Functional and morphological assessment of early impairment of airway function in a rat model of emphysema

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EMPHYSEMA IS THOUGHT TO BE a disease of the parenchymal structure deep in the periphery of the lung (27). However, emphysema and chronic bronchitis, the two main components of chronic obstructive pulmonary disease (COPD), can also occur together (26). Animal models are commonly used to study the pathogenesis of COPD (29). While parenchymal destruction in emphysema is often investigated in animal models such as elastase treatment of rodents, less attention is paid to the possibility of airway involvement. One of the reasons is that in most studies, little or no change in airway function such as airway resistance (Raw) is observed. Indeed, Raw has been reported to be similar in normal and emphyse-matous mice (10, 13); baseline pulmonary resistance in Brown-Norway rats returned to the control level after 3 wk of elastase treatment (2). This raises the question of the relevance of the elastase-treated rodent as a model of the human disease.

More recently, we reported measurements of Raw and tissue elastance (H) as a function of both thoracic gas volume (TGV) and transrespiratory pressure (Pr) in normal and elastase-treated mice (10). Interestingly, the results showed that Raw was different between the groups only when it was plotted as a function of TGV and this difference was attributed to the reduced elastic tethering in the treated mice. Indeed, several earlier studies reported a reduction in the number of alveolar attachments around small airways (2, 5, 7, 29). On the other hand, the mRNA levels of the two structurally most important components of the extracellular matrix, elastin and collagen, were upregulated in the airway wall within 6 h after elastase treatment of hamsters (17). If these events are manifested in remodeling, they likely alter airway wall stiffness and hence the balance between wall elasticity and tethering, which in turn could lead to a change in airway diameter and Raw.

The present study was undertaken to examine whether elastase treatment of rodents, the most common animal model of emphysema, induces pure parenchymal destruction or if it also involves airway abnormalities. To this end, rats were treated with porcine pancreatic elastase (PPE) and the functional changes were characterized by detailed assessment of airway and tissue mechanics using the forced oscillation technique. The pressure-volume (P-V) curve and crackle sound were also measured during inflation from the in vivo degassed state, which provide separate and additional information on paren-chymal and airway mechanics. To evaluate the structural changes underlying the functional alterations, the morphological properties of the parenchyma including alveolar attachments around small airways were assessed and elastin as well as collagen in the septal and airway walls was visualized and quantified. Our results suggest that the PPE-induced severe alveolar destruction is accompanied by subtle remodeling of the airway wall with a potential change in airway function.

METHODS

Animal preparation. The study protocol was approved by the Institutional Animal Care and Use Committees of the University of Szeged and Boston University. The studies were performed in 14 male adult (10 wk old) Sprague-Dawley rats. The animals were anestheized with a single intraperitoneal injection of chloral hydrate (350 mg/kg). Supplemental doses of anesthesia (50 mg/kg) were adminis-tered during the measurement as needed.

Rats were intubated with a polyethylene cannula (14-gauge, Braun, Melsungen, Germany) under illumination with a cold light source (model FLQ85E, Helmuth Hund, Wetzlar, Germany) as described in detail previously by Brown et al. (4). Eight animals were treated with
intratracheal instillation of 50 IU porcine pancreatic elastase (PPE, Affymetrix/USB, Cleveland, OH) in 0.5 ml saline, and six rats were given only saline.

Six weeks after the treatment, rats were reanesthetized as described before, tracheotomized, and cannulated with a 1.7-mm-ID polyethylene tube. The rats were placed in the supine position in a custom-built 2.8-liter body plethysmograph and mechanically ventilated with a small-animal respirator (Harvard Apparatus, South Natick, MA) at a rate of 80 min\(^{-1}\) and tidal volume of 8 ml/kg. Following the measurements, the animals were killed with an overdose of anesthetics and the lungs were removed for histopathological evaluation.

There were no significant differences in body weight between the control (C) and the PPE groups either at the time of treatment (318 ± 34 vs. 314 ± 25 g) or 6 wk thereafter (468 ± 52 vs. 471 ± 58 g).

**Measurement of lung volumes.** Functional residual capacity (FRC) was measured with the body plethysmographic technique (6). Briefly, the respirator was stopped at end expiration and the breathing efforts against the occluded tracheal cannula were recorded for 6 s. Box pressure (Pbox) and tracheal pressure (Ptr) were measured with a Validyne MP-45 (± 2 cmH\(_2\)O) pressure sensor (Validyne, Northridge, CA) and a miniature pressure transducer (model 8507C-2, Endevco, San Juan Capistrano, CA), respectively. FRC was estimated from the Pbox and Ptr relationship on the basis of Boyle’s principle and the thermal characteristics of the plethysmograph (15).

Inspiratory capacity (IC) and expiratory reserve volume (ERV), respectively, were defined as the volume changes accomplished by decreasing Pbox to −35 cmH\(_2\)O and increasing it to 20 cmH\(_2\)O. The IC and ERV maneuvers were shorter than 35 and 15 s, respectively. The volume changes were measured by means of the pressure drop through the wave tube (see below). Total lung capacity (TLC) and residual volume (RV) were calculated as TLC = FRC + IC and RV = FRC − ERV.

**Measurement of respiratory impedance.** Low-frequency impedance of the total respiratory system (Z\(_{rs}\)) was measured with the wave tube pseudorandom forced oscillation method (12, 28) between 0.5 and 16 Hz at FRC. Z\(_{rs}\) was determined as the load impedance of a 100-cm tube (ID = 2 mm) during 6-s interruptions of mechanical ventilation.

Newtonian resistance (R\(_N\)), tissue elastance (H), tissue damping (G), and inertance (I) were estimated by fitting the constant-phase model (11) to the average Z\(_{rs}\) data obtained from 5–6 successive recordings. Hysteresivity (η), which is the ratio of the dissipative and elastic parameters of the tissue impedance (14) was calculated as η = G/H. Contribution of the resistance and the inertia of the tubing including the tracheal cannula to R\(_N\) and I were subtracted. The remaining values of I are considered physiologically unimportant in small animals and are not reported.

**Crackle sound recordings.** Intratracheal sounds were recorded during slow (~20-s) inflations from the degassed state of the lungs to

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Fig. 1. Definition of lung slices for histological evaluation (top) and illustration of the areas in every slice used for alveolar and airway morphometry (bottom).
ducts were manually excluded from the analysis. The equivalent airspace was measured. Large airways, blood vessels, and alveolar vessels were chosen in 3–4 measurements at random locations along the perimeter and the number of septal attachments (Nv) were manually determined for each airway. Septal attachment density was calculated as Nv/Pb.

Visualization and quantification of elastin and collagen. To visualize the elastin content in the airway wall, the established method of Verhoeff-Van Gieson staining for elastic fibers (adapted from IHC World; http://www.ihcworld.com/_protocols/special_stains/vvg.htm) was used with slight modifications. The omission of the counter Van Gieson staining provided blue color only of elastic fibers and was used for quantification. To visualize collagen, the Mason’s trichrome was used. Quantitative analysis of elastin and collagen density was made on randomly selected lung sections using custom-made software. A total of 51 and 52 airways for elastin and 51 and 57 airways for collagen were examined quantitatively in the control and PPE groups, respectively. Once an airway was manually chosen, the algorithm determined the inner boundary of the airway and created an outer boundary by moving along the outward normal to the inner boundary by 9 μm. This procedure automatically defined a band inside the airway wall (Fig. 2). The thickness of 9 μm was chosen so that the band was always inside the bronchial wall. The pixels within the band were then split into two colors, white and blue, with blue representing elastin or collagen. The mean and SD of the grayscale values for the pixels that were identified as blue were determined.

Statistical analysis. The differences in lung volumes and mechanical parameters between the control and elastase-treated rats were compared by using Student’s t-test. All the results are expressed as means ± SD. Distributions were compared using the Kolmogorov-Smirnov test. Dependencies of Tw on Db in the two groups were tested by using Student’s t-test. All the results are expressed as means ± SD. Distributions were compared using the Kolmogorov-Smirnov test. Dependencies of Tw on Db in the two groups were tested with the analysis of covariance. A P value of less than 0.05 was considered significant.

RESULTS

Lung volumes. FRC and RV were statistically significantly higher in the PPE group compared with the controls (by 38% and 53%, respectively); however, the increase in TLC in the treated rats (5%) was statistically not significant (Fig. 3).

Respiratory mechanics. Table 1 summarizes the respiratory mechanical parameters estimated from the impedance data. G

Fig. 2. Inner and outer boundary of the airway wall determined automatically for the calculation of elastin density.

TLC, similarly as described previously in mice (10). In short, degassing was accomplished with a 10-min ventilation with 100% O2 and subsequent ERV maneuver followed by tracheal occlusion for 10 s; the degassing-reinflation maneuver was repeated 2 more times in each animal. A 15-mm metal tube (0.7-mm OD) attached to the microphone was positioned in the tracheal cannula. Sound was recorded at a sampling rate of 44 kHz with 16-bit resolution and was high-pass filtered at 2 kHz to eliminate the cardiac noise. Individual crackle events were identified in 0.33-ms time windows, if the sound energy in a window was higher than a threshold and increased with a given step compared with the previous window, as described previously in detail (22).

Morphometry. After the measurements, rats were euthanized with an overdose of anesthetics, and the heart and lungs were removed from the chest en bloc. The isolated lungs were then hung in an airtight glass box, and the tracheal cannula was led through the lid of the bottle and attached to a formaldehyde-filled container outside. Lungs were filled with 4% buffered formaldehyde via the tracheostomy tube from a height of 10 cm while maintaining a pressure of −15 cmH2O around the lungs. The trachea was then ligated at this fixation pressure (25 cmH2O), and the lungs were stored in formaldehyde for 7 days before embedding in paraffin. In each lung, three transversal sections of 4-μm thickness were made at 25, 50, and 75% lung height for hematoxylin and eosin (H&E) staining, and eight, five, and four areas, respectively, of 2,085 pixels that were identified as blue were determined.
and H, respectively, were statistically significantly smaller in the PPE-treated rats than in the controls (76 and 62%). Because of the larger differences in H, tissue hysteresivity (η = G/H) was elevated (129%) in the treated rats. There was no difference in Rs and the error of model fitting between the groups.

Crackles. The lower knee of the inflation P-V curves from the degassed state (as defined by the intercept of the lines fit to the P-V curve between 5 and 15, and the maximum slope projected to the P axis) was shifted to higher pressures (19.3 ± 1.0 vs. 17.8 ± 1.2 cmH2O; P = 0.021) and the asymptotic volume level (TLC) was slightly larger in the PPE group than in the control rats (Fig. 4). The crackle numbers (N) per inflation were not different between the two groups (1,152 ± 216 vs. 1,344 ± 305; P = 0.37 ± 0.04); however, their distributions expressed as relative frequencies (N/Ntotal) were statistically significantly different (P < 0.001). In the control rats, N peaked at lower pressures (~10 cmH2O) than in the PPE group (~17 cmH2O), and exhibited another maximum at ~25–30 cmH2O (Fig. 5A). This latter fact suggests a bimodal distribution of the sounds of reopening, which is also expressed by the log(N/Ntotal) vs. volume (V) graphs exhibiting a second peak at ~19–23 ml in the control group (Fig. 5B).

Alveolar and bronchial morphometry. Figure 6 compares the size distributions of Dalv pooled from all regions and animals in either group. The average number of alveoli evaluated in all regions of an animal was 8,091. In the PPE group, the distribution was shifted to higher Dalv values and, according to the Mann-Whitney rank sum test, the median of Dalv was significantly higher in the treated group (68.4 vs. 61.8 μm; P < 0.001).

The number of bronchial cross-sections analyzed in each rat ranged between 40 and 50. The distributions of Db were not different between the groups (Fig. 7A). The attachment density N/Pb (Fig. 7B) was mildly but statistically significantly lower in the PPE group (median 0.0175 vs. 0.0189 μm−1; P < 0.001). Regression analysis showed that Tw did not depend on Db in either group (Fig. 7C). However, the mean value of Tw was higher in the PPE group compared with the controls (12.4 ± 4.05 vs. 10.8 μm; P < 0.0001).

Elastin and collagen density in the bronchial wall. Similarly to the bronchial morphometry, there was no difference between the Db of the airways of control and PPE-treated animals for which elastin and collagen densities were evaluated. The mean and SD of elastin grayscale representing the average and the spatial variability of elastin density within the bronchial wall, respectively, were not different in the groups. The sum of all grayscale values, which represents the total elastin in the 9-μm band inside the bronchial wall, was also not different between the groups. The density of elastin and collagen did not correlate with Dalv. The inter-airway variance of the elastin density was also not different between control and treated animals. Interestingly, the collagen density as well as its intrabronchial SD were increased by 12 and 17% in the treated group (P < 0.05). Furthermore, the inter-airway variance of collagen density was substantially higher (67%, P < 0.01) in the treated animals. Figure 8 illustrates the difference in collagen wall structure between the control and treated animals.

DISCUSSION

In this rat model of emphysema, we aimed at investigating the alveolar and bronchial structural changes underlying the alterations in lung volume and mechanics, and the acoustic manifestations of airway function. The combination of structural and functional measurements revealed that 1) the PPE treatment caused significant increases in FRC and RV, whereas no change in TLC was observed; 2) the tissue mechanical parameters were significantly lower in the treated group, whereas there was no detectable alteration in the total airway resistance as measured at FRC; 3) while the number of crackles per inflation was similar, the distributions of crackles as a function of transpulmonary pressure or lung volume were statistically significantly different between the PPE-treated and control groups, and the lower knee of the inflation P-V curve moved to higher P in the treated rats; 4) the distribution of alveolar diameters was shifted toward higher values in the PPE group with a slightly higher median alveolar diameter; 5) bronchial morphometry revealed that compared with controls, alveolar attachment density was 7% lower and wall thickness was 15% higher in emphysematous animals; and 6) while no difference was found in the elastin content per unit wall

Table 1. Mechanical parameters of the lungs in the control animals and those treated with porcine pancreatic elastase

<table>
<thead>
<tr>
<th></th>
<th>Rs, cmH2O·s/l</th>
<th>G, cmH2O/l</th>
<th>H, cmH2O/l</th>
<th>η (= G/H)</th>
<th>F%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>39 ± 8.5</td>
<td>653 ± 99</td>
<td>2,178 ± 305</td>
<td>0.30 ± 0.01</td>
<td>2.40 ± 0.40</td>
</tr>
<tr>
<td>PPE</td>
<td>35.5 ± 8.5</td>
<td>496 ± 66</td>
<td>1,344 ± 216</td>
<td>0.37 ± 0.04</td>
<td>2.53 ± 0.34</td>
</tr>
<tr>
<td>P</td>
<td>0.451</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.512</td>
</tr>
</tbody>
</table>

Values are means ± SD. C, control animals; PPE, animals treated with porcine pancreatic elastase; Rs, Newtonian resistance; G, tissue damping; H, tissue elastance; η, hysteresivity; F%, average fitting error of the model; P, level of significance for differences between groups.

Fig. 4. Comparison of the inflation pressure-volume (PV) curves of control (thin line) and PPE (thick line) groups from degassed state (means ± SD).
thickness of the bronchial wall, collagen content was higher and more heterogeneous in the treated animals.

Changes in lung volumes and parenchymal mechanics. The increases in lung volumes observed in the current study are in accord with the effects of elastase treatment in rats, with regard to FRC (2, 8, 16, 18, 23, 30) and RV (18, 30). However, we observed no change in TLC whereas previous studies found significant increases in TLC (8, 16, 18, 23, 30). Often the changes in TLC are more variable than those in RV and FRC and depend on the definition of TLC, the dose of elastase, and the time period after treatment. For example, Yokoyama et al. (30) reported no change in TLC until 7 wk after treatment, in agreement with our results. Our finding that H as measured at FRC decreased following treatment is also in line with the reported elevations in compliance (8, 16, 18, 23, 30). Total respiratory resistance has been found to decrease, probably via the smaller contribution of the tissue resistance, due to the elastolytic processes (8). We also observed a decrease in G, although to a lesser extent compared with that in H; this resulted in an elevation in \( \frac{H}{G} \) similarly to the effect of elastase treatment in mice (3, 10, 14), most likely reflecting the structural alterations in the parenchymal composition. Although the parenchymal changes may have been masked by the contributions of the chest wall impedance to G and H in both groups of rats, it is still unclear why the decrease in dynamic elasticity at FRC is not accompanied by a fall in quasi-static elastance at high transrespiratory pressures resulting in an elevation of lung volume.

Airway resistance, crackles, and morphometry. The shift in the inflation P-V curve was consistent with a delayed recruitment process also indicated by the crackle intensity. The mechanisms behind the bimodal crackle distribution in the control rats are unclear. Nevertheless, it is tempting to speculate that the altered shape of the crackle distribution in the PPE-treated animals is directly related to the high airway-to-airway variability of the remodeled collagen density and hence of bronchial wall stiffness (see below).

The \( R_N \) parameter was not different between the two groups suggesting that the overall resistance of the bronchial tree was not affected by the elastase treatment, with the provision that the contribution of the Newtonian resistance of the chest wall to \( R_N \) was similar in both groups. However, the knee of the P-V curve is shifted to the right in the treated animals by about 9%, which was statistically significant. Since the lower knee of the P-V curve signifies airway openings (24), this shift implies that the critical opening pressure at which massive airway opening allows the alveoli to start filling up was higher in the treated lungs. The critical opening pressure is influenced by many factors, including surface tension and viscosity of the air-liquid interface (9), airway wall stiffness, and parenchymal tethering (21). While surface tension appears to be normal in elastase-induced emphysema (19), little is known about how liquid film viscosity or airway wall stiffness might change with treatment.

In this study, we quantified the strength of parenchymal tethering by measuring the number of attachments per unit airway wall perimeter and found that the treatment reduced the average attachment density by 7%. This is similar to the findings of Collie et al. (5) who reported a decreased number of attachments and an increased average distance between attachments around the bronchi of sheep following elastase treatment. One might argue that if the wall is more compliant in the treated lungs, the decreased attachment density is simply a result of the larger airway diameters at the same fixation pressure in the treated animals. However, there was no difference in diameter between the two populations of airways included in our analysis, suggesting that the reduction in attachment density was likely due to loss of parenchymal walls around airways. The elastin density of the walls measured from the histologic images was similar in the two groups. We should point out that the elastin density was measured in a band of fixed width around the bronchi. Since the wall thickness in the treated animals was larger by 15%, it is likely that the total elastin content was also larger in the bronchial walls of the...
treated rats, implying cellular remodeling of the airway walls. However, collagen also contributes to stiffness especially at higher transmural pressures. The increased collagen density of the wall and the elevated intrawall heterogeneity suggest a disordered cellular remodeling following PPE treatment. It is thus likely that the incremental Young's modulus of the airway walls was also elevated in the treated animals especially at high lung volume where collagen is expected to contribute to stiffness more than elastin. Furthermore, the increased wall thickness would also result in a higher volumetric elastance of the airway wall (25). Thus these considerations imply a real decrease in tethering forces as well as a likely stiffening of the airway walls as a consequence of the PPE treatment.

Examining the results from the crackle sounds, we also found major differences in the distribution of the number of crackles as a function of airway pressure or lung volume (Fig. 5). The amplitude distribution of the crackle sounds followed a power law (not shown) in agreement with previous studies in normal dog lungs (1) and in elastase-treated mice (10). The exponent \( \alpha \) of the power law distribution has been shown to reflect the average bifurcation geometry of the airway tree (1). Specifically, it was shown that \( \alpha = \ln(2/b)/\ln(b) \), where \( b = 2A_1/(A_0 + A_1 + A_2) \) and \( A_0 \) is the cross-sectional area of the parent airway, \( A_1 \) is the cross-sectional area of the daughter branch where the crackles comes from, and \( A_2 \) is the cross sectional area of the other daughter branch. Assuming that on average, the bifurcation geometry is symmetric (i.e., \( A_1 = A_2 \)) and knowing \( \alpha \) and hence \( b \) from experiments, the average diameter ratio \( d_1/d_0 \) at bifurcations can be estimated. The values of \( b \) in the control and treated rats were 0.573 and 0.512, respectively, resulting in corresponding diameter ratios of 0.757 and 0.712. Although the assumption of symmetric bifurcations seems unwarranted, based on the theory published earlier (1), it is possible to show that similar results can also be derived using asymmetric bifurcations (unpublished data). This analysis then suggests that on average, airway diameters decrease faster along the airway tree in the treated animals than in the control animals, which seems to contradict the fact that \( R_N \) was not different between the two groups. However, whereas \( R_N \) characterizes air flow resistance around FRC, the distribution of crackle amplitudes mostly reflects diameters at much higher lung volumes than FRC. Furthermore, the reduced diameter ratio obtained from crackles is also in accord with both a stiffer wall due to increased collagen content and wall thickness, and a reduced parenchymal tethering as a result of the lower attachment density and septal stiffness. We thus conclude that the reduced diameter ratio inferred from crackles and the shift of the knee of the P-V curve imply an important deterioration in airway patency under conditions such as airway reopening and high lung volumes that the FOT-based \( R_N \) measured around FRC does not detect.

Conclusions. In this study, we have shown that elastase treatment of the rat lung that is often used to generate airspace enlargement as a model of human emphysema also leads to altered airway mechanics, which manifests in enhanced collapsibility and increased difficulty to reopen the airways. These results cannot be observed by evaluating lung and airway mechanics around FRC. Our results also imply that this loss of function is partly due to reduced alveolar tethering and partly due to increased stiffness of the remodeled bronchial wall. Since such deterioration in airway function also occurs in human COPD, the implications are important both in mechanical ventilation where airway collapse and the hindered ability to reopen the airways can lead to flow limitation and trapped gas, and in exercise where the reduced airway diameter ratios at higher lung volumes may limit the exercise capacity of patients.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.T., M.V.S., B.S., and Z.H. conception and design of

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