Mechanics, nonlinearity, and failure strength of lung tissue in a mouse model of emphysema: possible role of collagen remodeling

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ABSTRACT

Enlargement of the respiratory airspaces is associated with the breakdown and re-organization of the connective tissue fiber network during the development of pulmonary emphysema. In this study, a mouse (C57BL/6) model of emphysema was developed by direct instillation of 1.2 IU of porcine pancreatic elastase (PPE) and compared with control mice treated with saline. The PPE-treatment caused 95% alveolar enlargement (P = 0.001) associated with a 29% lower elastance along the quasi-static pressure-volume curves (P < 0.001). Respiratory mechanics were measured at several positive end-expiratory pressures in the closed chest condition. The dynamic tissue elastance was 19% lower (P < 0.001), hysteresivity was 9% higher (P < 0.05), and harmonic distortion, a measure of collagen-related dynamic nonlinearity, was 33% higher in the PPE-treated group (P < 0.001). Whole lung hydroxyproline content, which represents the total collagen content, was 48% higher (P < 0.01), and α-elastin content was 13% lower (P = 0.16) in the PPE-treated group. There was no significant difference in airway resistance (P = 0.7). The failure stress at which isolated parenchymal tissues break during stretching was 40% lower in the PPE-treated mice (P = 0.002). These findings suggest that following elastolytic injury, abnormal collagen remodeling may play a significant role in all aspects of lung functional changes leading to progressive emphysema.

214 words

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INTRODUCTION

Pulmonary emphysema is characterized by permanent destruction of the respiratory bronchioles, alveolar ducts, and alveolar walls, which leads to hyperexpansion and loss of elastic recoil (1). The most widely accepted hypothesis of how tissue destruction occurs in emphysema is that an imbalance of protease and anti-protase activity exists within the lung that ultimately leads to enzymatic degradation of elastin (38, 45). However, changes consistent with emphysema can also result from abnormality of the collagen matrix (4, 8, 12, 21, 23, 25, 35, 49). Recent studies have provided evidence that significant remodeling of the extracellular matrix (ECM) including collagen, elastin, and proteoglycans occurs during the development of emphysema both in humans (4, 12, 48, 49) and in experimental rodent models (12, 21, 25, 52). Therefore, besides elastin degradation, the biological breakdown and subsequent remodeling of collagen during the abnormal repair of the lung tissue would play a role both in the progress of this disease (24, 52) and the physiological functioning of the lung (21, 44).

Among the constituents of the ECM components, elastin and collagen appear to account for most of the viscoelastic mechanical properties of the lung tissue strips (33, 46, 53). Elastic fibers behave more linearly than collagen fibers (28, 36). Therefore, one would predict that the nonlinear behavior of the lung could change when alterations occur in the relative amounts of elastin and collagen in the connective tissue.

We hypothesized that if alterations occur in the collagen fiber network during the development and progress of emphysema, then both the nonlinear viscoelastic properties of the lung tissue and the strength of alveolar walls should change. To test this hypothesis, we measured the nonlinear and viscoelastic properties of the whole lung in a mouse model of emphysema induced by direct instillation of porcine pancreatic elastase (PPE) (26). To characterize lung elasticity and airway function, we measured the quasi-static
pressure-volume (P-V) curves and the forced oscillatory impedance of the respiratory system. We calculated a dynamic nonlinearity index, called harmonic distortion (42), as well as measured the failure stress of parenchymal tissue strips. Finally, to assess changes in composition, we also determined the total amounts of elastin and collagen in the lung.

METHODS

Animal Preparation

Two groups of male C57BL/6 mice (Charles River, Boston, MA), weighing 23 - 25 g were studied. Animals were treated with direct instillation of either 1.2 IU porcine pancreatic elastase (PPE) (Sigma, St. Louis, MO) dissolved in 90 µl of sterile saline (n = 18) or the same amount of saline (control) (n = 18) (26). Three weeks after the treatment, the following experiments were performed. All animal procedures were approved by the Animal Care and Use Committees of Boston University and Harvard Medical School.

Measurement of Respiratory Mechanics

The detailed methods are described elsewhere (20). The control (n = 8) and PPE-treated (n = 9) mice were deeply anesthetized by intraperitoneal injection of pentobarbital (70 mg/kg), tracheostomized, and cannulated in the supine position. The cannula was connected to a computer-controlled ventilator (Flexivent, SCIREQ, Montreal, Canada). Mice were mechanically ventilated with room air using a tidal volume of 8 ml/kg at a frequency of 240 breaths/min. After stabilization, the quasi-static pressure-volume (P-V) curve of the respiratory system was measured as follows. After inflation of the lungs to total lung capacity, defined as a tracheal pressure of 25 cmH2O, mice were mechanically ventilated under the same condition for 5 sec. Following 3 sec of delay, slow volume inflation (0.1 ml/sec, total 1.2 ml) starting from end-expiratory lung volume was
performed using the computer-controlled ventilator without applying positive
end-expiratory pressure (PEEP) (21). Dynamic respiratory mechanics were measured at
four different PEEP levels (0, 3, 6, and 9 cmH₂O) in the closed chest condition and
assessed by measuring impedance data during forced oscillations. To standardize volume
history, each measurement was preceded by two consecutive inflations of the lungs to
total lung capacity.

Impedance Measurements
Impedance data collection was made by interrupting mechanical ventilation for 6 s using
the optimal ventilation waveform (OVW), which is a broad-band waveform containing
energy from 0.5 to 15 Hz as described previously (27). The frequencies in the OVW are
selected according to a non-sum non-difference criterion, which eliminates harmonic
distortion and minimizes cross talk among the frequencies that are present in the input
flow waveform and hence provides smooth estimates of the input impedance (43). The
volumes delivered are similar to normal spontaneous tidal volume values and hence the
method provides information on the mechanical properties during conditions mimicking
breathing. In our experiment, we matched the peak-to-peak OVW amplitude to the tidal
volume delivered by the mechanical ventilator. The ventilator displacement and cylinder
pressure signals were low-pass filtered at 30 Hz and sampled at 256 Hz. With the use of
Fourier analysis, impedance spectra were calculated on overlapping blocks of pressure
and flow data as the ratio of the cross-power spectrum of pressure and flow and the
autopower spectrum of flow. The forced-oscillatory system was calibrated by measuring
the input impedance of known analogs including tubes and bottles with known
impedances. The frequency response of the system was obtained, and the measured
impedance spectra were off-line corrected for any phase difference between pressure and
flow. Additionally, the flow-dependent impedance of the tracheal cannula was characterized separately and removed from the respiratory impedance of the mice (20).

**Mathematical Modeling**

Data were analyzed with a model that allows for specific alterations in the lung tissue similar to what might be expected in emphysema. Specifically, we applied a heterogeneous tissue elastance model of the lung (20). Briefly, in this model, we represented the airway tree by a set of airway pathways arranged in parallel, where each compartment is composed of an airway resistance ($R_{aw}$), an inertance ($I_{aw}$), and a linear tissue impedance ($Z_{Lti}$) connected in series. Hantos et al. (16) introduced the constant-phase model and described the $Z_{Lti}$ as:

$$Z_{Lti}(\omega_n) = \frac{G - jH}{\omega_n^\alpha}, \text{ with } \alpha = \frac{2}{\pi} \text{arctan}(H/G) \text{ and } \omega_n = \omega/\omega_0 \text{ (Eq. 1)}$$

where $\omega_n$ and $\omega$ are the normalized and absolute circular frequency, $G$ and $H$ are the coefficients of tissue damping and elastance, respectively, $j$ is the imaginary unit, and the exponent $\alpha$ describes the frequency dependence of tissue resistance ($R_{ti} = G/(\omega/\omega_0)^\alpha$) and tissue elastance ($E_{ti} = H(\omega/\omega_0)^{1-\alpha}$). The normalization factor $\omega_0 = 1$ rad/sec is introduced in order to obtain meaningful units for the parameters $G$ and $H$ (3). The impedance of the airways ($Z_{aw}$) in each pathway is given by:

$$Z_{aw}(\omega_n) = R_{aw} + j\omega_nI_{aw} \text{ (Eq. 2)}$$

The $R_{aw}$, $I_{aw}$ and hysteresivity (14), defined as the ratio of $\eta = G/H$, of the tissue elements were the same in each pathway while the elastance ($H$) of the tissue elements followed a hyperbolic distribution between a minimum ($H_{\text{min}}$) and a maximum ($H_{\text{max}}$). The input impedance $Z$ of the network is obtained as (20):

$$Z_{in} = \frac{FZ_{aw}}{F + \ln \left( \frac{Z_{Lti,\text{min}} + Z_{aw}}{Z_{Lti,\text{max}} + Z_{aw}} \right)} \text{ (Eq. 3)}$$
where $F$, $Z_{ti,min}$ and $Z_{ti,max}$ are given by:

$$F = \ln(H_{\text{max}}/H_{\text{min}}), \quad Z_{ti,min}(\omega_n) = (\eta - j)H_{\text{min}}/\omega_n^\alpha,$$

and

$$Z_{ti,max}(\omega_n) = (\eta - j)H_{\text{max}}/\omega_n^\alpha.$$

By minimizing the root mean square difference between the model and the data (6) the five parameters ($R_{aw}$, $I_{aw}$, $\eta$, $H_{\text{min}}$, and $H_{\text{max}}$) were determined. The mean $H$ value ($H_{\text{mean}}$) was estimated as the expected value of the distribution function and was calculated from the estimates of $H_{\text{min}}$ and $H_{\text{max}}$ as:

$$H_{\text{mean}} = \frac{H_{\text{max}} - H_{\text{min}}}{F} \quad \text{(Eq. 4)}$$

The tissue damping, $G$, was calculated as $G = \eta H_{\text{mean}}$.

**Dynamic Nonlinearity of Respiratory Mechanics**

When applying a broad-band input, one way to probe system nonlinearity is to measure how the elastic and viscous moduli depend on the amplitude of the input. Another way of quantifying nonlinearity is via the so-called harmonic distortion index, which estimates the amount of both harmonic distortion and cross talk in the output signal resulting from system nonlinearities (42). For a broadband input, the coefficient of harmonic distortion $K_d$ is defined as (54):

$$K_d = \frac{P_{NI}}{P_{TOT}} \times 100 \% \quad \text{(Eq. 5)}$$

where $P_{TOT}$ is the total power in the output and $P_{NI}$ is the output power due to system nonlinearities only, i.e., the power at non-input frequencies. Since only nonlinearities and noise can produce output energy at non-input frequencies, the values of $K_d$ were also corrected for nonzero energy at non-input frequencies (54). The advantage of using $K_d$ is that it can be calculated from a single impedance measurement, whereas the traditional method of characterizing nonlinearity requires the measurement of the moduli at several
distinct amplitudes. The $K_d$ in a linear system is zero. In a nonlinear system driven by sinusoidals, the $K_d$ measures the distortion and crosstalk due to system nonlinearities.

**Measurements of Mechanical Failure of Lung Tissues**

To assess the strength of the alveolar wall in the parenchyma, failure test of lung tissue strips were carried out. Following the measurements of respiratory function, an additional dose of pentobarbital (70 mg/kg) was injected intraperitoneally to each animal ($n = 6$ in each group), the thorax was opened, and the animals were exanguinated by severing the inferior vena cava. The heart, lungs, and trachea were carefully resected en bloc and rinsed in phosphate-buffered saline (PBS) (Sigma). The experimental set up was described previously (53). Parenchymal tissue strips having dimensions of 2.0 - 3.0 mm x 0.7 - 1.0 mm x 0.7 - 1.0 mm in length, width and thickness, respectively, were carefully prepared from each lung and the pleura were removed with the use of a razor. Each end of the tissue strip was fixed by cyanoacrylate glue to small metal plates attached to straight steel wires. The assembly was placed in a horizontal tissue bath filled with PBS at room temperature, with one wire attached to a computer-controlled lever arm containing a force transducer (Model 300B, Aurora, ON, Canada). We followed the method reported by Tanaka and Ludwig (47). Briefly, each strip was stretched at a rate of 0.2 mm/sec until the sample separated into two pieces. Due to the limitation of the maximum displacement of the level arm, the strips were first stretched to approximately 3 times their unstretched length before the recording of the displacement and force signals was started. The strain was defined as the total displacement in length normalized by the unstretched length of the samples. A transient decrease in the force (see arrows in Fig. 8) was defined as “the failure stress”, indicating that fibers in the alveolar walls started to break during
stretching. Two or three strips were prepared and measured from each animal, and the average value of the failure stress was used for statistical analysis.

**Bronchoalveolar Lavage**

Whole lung lavages were performed with 1 ml of PBS twice via the tracheal cannula following the impedance and P-V curve measurements (n = 5 in each group). The return volume was measured, and the bronchoalveolar lavaged fluid (BALF) was centrifuged. The cell pellet was re-suspended in red blood cell lysis buffer (0.01% NH₄Cl) and brought up to the initial lavaged return volume for total cell count by hemocytometry. Slides containing 500 - 1000 cells were prepared using a cytocentrifuge, and stained with rapid Wright’s stain. Differential cell analysis was performed by manual counting under a light microscope.

**Lung Histology and Morphometry**

After mediastinal dissection, the lungs were perfused with 10% buffered formalin via the tracheal cannula at an airway pressure of 25 cmH₂O for at least 20 min (n = 5 in each group). The fixed lungs were embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological analysis. We calculated the average distance between alveolar walls, the mean linear intercept (MLI), according to established methods (10) using a light microscope. For each pair of lungs, 10 histological fields were evaluated.

**BALF Elastase-like Activity**

Elastase-like activity in BALF was measured using the protocol by Dhami et al. (9). Briefly, BALF samples were lyophilized and reconstituted in water to make a fivefold
concentrated solution. An assay buffer of 0.2 M Tris · HCl, pH 8.0, was prepared and then, 100 µl of assay buffer, 50 µl of substrate (0.5 mg/ml of N-succinyl-Ala-Ala-Ala-\(p\)-nitroanilide), and 50 µl of BALF were added. All samples were assayed in duplicate. EDTA (10 mM) was added to the samples to inhibit metalloelastase (13). Negative control of 150 µl of assay buffer and 50 µl of substrate were used. We assessed background absorbance of each BALF sample by incubating 150 µl of assay buffer with 50 µl of each sample. This value was then subtracted from the absorbance of the test wells. The absorbance of the wells is measured at 405-nm wavelength using a spectro-photometer.

**Whole Lung Collagen Content**

Collagen content was assessed by measuring the hydroxyproline content of the tissue as previously described by Woessner (51). Lungs from the control (n = 7) and the PPE-treated (n = 7) mice were lyophilized for 12 h to dry weight, measured, minced. The lung sample was then hydrolyzed with 4 ml of 6 N HCl at 100°C for 6 h. One ml of the hydrolysate was then taken and evaporated. The powder was reconstituted with 1 ml of distilled H\(_2\)O (dH\(_2\)O) and re-evaporated. The powder was then reconstituted with 5 ml of dH\(_2\)O. Hydroxyproline (Sigma) standard solutions of 0 - 10 µg/ml were prepared. Sample solution (2 ml) was taken and oxidized with 1 ml of Chloramine-T (Sigma) for 20 min. The reaction was then stopped with 1 ml of 3.15 M perchloric acid. After 5 min, 1 ml of \(p\)-dimethylaminobenzaldehyde solution was added. The sample was vortexed, incubated in a 60°C bath, and then cooled under tap water for 5 min. The absorbency of the solutions was determined at 557 nm using a spectro-photometer. The hydroxyproline concentration was determined from the standard curve.
Whole Lung Elastin Levels

The lyophilized was placed into 1 ml of 0.25 M oxalic acid. The suspension was then heated at 100°C for 1 h. The specimen was centrifuged, and the supernatant was collected. The above procedure was repeated for a total of five times until all the insoluble elastin had been converted into a soluble product (α-elastin) (7). One ml of the collected supernatants for each mouse was then dialyzed against water using 15,000 molecular-weight cutoff dialysis membrane (Spectrum, Huston, TX). The levels of α-elastin were then determined using Fastin-elastin assay (Biocolor, Belfast, N. Ireland) following the specific protocol outlined in the kit.

Statistical Analysis

All data were expressed as means ± standard deviation (SD). Student's t-test and repeated-measure two-way analysis of variance were used to evaluate the significance of differences between means and variances, with P < 0.05 as the level of significance.

RESULTS

Histopathology

Fig. 1A and B shows representative alveolar structures of a control and a PPE-treated lung stained with hematoxylin and eosin three weeks following initial treatment. Significant enlargement of the alveolar air spaces was observed in lung samples from PPE-treated mice compared to control. Both the mean and the SD of the MLI in the PPE-treated group (83 ± 21 μm) were significantly larger than in the control group (43 ± 3 μm)(P = 0.001). There were no significant differences in body weight between PPE-treated (24.9 ± 0.9 g) and control groups (25.2 ± 1.1 g).
**BALF Analysis**

There were no significant differences in BALF total cell numbers between PPE-treated (3.4 ± 1.0 x 10⁴ /ml) and control groups (3.8 ± 0.9 x 10⁴ /ml) and over 95% of the cells were macrophages in both groups. No eosinophils and only 0.5% neutrophils were observed in both groups. Additionally, no elastase-like activity was detected in BALF of the control and PPE-treated mice, suggesting that direct elastolytic activity of PPE had already diminished at three weeks following treatment as reported previously (40).

**Pressure-volume Curves and Dynamic Respiratory Mechanics**

Fig. 2 compares the quasi-static P-V curves in the two groups obtained during volume-controlled inflation from end-expiratory volume. The pressure was significantly lower in the PPE-treated mice than in the control (P < 0.001). The average quasi-static elastance value, defined as a slope between 0 and 1.15 ml of inflated volume, was significantly lower in the PPE-treated mice (18.8 ± 2.8 cmH₂O/ml) than in the control (26.5 ± 1.7 cmH₂O/ml) (P < 0.001).

Fig. 3 shows representative cases of the dynamic respiratory system resistance and elastance (calculated from the reactance where elastance = -2πf * reactance) as a function of frequency, and the fits of the mathematical model (20) to the data in representative control and PPE-treated mice at PEEP = 3 cmH₂O. The measured data were fit well by the model at all PEEP levels in both groups. As a function of PEEP, all the values of tissue elastance (H) parameters, H_{min}, H_{max} and H_{mean}, were significantly PEEP-dependent (P < 0.001) and decreased with increasing PEEP in both groups (Fig. 4). The values of H_{min}, H_{max}, and H_{mean} were significantly lower in the PPE-treated group than in the control (P < 0.001)(Fig. 4A and B). The largest difference in H_{max} between the
control and the PPE-treated mice was at the highest (9 cmH\textsubscript{2}O) PEEP. Hysteresivity $\eta$ was significantly PEEP-dependent in the PPE-treated mice ($P < 0.001$) but not in the control, and was significantly higher in the PPE-treated mice at PEEP $\geq 6$ cmH\textsubscript{2}O (Fig. 5A). Airway resistance $R_{\text{aw}}$ was significantly PEEP-dependent ($P < 0.001$), but there was no significant difference between the groups ($P = 0.78$)(Fig. 5B). The tissue damping ($G$) was significantly PEEP-dependent ($P < 0.001$) and significantly lower in the PPE-treated mice ($P < 0.001$) (Fig. 5C).

**Dynamic Nonlinearity**

The harmonic distortion $K_d$, a measure of dynamic nonlinearity of the respiratory system, was significantly PEEP-dependent ($P < 0.001$) and decreased implying more linear behavior when PEEP was increased in both groups (Fig. 6). The $K_d$ was significantly larger in the PPE-treated group than in the control ($P < 0.001$). Figure 7 shows the linear relationships between $H_{\text{min}}$ or $H_{\text{max}}$ and $K_d$ including data from all PEEP levels. There were significant correlations between $H_{\text{min}}$ and $K_d$ both in the PPE-treated ($K_d = 0.51H_{\text{min}} - 1.56$) ($P < 0.001$, $r = 0.83$) and control groups ($K_d = 0.36H_{\text{min}} - 2.21$) ($P < 0.001$, $r = 0.90$) (Fig. 7A). The slopes of the two lines were significantly different ($P < 0.05$). There were also significant correlations between $H_{\text{max}}$ and $K_d$ both in the PPE-treated ($K_d = 0.13H_{\text{max}} - 2.44$) ($P < 0.001$, $r = 0.86$) and the control mice ($K_d = 0.14H_{\text{max}} - 0.82$) ($P < 0.001$, $r = 0.86$) (Fig. 7B). The two lines were nearly parallel and there was no significant difference in slopes between the two lines ($P = 0.48$) but the intercept of the PPE-treated group was relatively higher which nearly reached a statistical level ($P = 0.07$). There were also significant correlations between $H_{\text{mean}}$ and $K_d$ both in the PPE-treated ($K_d = 0.29H_{\text{mean}} - 2.04$) ($P < 0.001$, $r = 0.93$) and the control groups ($K_d = 0.23H_{\text{mean}} - 3.00$) ($P < 0.001$, $r = 0.94$), and the slopes of the two lines were significantly different ($P < 0.05$).
Following the PPE treatment, each point moved to the left at an equivalent PEEP, implying stronger nonlinearity per unit tissue elastance in the PPE-treated group.

**Lung Tissue Failure Tests**

Fig. 8 shows representative traces of parenchymal tissue strip failure tests from a PPE-treated and a control mouse. The stress in each tissue strip increased with strain until a maximum was reached. Next, the stress started to drop in discrete steps suggesting that groups of alveolar walls broke within the tissue. Finally, the stress levels returned to zero when the samples separated into two pieces. Table 1 shows the summary of failure test results for all parenchymal tissue strips. The stress that developed just prior to the strips failing, defined as the failure stress, was significantly lower by 40% in the PPE-treated group than in the control (P = 0.002), demonstrating that the PPE-treated parenchymal tissues were weaker than control tissues. There were no significant differences in the strain values at failure (P = 0.56).

**Lung Hydroxyproline and Elastin Levels**

The levels of hydroxyproline and \( \alpha \)-elastin are expressed as µg/mg dry weight of lung tissue (Table 2). There were no significant differences in dry lung weight between the control and the PPE-treated mice (P = 0.81). The levels of hydroxyproline, a measure of total collagen, were significantly higher in the PPE-treated mice by 48% than in the control (P = 0.002). The levels of \( \alpha \)-elastin were lower by 13% in the PPE-treated mice, but the difference was not statistically significant (P = 0.16).

**DISCUSSION**
The primary findings of this study are that following PPE-treatment of mice (1) lung elastance decreased and hysteresivity increased, (2) parenchymal tissue fibers failed at a lower stress than in control, (3) dynamic nonlinearity characterized by harmonic distortion ($K_d$) increased, and (4) total collagen content increased while there was a tendency of loss of elastin of whole lungs in the emphysema group. These physiological observations support the hypothesis that exposure of the lung to PPE leads to a remodeling of the fiber network that significantly alters the mechanical properties and the nonlinear mechanical behavior of the whole organ.

**Collagen Remodeling in Emphysema**

In emphysema, the loss of elastin from the alveolar walls appears to be a major event in clinical pathology (1). The breakdown and the degradation of collagen fibers have also been reported both in human patients (39) and in rodent models of emphysema (9, 35, 52). Additionally, several studies have demonstrated that after the destruction of alveolar walls, remodeling of collagen fibers as a result of an abnormal repair process contributes to the pathogenesis of emphysema (12, 17, 21, 22, 25, 49). Increases in total amount of collagen of the lungs have been reported in human patients (23, 35). In the present mouse model of emphysema, we also observed a 45% statistically significant increase in total collagen content and a 13% decrease in total elastin content of the whole lung (Table 2), suggesting that collagen remodeling within the lung was indirectly triggered following the elastolytic injury. Previous studies have shown that following the onset and initial progression of emphysema due to the proteolytic injury caused by PPE, synthesis of collagen by lung fibroblasts is considered to be upregulated as part of the repair process of the damaged lung (15, 22). However, the repair process does not restore normal structure and function to the lung leading to pathology and altered physiology (24). Since
the extracellular assembly of collagen molecules to fibrils and fibers is sensitive to the composition of the surrounding matrix (22, 24), it is conceivable that the structure and mechanical properties of the newly synthesized collagen differs from those that occur during normal growth. While the details of this abnormal repair process at the molecular level is beyond the scope of the present study, it is important to discuss the physiological consequences.

Alveolar Wall and Fiber Failure in Emphysema

It has long been proposed that mechanical failure of the alveolar walls plays a pivotal role in the progression of emphysema (50). Recently, Kononov et al. (21) observed the failure of a single alveolar wall in a rat model of PPE-induced emphysema. However, to our knowledge, this is the first study to quantify the failure stress of parenchymal tissue strips from emphysematous lungs. We found evidence that the emphysematous lung tissue breaks at the same strain, but at a stress 40% smaller than the normal tissue (Table 1). In an attempt to interpret these results, we first note that normal collagen fibers are stiffer and stronger than other connective tissue constituents (36). As a consequence, the amount and organization of collagen in the alveolar walls should play a crucial role in determining the stiffness and the failure properties of the lung tissue. For example, when the collagen content of normal lung tissue strips was decreased via \textit{in vitro} digestion using collagenase, the stiffness of the tissue dropped to 40% of its value before digestion (53). Alternatively, normal developmental changes of the lung increase both the stiffness and the failure strength of the alveolar walls as well as collagen contents during maturation. Indeed, Tanaka and Ludwig (47) reported that the failure stress of normal lung tissue from baby rats was 18 kPa which increased to 28 kPa in adult rats. At the same time, in a follow-up study, Tanaka et al. (46) also found that the collagen content of the
lung increased from 18% to about 38% during this normal maturation process. Thus, since in this model of emphysema we found a 45% increase in lung collagen (Table 2), one would expect the stiffness and the failure stress of the tissue to increase. Surprisingly however, our data showed just the opposite behavior: despite the increase in collagen content, both lung elastance and the failure stress decreased by about 30% (Figs. 2 and 4) and 40% (Table 1), respectively, indicating that the total amount of collagen in the lung tissue is not the primary determinant of the mechanical properties in the diseased state.

In an attempt to resolve the apparent contradiction between increased collagen content and decreased elastance and failure stress in the emphysematous tissue, we first discuss the relation of lung stiffness and alveolar structure. Since the MLI nearly doubled in the emphysematous group, the number of alveolar walls per unit volume that can resist the deformation of the tissue strip could have decreased. This mechanism alone could account for the lower elastance and lower failure stress even if the mechanical properties of the alveolar walls were similar to those of the normal tissue. However, it is likely that not all of the increase in MLI is due to alveolar wall rupture. The PPE treated lung is softer and at the fixation pressure of 25 cmH₂O the alveoli would be more extended than those in the normal lung. Thus, the number of alveolar walls per unit volume in the tissue strip is not necessarily smaller in the PPE treated lung than in the normal. Using microscopic imaging, Brewer et al. (3) recently reported that individual alveolar walls from PPE-treated rats, which also involved collagen remodeling (21), appeared softer and more extensible than those from normal rats. These observations suggest that despite the increased collagen content, the alveolar walls and the collagen fibers are likely to be weaker in the emphysematous lung as a consequence of the process of degrading and remodeling. Indeed, the ultrastructure of collagen from human emphysematous lungs reveals thickened and disorganized fibrils after remodeling (12). Our data then suggest
that the stiffness and the failure properties of the remodeled fibers must decrease compared to normal collagen fibers.

The reduction in the failure stress of collagen has an important effect on how the structure of the lung evolves during the progression of emphysema. Suki et al. (44) recently developed a fiber network model and argued that since mechanical forces influence the process of tissue breakdown, the alveolar structure must be very heterogeneous and the alveolar walls around the perimeter of severe emphysema lesions or the walls that separate such lesions may be overstretched. In agreement with these predictions, the heterogeneity of the alveolar dimensions was found to be much larger in the emphysematous than in the control lungs, both in the current study and in previous studies (10, 20, 34). Therefore, the alveolar walls in the emphysematous lung may have to oppose larger stresses locally, and as a result, the increased local stresses can promote rupture of the remodeled walls (44), which in turn results in a decreased failure stress observed in Table 1. We thus conclude that mechanical forces are expected to play an important role in the progression of emphysema once the collagen matrix has undergone a critical amount of remodeling.

**Lung Mechanical Properties**

The consequences of alterations in the ECM of alveolar walls can be traced to organ level changes in the mechanical properties of the lung. Since the chest wall is very soft in the mouse, at least 90% of H is due to the lung parenchyma (37). Additionally, any change in H must be related to a change in lung mechanics and hence in the discussion that follows we assume that changes in H largely reflect changes in lung mechanics. We have recently developed a new mathematical model which assumes a continuous distribution of H between a minimum and a maximum value (H_{\text{min}} and H_{\text{max}}, respectively) (20). We found
that all H-related parameters (Fig. 4) as well as the static elastance (Fig. 2) decreased in the emphysematous mice compared to controls. The $H_{\text{max}}$ represents the stiffest regional elastance in the lung and the collagen should be the most important determinant of its value. Thus, the lower $H_{\text{max}}$ values in the PPE-treated mice suggest that the ultrastructural changes of remodeled collagen weakens the fibers and the alveolar walls in agreement with the analysis of the failure tests. On the other hand, we speculate that $H_{\text{min}}$ represents the softest regional elastance, which may be related to the loss of alveolar walls in that region. Thus, it is likely that the lower value of $H_{\text{min}}$ is a functional consequence of the increased MLI in the emphysematous mice.

The hysteresivity is a material property of the lung tissue (14) and it also depends on the microscopic constituents of the alveolar walls. Indeed, changes in ECM composition can cause a change in the hysteresivity in the parenchymal tissue level (33, 53). In the present study, hysteresivity of the PPE-treated mice was higher than that of the control mice, as observed in TGF-α transgenic emphysematous mice (32), in mild emphysematous mice induced by nebulized PPE-treatment (20) as well as in rats (3). In parenchymal tissues of normal guinea pigs, hysteresivity after in vitro digestion with collagenase was significantly higher than that after digestion with elastase (53). This suggests that in the normal lung tissue, the larger the elastin-to-collagen ratio the larger the value of hysteresivity. However, compared to controls, hysteresivity of the emphysematous mice increased (Fig. 5A) while collagen content also increased (Table 2). Taken together, the hysteresivity and elastance results suggest that remodeling in emphysema produces weak and viscous alveolar walls that also fail at lower stresses than those of the normal lung.

The values of $R_{\text{aw}}$ decreased with increasing PEEP most likely due to the increasing diameters of the airways with lung inflation in both groups (Fig. 5B).
Although increased $R_{aw}$ values, which suggest an underlying airway obstruction, were reported in sheep with experimental emphysema following papain treatment (19), $R_{aw}$ values were the same between the groups, perhaps because the effects of PPE treatment on the airways used in this murine model is distinct from that of papain that was used in the ovine model. To our knowledge, increases in $R_{aw}$ have not been reported in mouse models of emphysema yet. In another emphysema model of surfactant protein D deficient mice, $R_{aw}$ became lower than in control (5). Thus, the physiological feature of the present model is similar to the classical physiological alterations in patients with $\alpha_1$-antitrypsin deficiency (2), in which airway conductance was within normal limits, and the primary physiologic defect was a loss of elastic recoil.

Since surfactant plays an important role in the mechanical properties of the normal lung, it is conceivable that alterations in surfactant properties in the emphysematous lung could influence our results. Because fewer type II pneumocytes that secrete surfactant were observed in the lungs of human emphysema patients (31), it has been suggested that surfactant plays a protective role against the development of pulmonary emphysema. In PPE-treated mice, administration of surfactant prevents the development of emphysema (30). However, it is not known whether the composition and biophysical properties of lung surfactant change as a consequence of the development of emphysema. While the expression of surfactant protein A messenger RNA is increased in the lungs of the klotho mouse model of emphysema (41), this protein plays a less important role in stabilizing the alveoli than the hydrophobic surfactant proteins (18). One could argue that due to the reduction in lung tissue recoil, surface tension may become even more important in emphysema than in the normal lung. However, emphysema is associated with pronounced heterogeneity at the alveolar level, and it is unclear whether abnormalities in surfactant actually contribute to the development of the
disease. The extent to which lung surfactant contributes to recoil in emphysema has also not been well characterized. Further studies would be needed to clarify the role of surfactant in emphysema.

**Dynamic Nonlinearities**

Another important physiological finding of this study is that the tissues responsible for generating elastic recoil in the emphysematous lungs also displayed significantly greater nonlinear behavior than control lungs. The mechanical behavior of the normal lung tissue has been characterized as nonlinear (28, 29, 42, 53), and the origin of dynamic nonlinearity has been investigated in various organs (11). In the respiratory system, dynamic nonlinearity is likely related to the ECM components, including the nonlinearily viscoelastic collagen and its interactions with the linear viscoelastic elastin, and the viscous ground substance including mainly proteoglycans (29). Since elastic fibers behave more linearly than collagen fibers (28, 36), tissue nonlinearity could be more related to collagen and in particular, the extent to which collagen fibers are stretched in the alveolar walls. Thus, the dynamic nonlinear behavior of the lung tissue can be considered as a global *in vivo* assay of collagen function in the intact lung. While the nonlinearity is certainly related to collagen, it is also possible that the physical interaction between collagen and elastin also influences nonlinear behavior.

In the present study, we demonstrated for the first time that dynamic nonlinearity of the lung, as characterized by the harmonic distortion index $K_d$ (42), is linearly related to lung elastance parameters ($H_{\text{min}}$ and $H_{\text{max}}$) both in the PPE-treated and the control mice (Fig. 7), as found for normal tissue strips (53). More importantly, despite a decrease in $H$, the $K_d$ increased in emphysema compared to normal lungs. Specifically, the relationship between $K_d$ and $H_{\text{min}}$ as well as $H_{\text{max}}$ shifted to the left in emphysema. This can be
accounted for by changes in ECM components. As discussed above, a decrease in elastance and an increase in collagen content suggest that the new collagen in the remodeled alveolar walls must be less stiff than the normal collagen. An increase in $K_d$ on the other hand, suggests that the collagen fibers are either more stretched or inherently different from normal with respect to their nonlinear mechanical behavior in the emphysematous alveolar wall.

One may argue that in contrast to tissue strips, in the whole lung airway closure also contributes to harmonic distortion. If a significant portion of the lung is blocked by airway closure and tidal volume remains the same, then a smaller lung will receive the same tidal volume and the lung becomes overstretched. In fact, the $K_d$ was largest at PEEP = 0 in both groups (Fig. 6), which is the condition where recruitment and derecruitment could occur most during oscillations. Furthermore, the decrease in $K_d$ with PEEP implies gradual recruitment. However, neither lung elastance nor $K_d$ decreased when PEEP was increased from 6 to 9 cmH$_2$O. Thus, these data suggest that above 6 cmH$_2$O PEEP, recruitment did not occur and hence it could not influence our data. Therefore, we believe that the relation between $K_d$ and H and consequently the above interpretation of the results, is insensitive to airway closure at least at the higher PEEPs included in this study.

In summary, we have characterized the respiratory and lung mechanical properties of a mouse model of emphysema induced by PPE. We observed a decrease in lung elastance and failure strength of the alveolar walls as well as an increase in hysteresivity, dynamic nonlinearity and total lung collagen content. These results suggest that significant collagen remodeling takes place within the alveolar wall, which produces weak but more nonlinear fibers and alveoli that are locally overstretched and hence prone
to mechanical failure. These alterations in the micromechanics of the alveolar walls significantly affect organ level lung function in the mouse.

ACKNOWLEDGEMENT

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REFERENCES


FIGURE LEGENDS

Fig. 1.
Examples of the alveolar structures of lung tissues from the control (A) and the porcine pancreatic elastase (PPE)-treated (B) lungs. Photographs were taken at an original magnification of x 50 from slides stained with hematoxylin and eosin.

Fig. 2.
Mean ± SD of quasi-static inspiratory pressure-volume relationships from end-expiratory lung volume of the control and the PPE-treated mice. Values are means ± SD. * P < 0.05.

Fig. 3.
Representative examples of respiratory system resistance (A) and elastance (B) of the control and the PPE-treated mice calculated from the impedance data and corresponding fits of the model to the control and PPE-treated data at a positive end-expiratory pressure (PEEP) of 3 cmH2O.

Fig. 4.
Dynamic tissue elastance (H) parameters as a function of PEEP. Means ± SD of minimum (Hmin), maximum (Hmax) (A), and mean H (Hmean; B) in the control and PPE-treated mice. * P < 0.05.

Fig. 5.
Means ± SD of hysteresivity (A), airway resistance (Raw) (B), and tissue damping (G)(C) in the control and PPE-treated mice as a function of PEEP. * P < 0.05.
Fig. 6.
Harmonic distortion, an index of dynamic nonlinearity of the respiratory system, of the control and the PPE-treated mice as a function of PEEP. * $P < 0.05$.

Fig. 7.
Correlations between $H_{\text{min}}$ (A) or $H_{\text{max}}$ (B) and harmonic distortion. Data were obtained from the control ($n = 8$) and the PPE-treated ($n = 9$) mice at PEEP = 0, 3, 6, and 9 cmH$_2$O.

Fig. 8.
Representative examples of parenchymal tissue failure tests of the control and the PPE-treated mice. Notice that the strain axis starts at 2.0. Arrows indicate failure points.
Table 1. *Tissue fiber rupture test*

<table>
<thead>
<tr>
<th></th>
<th>Failure stress (kPa)</th>
<th>Strain</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>11.9 ± 1.4</td>
<td>3.33 ± 0.49</td>
</tr>
<tr>
<td>PPE-treated</td>
<td>7.2 ± 2.4</td>
<td>3.59 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>P = 0.002</td>
<td>P = 0.56</td>
</tr>
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</table>

Data are expressed as means ± SD (n = 6 in each group). PPE; porcine pancreatic elastase.
Table 2. *Lung collagen and elastin contents*

<table>
<thead>
<tr>
<th>Dried lung weight (mg)</th>
<th>Hydroxyproline (µg/mg)</th>
<th>α-Elastin (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5 ± 3.8</td>
<td>4.38 ± 0.83</td>
</tr>
<tr>
<td>PPE-treated</td>
<td>13.1 ± 2.4</td>
<td>6.38 ± 1.33</td>
</tr>
<tr>
<td><strong>P</strong> = 0.81</td>
<td><strong>P</strong> = 0.002</td>
<td><strong>P</strong> = 0.16</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. Hydroxyproline and α-elastin contents are expressed as µg/mg of dried lung weight (n = 7 in each group).
Fig. 1A and B

A

B
Fig. 2

![Graph showing the relationship between volume (ml) and pressure (cmH₂O) for Control and PPE-treated conditions. Control data is represented by solid circles, while PPE-treated data is represented by open circles. The graph includes error bars for each data point.](image-url)
Fig. 3A and B

A

Resistance (cmH$_2$O$^\times$s/ml)

Control data
Control model
PPE-treated data
PPE-treated model

Frequency (Hz)

B

Elastance (cmH$_2$O/ml)

0
10
20
30
40

Frequency (Hz)
Fig. 4A and B

A

H_{min} and H_{max} (cmH_2O/ml)

---

H_{max} (Control)

H_{max} (PPE-treated)

H_{min} (Control)

H_{min} (PPE-treated)

B

H_{mean} (cmH_2O/ml)

---

Control

PPE-treated

*
Fig. 5A and B

A

PEEP (cmH\textsubscript{2}O)

Hysteresivity

- Control
- PPE-treated

B

Raw (cmH\textsubscript{2}O*s/ml)

- Control
- PPE-treated

$R_{aw}$ (cmH\textsubscript{2}O*s/ml)

PEEP (cmH\textsubscript{2}O)
Fig. 5C

![Graph showing the relationship between G (cmH₂O/ml) and PEEP (cmH₂O) for Control and PPE-treated groups.](image)

- **Control**
- **PPE-treated**

* indicates significant difference.
Fig. 6

![Graph showing PEEP (cmH2O) vs. Kd (%). The graph compares Control and PPE-treated groups. The y-axis represents Kd (%) ranging from 0 to 10, and the x-axis represents PEEP (cmH2O) ranging from 0 to 9. The Control group is represented by filled circles, and the PPE-treated group is represented by open circles. There are significant differences indicated by asterisks (*) at certain PEEP levels.]
Fig. 8

Stress (kPa)

Control

PPE-treated

Strain

10 s