responses (16; 17; 22) (23). Others have used 0.75% to 1% agarose to inflate rodent lungs prior to cutting into slices 500-1000 µm thick (16), or 250 µm thick (22). This approach was then modified using 2% agarose to inflate and stiffen the lungs prior to cutting into slices approx. 100 µm thick (19). Adler et al. have previously demonstrated that 2% agarose-filled lungs have a shear modulus similar to that of air-filled lungs (24).

However, those earlier studies have by and large examined only the absolute magnitudes of airway narrowing, paying little or no attention to the dynamics of those changes. Important information pertaining to the connective tissue matrix is likely present in the time-course of airway narrowing/opening. In this paper, we have extended the technique to include estimations of instantaneous velocities of narrowing, and showed that the latter are a facile and close estimate of the velocities of smooth muscle shortening. This technique has another advantage over other in vitro techniques for measuring airway function: the latter are carried out under either isotonic or isometric conditions, whereas the airways in the lung slices and in the lungs in vivo contract auxotonically (neither isometrically nor isotonically).

For all these reasons, this approach could prove to be invaluable in studying the interactions between the ASM and its surrounding parenchymal matrix. Duguet et al. have reported that airway constriction and velocity of narrowing is governed by the elastic load on the airway wall (25). The primary elastic loads that limit ASM shortening are the lung parenchyma (via the alveolar attachments to the airway wall), the airway wall itself, and the internal resistance to shortening of the smooth muscle cell (26-28).

COPD is characterized in part by lung parenchymal destruction and enlargement of the air spaces with loss of functioning alveoli. There is considerable evidence that this parenchymal...
destruction is secondary to an excess of proteolytic activity, which in turn is related to airway inflammation (29; 30). Many of these changes are reproduced in murine models of elastase-induced parenchymal destruction. However, while the histological and inflammatory indices referred to above are easily examined in such rodent models, the also highly clinically relevant changes in airway function brought on by altered tethering forces are not so easily examined. The thin lung slice technique offers the opportunity to easily monitor mechanical responses of even very small airways with high temporal and spatial resolution while they are still in their native environment.

We hypothesized that parenchymal destruction in this rodent model of emphysema would manifest as larger and faster contractions, with slowed airway re-opening upon induction of relaxation. Contrary to our expectations, we found the cholinergic responses in the animals treated in vivo with elastase to be consistently and markedly decreased compared to matched naïve animals. In fact, in animals exposed to the higher concentrations of elastase used here (15 and 20 IU), cholinergic responsiveness was negligible. The decreased magnitudes of cholinergic responsiveness were accompanied by decreased velocity of airway narrowing, which we predicted would herald increased tethering forces within the tissue. It may be that elastase inhalation leads not only to the emphysematous changes in the parenchyma, but also fibrotic remodeling of the airway walls per se. Enzymatically-digested elastin is repaired by lung-derived cells (31), and even the first study using this rodent model (10) noted a net increase (by 30%) in lung elastin levels in addition to the patchy emphysematous changes. Brewer et al. (32) have shown that alveolar walls exhibit more hysteresis following elastase treatment. On the other hand, there may be collagen-related remodeling: Ito et al. (33; 34) found the airspace enlargement and loss of parenchymal elastin in this murine elastolytic model to be accompanied by increased (48%) collagen content. We would also acknowledge the possibility that our experimental approach might bias against studying those slices/airways with the most emphysematous changes, due to airway collapse and tearing of the lungs during slicing, and
instead predispose towards the study of airways which have become fibrotic (another feature of COPD which can be reproduced in this murine model), since they are more robust during the slicing procedure. Another possibility is that the in vivo elastase injury includes a change within the smooth muscle itself: a loss of the contractile phenotype. In this light, it is possible that the inflammatory and/or reparative processes which occur during the 2 weeks following elastase instillation induce a transition within the smooth muscle towards a more secretory phenotype (with concomitant loss of the contractile phenotype). We would discount an alternative explanation that elastase-treated tissues express more/different proteins on the alveolar walls which make them more adherent to the glass surface used in our in vitro apparatus, since we would expect to have at least seen the airway straining inwards (this has been our experience when we watched airways contract against an agarose plug within the lumen) and/or portions of the airway tearing away suddenly from the glass: we saw neither.

When we examined the concentration-response relationship for Ach, we found no differences between the elastase-treated and naïve tissues: as such, the inherent sensitivity of the ASM was not affected. One interpretation of these concurrent changes is that elastase-exposure in vivo led first to tissue injury/destruction followed by a repair process, which left the airways scarred and fibrotic. We are now adapting our histological assays in order to quantify elastin/collagen content in the very same thin slices from which videorecordings were made: it is difficult to cut histological sections of these already very thin slices, and the agarose within the tissues may alter the staining assay per se. Alternatively, elastase treatment may have led to loss of ASM mass and/or otherwise compromised ASM function.

Tissues pretreated overnight with elastase, on the other hand, exhibited the expected changes: tearing of the airway wall from the surrounding parenchyma and slower and less complete relaxations back to resting diameter upon wash-out of Ach; the magnitude and velocity of cholinergic contractions, on the other hand, remain unaltered with respect to control slices. These observations are
consistent with decreased tethering forces within the tissues brought on by the acute treatment with protease: elastin is vital for elastic recoil of the small airways and their ability to resist negative pressure collapse (35). Overnight treatment with this protease would not have allowed sufficient time for tissue remodeling events such as fibrosis or smooth muscle hyperplasia/hypertrophy (nor transition of the latter towards the secretory phenotype).

Altogether then, this experimental approach allows one to directly visualize small airway narrowing with high spatial and temporal resolution while still in their native extracellular environment, providing valuable insight into the kinetics of airway narrowing and the effects of altered tethering. Using this approach, it was possible to discriminate differences in airway kinetics due to different degrees of smooth muscle activation ($10^{-7}$ versus $10^{-6}$ M Ach) or even strain of animal (Balb/C versus C57 mice; data not shown here). It is otherwise not possible to visualize airways of this size with the same resolution (e.g., CT/PET). Nonetheless, we recognize some of the limitations of this technique. The absolute kinetics of airway narrowing could be affected by friction between the cover slip and the tissue slice, as well as by the agarose itself which remains in the alveoli in a gel-state throughout the experiment. However, these effects should be constant between treatment groups, and thus relative comparisons can still be made. Also, longitudinal forces are missing in this experimental arrangement.

In future studies, it will be important to examine the potential contributions of protease-activated receptors to the changes described here. Also, it will be intriguing to extend this model to the human condition by comparing magnitudes and velocities of airway narrowing in lung slices obtained from surgical specimens of humans with/without COPD.
Acknowledgements: We would like to thank Dr. Michael Sanderson and Dr. Yan Bai (Department of Physiology, University of Massachusetts Medical School, Worcester, MA) for their help with the lung slice technique. These studies were supported by a grants-in-aid provided by the Canadian Institutes of Health Research and AstraZeneca International.


15. **Siafakas NM, Vermeire P, Pride NB, Paoletti P, Gibson J, Howard P, Yernault JC, Decramer M, Higenbottam T, Postma DS and .** Optimal assessment and management of


23. **Kott KS, Pinkerton KE, Bric JM, Plopper CG, Avadhanam KP and Joad JP.**


Table 1

Dependence of measurements upon concentration and volume of agarose used to inflate lungs prior to slicing.

<table>
<thead>
<tr>
<th></th>
<th>Decrease in airway area (%)</th>
<th>Velocity of contraction (%/sec)</th>
<th>Velocity of relaxation (%/sec)</th>
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<td><strong>Concentration of agarose</strong></td>
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</tr>
<tr>
<td>1%</td>
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<td>17.5 ± 2.3</td>
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</table>
Figure Legends

Figure 1 Lung slices from mice inoculated intranasally with 5 (B), 10 (C), or 20 (D) IU porcine pancreatic elastase per 100 g body weight; (A) shows a naïve lung for comparison. Airspace enlargement was quantified and expressed as mean chord length (E). Data displayed show mean ± SE (n=3). *, p<0.05, ANOVA.
**Figure 2** (A) Single frame images of the airway before (*i*) and during (*ii*) stimulation with ACh (10⁻⁵ M).  (B) Measurements of airway diameter during bolus addition of a supramaximally-effective concentration of acetylcholine (ACh; 10⁻⁵ M), followed by wash-out of ACh, and then re-introduction in log unit increments beginning at 10⁻⁸ M. (*i*) and (*ii*) correspond to images in (A). (C) First order derivatives of data in (B), giving velocities of lumen narrowing (thin black line) or of airway smooth muscle shortening (thick grey line; obtained by estimating lumen radius on a frame-by-frame basis; see Methods). Tissue was obtained from a naive mouse, and was incubated overnight in DMEM.
Figure 2

(A) (i) (ii)

(B) Ach 10^{-5} Ach 10^{-6} 10^{-7} 10^{-8} 10^{-5}

(i) (ii)

(C) Velocity of narrowing (pixel/second) Velocity of shortening (pixel/second)

Time (s)
Figure 3  Effect of elastase on cholinergic mechanical responses. (A) Representative images of airways treated with 10 IU or 20 IU elastase in vivo, before and during challenge with Ach (10⁻⁵ M). Panels B to E summarize the magnitudes of contraction (B), velocities of contraction (C), velocities of relaxation (D), and ratios of the two velocities (E) in airway slices from naïve mice and those treated with elastase in vivo (5, 10, 15 or 20 IU) or in vitro (2.5 µg/ml), as indicated; data from individual slices are indicated by “X”, whereas mean +/- S.E. (n>5 for all) are indicated by “●”. *, p<0.05, ANOVA.
Figure 3

(A) 10 IU 10 IU 20 IU

at rest

+ACh

(B) Change in airway area (% of initial)

(C) Velocity of contraction (Cél/cm)

(D) Velocity of relaxation (Cél/sec)

(E) VcnMx (Cél/sec)

Airway condition

Naïve 5 IU 10 IU 15 IU 20 IU 2.5 ug/ml Elastase

ND ND
Figure 4  Mean concentration-response relationship for ACh obtained in lung slices from naïve mice and those treated in vivo with elastase (5 or 10 IU), as well as naïve slices incubated overnight with 2.5 µg/ml elastase (●), as indicated. n>5 for all.
Figure 5 Magnitudes of contraction (top), velocities of contraction (middle) and velocities of relaxation (bottom) did not correlate with initial airway size.
Figure 5
Figure 6  Raw tracing showing airway diameters (A) and velocity of contraction (B) in a naïve lung slice incubated overnight with DMEM containing 2.5 µg/ml elastase. Inset: individual frames immediately before application of bolus concentration of Ach ($10^{-5}$), as well as at the peak of that contraction: note tearing of the airway away from the parenchyma.
Figure 6