Alveolar Dynamics during Respiration

Are the Pores of Kohn a Pathway to Recruitment?

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The change in alveolar size and number during the full breathing cycle in mammals remains unanswered, yet these descriptors are fundamental for understanding alveolar-based diseases and for improving ventilator management. Genetic and environmental mouse models are used increasingly to evaluate the evolution of disease in the peripheral lung; however, little is known regarding alveolar structure and function in the fresh, intact lung. Therefore, we have developed an optical confocal process to evaluate alveolar dynamics in the fresh intact mouse lung and as an initial experiment, have evaluated mouse alveolar dynamics during a single respiratory cycle immediately after passive lung deflation. We observe that alveoli become smaller and more numerous at the end of inspiration, and propose that this is direct evidence for alveolar recruitment in the mouse lung. The findings reported support a new hypothesis that requires recruitable secondary (daughter) alveoli to inflate via primary (mother) alveoli rather than from a conducting airway.

Keywords: alveolar recruitment; mechanics; mouse lung; confocal microscopy; collateral ventilation

Mechanical changes in alveolar structure during respiration also known as alveolar mechanics have been widely discussed, yet no established unifying hypothesis exists. Much of the uncertainty has been due to the difficulties in documenting alveolar mechanics, given their small size and the large movement of the lung during breathing. Alveolar mechanics have largely been inferred from pulmonary function tests and static histopathology of one form or another, which, although providing a better understanding of whole lung mechanics, are not specific (1). To better understand alveolar-based diseases and for further improvement of ventilator management, direct evidence regarding alveolar mechanics is required.

The process of recruitment and de-recruitment of alveoli has been controversially discussed, with no unifying theory on its mechanism. There are several common hypotheses not restricted to: balloon-like expansion of alveoli, limited expansion of alveoli with major expansion in the airways and ducts, or continual recruitment and de-recruitment of alveoli (2).

Further advancement in techniques for investigation into alveolar mechanics are needed and ideally would occur *in vivo* with the alveoli visible in three dimensions. The technology required to provide the spatial and temporal resolution to perform such a task is still in development.

There have been light-scattering techniques employed by Suzuki and coworkers (3), Miki and colleagues (4) and Butler

CLINICAL RELEVANCE

The research presented provides new insight into alveolar mechanics in fresh intact mouse lungs and has a significant impact on the understanding of alveolar-based diseases and current clinical ventilator management practices.

and coworkers (5, 6) in which the pattern of light backscattered by lung tissue below the pleura was used to calculate the size of respective airspaces in dynamic studies. Although a very elegant technique based on sound optics theory, no visualization is performed, and leaves open questions about the tissue under analysis.

The most recently proposed alternative for imaging sub-pleural alveolar structure is Fourier domain optical coherence tomography (7). In their feasibility study, Popp and colleagues initially evaluated formalin fixed rabbit lung followed by isolated fresh lungs. Subpleural alveolar walls could be identified, although changes in individual alveoli could not be seen with certainty because of limitations in resolution and acquisition speed. No measurements were presented in this study. Also, tissue subtypes cannot be discriminated due to the current technical limitations. Nevertheless, this method has great potential if improved upon and in the future may provide further valuable insight.

There have also been a substantial number of direct dynamic studies imaging the pleural surface aspect of alveoli using two-dimensional *in vivo* video microscopy techniques (8–15). These studies evaluate the alveolar dimensions as seen through the overlying pleural surface with the expected lack of detailed morphology and measurement. These studies have been informative in describing novel behaviors of the alveolar structures, usually in the pig and rat lung. This methodology is limited to a superficial view of the alveoli through a glass plate suction adhered to the pleura, leaving unanswered information regarding the underlying structures.

Recently, through such *in vivo* studies along with focused histologic examination, there is a growing consensus suggesting that alveoli are stable during normal lung tidal breathing, and changes in lung volume are the result of a greater number of open alveoli (16). Smaldone and coworkers observed recruitment of smaller alveoli (17), and in a later study Lum and colleagues, through histologic measurements of gerbil lungs fixed at various volumes, concluded that lung volume change was the result of alveolar recruitment/de-recruitment (18). Escobar and coworkers have also demonstrated that lung volume change is largely due to the number of alveoli, and they further postulated that the hysteresis between the inflation and deflation limb commonly observed in pressure–volume curves was the result of the number of open alveoli (19). Although concepts presented in these studies are gaining acceptance, understanding the underlying mechanisms responsible requires further investigation.

Repeated quantitative visualization at the alveolar level in unfixed breathing lung, without the overlying pleura partially

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obstructing the view, has yet to be accomplished and would be a significant contribution (14). Furthermore, development of techniques for direct bedside monitoring of alveolar dynamics would be a major step forward in clinical ventilator management (20).

MATERIALS AND METHODS

Animal Preparation

Animal experiments performed in this study were approved by the University of Iowa Animal Care Committee. C56BL/6 mice ($n = 5$) (Harlan, Indianapolis, IN) of 5 to 6 weeks in age, weighing 19 to 21 g, were initially sedated using 3 to 5% isoflurane, weighed, and anesthetized with an intraperitoneal injection of 87.5 mg/kg ketamine and 12.5 mg/kg xylazine. After a negative pedal reflex, 50 mg/kg of fluorescein was administered intraperitoneally to provide a reasonable fluorescence tissue signal. Ten minutes was allowed for the uptake of fluorescein throughout the body, and the mice were subsequently killed through an overdose of 150 mg/kg pentobarbital, followed by transection of the abdominal aorta once respiration had ceased. A tracheotomy was performed via a midline incision on the trachea and insertion of a 20-gauge catheter, and the lungs were carefully excised. The intact lungs were placed into a custom-made chamber (Figure 1E) filled with PBS. The lungs were connected via the tracheal catheter to a custom electronic pressure controller. A single active inflation of the lungs through a tracheal pressure to 30 cm H₂O while immersed in PBS was completed to assess for punctures in the tissue. This inflation was maintained for approximately one second, and then passively deflated to 0 cm H₂O pressure until the imaging routine had been initiated. In general this time frame was 20 minutes.

Laser Scanning Confocal Microscope

A Bio-Rad 1024 Radiance system (Bio-Rad, Hercules, CA) using a 488-nm Krypton-Argon laser was used for imaging in this study. Automated z sections were obtained on a $1,024 \times 1,024$ grid, with a pixel size of 1.2 μm and section thickness of 5 μm using a $\times 10$ Plan Fluor objective.

Ex Vivo Lung Chamber

A custom chamber was developed for housing the lungs during image acquisition (Figure 1E). The chamber contains an area for a mouse

lung to bathe in PBS. The tracheal catheter is then connected to a tube that passes through the chamber parallel to the image plane, and a 45×50 -mm coverslip is mounted over the upper void of the chamber to water and air seal the chamber. The chamber provides stability during imaging, prevents the lung dehydrating when imaging under the laser scanning confocal microscope (LSCM), and reduces the effects of fluorescent quenching due to pH changes. The chamber is watertight and allows accurate tracking of the change in lung volume as it is inflated.

Pressure Controller

The catheter is connected to a custom electronic pressure controller, which maintains a user-defined pressure level within the lung. This system is made up of a piezo electric double pneumatic solenoid, controlled using a proportional integrative derivative (PID) microcomputer, which can be set to any pressure between 0 and 50 cm H₂O. This system allows highly accurate and constant (iso) pressures with a tight tolerance to be fed and maintained through the entire imaging period. The microcomputer controller enables the accurate repeatability needed between studies. An Omega HHP680 electronic monometer (Omega Engineering, Stamford, CT) is also connected between the tracheal tube and the controller to monitor the inline pressure.

Imaging Routine

Lungs were inflated to the desired pressure levels sequentially (0, 5, 10, 15, 20, 25, 30, and 35 cm H₂O) and a 30- μm z series was acquired, followed by a deflation and acquisition in the reverse stepwise manner. A schematic of the LSCM *ex vivo* mouse lung imaging setup is shown in Figure 1. Figure 2 represents an example of LSCM cross-sectional images acquired for a complete inflation–deflation sequence.

Morphometric Analysis

In this study we calculate mean chord length (MCL) of the alveolar airspace and walls, along with alveolar airspace number using an automated custom application developed using Matlab (Mathworks, Natick MA). The location of the beginning (blue cross) and end (red cross) of each wall intercept is displayed and logged, to compile data on the airspace and wall chord metrics as shown in Figure 3. Figure 4 represents the results for five mice using the analysis techniques described here. The Mean Chord Length of the airspace (MCLa) and wall (MCLw) is calculated using the following formulas:

Figure 1. *Ex vivo* mouse lung imaging schematic, consists of a custom iso-pressure system for inflating the lung to the desired pressure, a commercial Bio-Rad laser scanning confocal microscope, and a custom *in vitro* air- and watertight lung imaging chamber.

Figure 2. Confocal images of the same mouse lung throughout an inflation/deflation cycle. *Panels a through h* represent 5- μ m-thick laser scanning confocal microscopy sections from the same mouse lung inflated through pressures 0 to 35 cm H₂O in 5 cm H₂O increments, respectively; *j through o* show results for deflation. All images were acquired using a $\times 10$ objective and field of view of 1.2×1.2 mm.

$$MCLa = \frac{\sum_{i=1}^n CLairS_i}{n}, CLairS = \{s \in CLair | s > 6 \wedge s < 120\} \quad (1)$$

$$MCLw = \frac{\sum_{i=1}^n CLwallS_i}{n}, CLwallS = \{s \in CLwall | s > 2.5 \wedge s < 35\} \quad (2),$$

where *CLair* and *CLwall* represent the chord length for the airspace and walls, respectively, and *CLairS* and *CLwallS* represent their subsets, which fall within a specified range. Airspace chord lengths less than 6 μ m and greater than 120 μ m, and wall chord lengths less than 2.5 μ m and greater than 35 μ m, were excluded from the mean calculation, as depicted by the limits imposed on the subsets in equation 1 and 2. The lower limit of 6 μ m for the airspace was chosen to reduce the influence of macrophages within the airspace and red blood cells between the walls, as cross-sections of either result in dark patches similar to airspaces. The upper limit of 120 μ m was chosen empirically where very few chord lengths surpassed this value. The lower limit of 2.5 μ m for the wall chord lengths was chosen to remove the effects of spurious noise, and the upper limit of 35 μ m was chosen again based on empirical data. Figures 5c and 6c represent the histogram of the raw airspace chord lengths for both the inflation and deflation series, respectively. The *red line* indicates the median value for each distribution, while γ_1 and γ_2 represent the skew and kurtosis values, respectively.

The airspace size and number are calculated using a series of image-processing steps. An illumination correction based on equalizing the local mean and variance is initially applied to each confocal image. A threshold is then applied based on an optimal iterative separation algorithm (21). The binary image is then labeled based on an 8-connected object model resulting in the segmentation of the airspace regions. Regions less than 70 μ m² were considered as noise, since they representing circular objects smaller than 10 μ m in diameter, and were therefore removed. Finally, areas touching the border were also excluded. In Figures 5b and Figure 6b, each airspace area has been identified and color-coded; *blue* represents the largest ($\sim 4,000$ μ m²) and *red* represents the smallest (~ 400 μ m²) airspace areas.

Catheter-Based Confocal Microscopy

A catheter-based confocal microscope (CBCM) system (Mauna Kea Technologies, Paris, France) has also been used for analysis of the alveolar structure and function in mice. The system uses a fiberoptic imaging bundle, a high-speed laser-scanning unit, and high-sensitivity photon detection system. The distal end of the fiber bundle is brought into contact with the tissue of interest and an image is acquired through the proximal end using a high-speed raster scan. The system can be used to image lung structure *in vivo* during spontaneous breathing or through a controlled breath-hold routine (22).

RESULTS

LSCM images of freshly excised mouse lungs ($n = 5$) inflated to 0, 5, 10, 15, 20, 25, 30, and 35 cm H₂O pressures and then deflated in the reverse stepwise order were acquired. Figure 2 illustrates LSCM cross-sections, at 25 μ m depth below the pleural surface, from the same mouse lung through the inflation and deflation sequence. Images at each pressure were used for morphologic analysis of mean alveolar airspace chord length, mean wall chord length, and total number of alveolar airspaces per field of view (FOV). The change in lung volume versus pressure curve (static P/V loop) as shown in Figure 4a indicates a hysteresis between the inflation and deflation limb that does not close, a result of the first breath phenomenon. During inflation, the number of alveolar airspaces in a given FOV versus pressure exhibits a U-shaped curve (Figure 4b), where initially the number of alveolar air spaces in an FOV decreases, stabilizes at approximately 25 cm H₂O, and is followed by an increase in alveolar airspace number as the pressure exceeds 30 cm H₂O. Simultaneously, mean alveolar airspace chord length (Figure 4c) steadily increases during inflation from 5 to 20 cm H₂O, begins to stabilize at 20 to 25 cm H₂O, and as the inflation pressure increases above 25 cm H₂O the alveolar airspace size remarkably begins to decrease. Also, in Figures 5c and 6c the chord length histograms reveal a shift from smaller airspace

Figure 3. Example of automated intercept labeling. Beginning of a wall is represented by a blue cross and end of a wall by a red cross. Logging of intercepts allows accurate calculation of airspace and wall chord lengths.

chord lengths at lower inflation pressures, toward a mix of small to large chord lengths (at least two groups) at mid-inflation pressures, and finally a consolidation and shift toward small to medium-sized chord lengths at the higher pressures. This phenomenon is also evident in the color-coded alveolar areas

in Figures 5b and 6b. The dramatic and counterintuitive demonstration of alveolar airspace size reduction and increase in number at higher inflation pressures is direct evidence of alveolar recruitment.

Catheter-based confocal microscopy (CBCM) images were acquired on C57BL/6 mice expressing green fluorescent protein (GFP) for visualization of the lung parenchyma. Figure 7 represents two examples using this technique through the mouse lung pleura. Here a series of sequential images was acquired over 250 milliseconds. In both examples we can clearly observe the “popping” open and recruitment of an alveolus, as indicated by the red arrows. A video of both sequences has also been posted in the online supplement.

DISCUSSION

The laser scanning confocal technique outlined in this article presents a new opportunity to visualize the lung without fixation artifacts using optical cross-sectioning capabilities. Although the technique is limited by the depth of imaging ($\sim 50\ \mu\text{m}$), due predominantly to the scattering of light at the air–tissue interface, the high-resolution cross-sectional view of the alveoli provides a clearer depiction of alveolar size, shape, and number during respiration. In addition, with vital fluorescent stains cellular events can be imaged in three dimensions and in four dimensions.

To counteract or reduce the compression of the lung surface at the coverslip interface due to buoyancy, the lung has been tethered to the imaging chamber from the side via the trachea. Three-dimensional reconstructions of the subpleural alveoli at 10 cm H₂O and 35 cm H₂O have been made (see Figure E2E in the online supplement) and reveal minimal compression of the alveolar structure at the pleural interface.

The qualitative and quantitative results presented demonstrate in the mouse lung that during an inspiration the mean alveolar size progressively increases, then decreases. Simultaneously, there is a decrease followed by an increase in alveolar airspace number per FOV with a similar trend reflected in the alveolar wall thickness. These results are best explained by

Figure 4. (a) Change in lung volume versus pressure. (b) Alveolar airspace number in field of view (1.44 mm²) versus inflation pressure. (c) Mean chord length of alveolar airspace versus inflation pressure. (d) Mean chord length of alveolar walls. Error bars represent $\pm$ SD for five mice.

Figure 5. (a) Inflation 0 to 35 cm H₂O, with wall intercepts. (b) Inflation 0 to 35 cm H₂O, clustered by color-coded area (μ^2). (c) Inflation 0 to 35 cm H₂O, histogram of airspace chord lengths (μ). γ_1 = Skew, γ_2 = Kurtosis, red line = median value.

recruitment of alveoli during high inflation pressures (similar to results presented by Lum and coworkers [18]), since there is an increase in alveoli number (Figure 4b), and mean alveoli airspace size is decreasing (Figure 4c). Simultaneously, the lung volume more than doubles during the change in inflation from 25 to 35 cm H₂O pressure (Figure 4a). The results are examined

further in Figures 5b and 6b, where the alveoli from the same mouse lung through an inflation and deflation cycle have been color-coded against airspace size. It can clearly be seen that the change in size is not homogenous. From Figure 5c, where the distribution of the airspace chord lengths has been plotted, the skew (γ_1) and median value depicted by the red line is shifting

Figure 6. (a) Deflation 35 to 0 cm H₂O, with wall intercepts. (b) Deflation 35 to 0 cm H₂O, clustered by color-coded area (μ^2). (c) Deflation 35 to 0 cm H₂O, histogram of wall chord lengths (μ). γ_1 = Skew, γ_2 = Kurtosis, red line = median value.

Figure 7. Two examples of alveoli “popping” open in C57BL/6 mouse lungs expressing green fluorescent protein. Each frame was captured over 50 milliseconds. Image was acquired using a catheter-based confocal microscopy technique.

toward a normal distribution ($\gamma_1 \rightarrow 0$). Simultaneously, the kurtosis (γ_2) is shifting from a leptokurtic (small tail) distribution toward a platykurtic (large tail) distribution. This reveals an increase in heterogeneity of the chord lengths through the inflation phase. Also, Figure 5b demonstrates that during the higher inspiratory pressures the alveoli are becoming smaller, again evident from the shift in chord length distribution and the median value toward the left seen in Figure 5c. Detection of smaller alveoli during inflation is in agreement with a previous study by Smaldone and coworkers (17), and the increase in the number of alveoli is also in agreement with studies performed by Escolar and colleagues (19). Our results also show high heterogeneity between alveoli during inflation, and this is in some agreement with a previous study that had proposed three types of alveoli: those that do not change with inflation, those that do change, and those that collapse at the end of inspiration (12). The videos available in the online supplement further confirm this heterogeneity.

At the end of this first inflation–deflation maneuver, the mean alveolar airspace has increased, evident by the color-coded images in Figures 5b and 6b. The distribution of the airspace chord lengths in Figures 5c and 6c also reflects this increase, where the distribution is less skewed at the end of deflation (0 cm H₂O) when compared with the initial inflation image (0 cm H₂O). The lung volume is also greater at the end of the maneuver as evident in Figure 3a, suggesting recruitment. It is quite remarkable that alveoli become smaller and more numerous at pressure gradients greater than 25 cm H₂O, as pressure levels of 15 to 25 cm H₂O should be sufficient for the majority of recruitment to occur. There seems to exist a complex mechanism that recruits large numbers of alveoli at high inflation pressures.

To explain these findings, we propose a mechanism by which there are main or mother alveoli directly connected to the conducting airways and daughter alveoli connected to the mother alveoli, recruited via the pores of Kohn. During inflation up to 25 cm H₂O, as the alveolar size increases and the walls stretch, we propose that the diameter of the pores of Kohn also increases leading to a thinning of the surfactant layer that has been shown to normally cover the pores of Kohn (23). When the pressure gradient between the mother and daughter alveoli becomes greater than that which the thinning surfactant layer over the pores of Kohn can withstand, air passes into the daughter alveoli, which are then recruited. Until this set of conditions is met, the pathways to recruiting the daughter alveoli are closed. The “popping” open of daughter alveoli has been captured in GFP-expressing mice lungs and is illustrated in two examples in Figures 7a and 7b. The original video capturing this phenomenon is also available on the online supplement. We propose that during deflation the pressure reduces in the mother and daughter alveoli simultaneously until the pores of Kohn reduce in diameter

and the surfactant layer reforms its seal, trapping the remaining air inside the daughter alveoli. This process would then be step-wise or irregular during inflation and smooth or linear during deflation, in agreement with the empirical data obtained here.

Current understanding of lung anatomy indicate ventilatory units within an acinus consisting of the alveolar duct and subtended alveoli (24). However, stacking these structures into an organ against a boundary such as the pleura means that these structures cannot occupy all of the space. Therefore alveoli that are air filled directly from other alveoli could fill this space.

Subsequent breaths would then behave differently from a breath taken from passive deflation, and this needs further investigation. If all alveoli were connected to a branching airway, a longstanding hypothesis arrived at from anatomical studies, they should all inflate uniformly unless their compliances were some how different or unless they had a mechanism for closure of their mouths to prevent airflow. However, as seen in this study and those reviewed by Gatto and Fluck (16), the alveoli are not isotropically expanding; there is a complex process combining expansion and contraction of alveoli, recruitment and de-recruitment, and static alveoli throughout the respiratory cycle.

Surfactant integrity would clearly play a pivotal role in this mechanism for recruitment/de-recruitment of alveoli, consistent with previous studies in which instability of alveoli was linked to the integrity of the surfactant (10, 12, 13, 15). The results of a recently presented study indicate that alveolar instability is greater with low PEEP (5 cm H₂O) and high tidal volume, while alveolar stability can be achieved with high PEEP (20 cm H₂O) and low tidal volume (8). This observation is also consistent with the mother/daughter hypothesis, where at low PEEP and high tidal volume, the pores of Kohn would be continuously opening and closing during inspiration and expiration, leading to instability as a result of the increase in recruitment/de-recruitment (i.e., increase in mechanical stress). With the high PEEP and low tidal volume, the pores of Kohn would remain open during inspiration and expiration, which would maintain recruitment and decrease the likelihood of de-recruitment, leading to overall stability of the alveoli. The heterogeneity of the recruitment process could also be linked to the previously observed heterogeneous number and regional distribution of the pores of Kohn (25).

The mother/daughter hypothesis also makes sense if we think about the structural benefit of having such daughter alveoli, which are recruited only at high pressures, at a time when the stress on the already inflated alveoli would be extremely high (26). With the recruitment of the daughter alveoli and subsequent reduction in the average size of the mother alveoli as more lung volume is distributed, the tension in all interconnected walls would be reduced. The results presented along with the hypothesis could also lead us to better understand why mice have

an irregular double-humped inflation curve, a longstanding mouse lung physiology question.

The pores of Kohn in the hypothesis would not only facilitate alveolar recruitment but would also be available for collateral ventilation at high lung volumes, such as seen at deep inspiration and in diseases associated with lung overinflation such as pulmonary emphysema.

Current fixation and evaluation of lung morphology through two-dimensional histopathology cannot easily demonstrate mother/daughter alveoli, since the process of fluid-filled fixatives in the airways would result in the expansion of both the mother and daughter alveoli without the need for high pressure gradients, as seen in saline-filled lungs. Although vascular perfusion following labor-intensive serial sectioning at various points along the pressure-volume curve have and can provide greater insight into the three-dimensional mechanics of the alveoli (27), more sophisticated microscopy techniques are still needed to produce further anatomical evidence of the proposed hypothesis.

From this study we can make the following conclusions: from a deflated lung undergoing inflation the alveoli are initially unstable. After one inflation-deflation maneuver both the lung volume and mean alveolar airspace increases. The increase in open/recruited alveoli increases the stability of the lung. From this we hypothesize that subsequent cycles would progressively increase the stability of the alveoli until eventually the majority become stable and exhibit minimal change in size during tidal breathing, as postulated by Mead and coworkers (28) and shown by Nieman and colleagues (8, 10–15, 29–32) and Escolar and coworkers (19).

The mother/daughter alveoli hypothesis, arising from direct observation and measurement of the subpleural alveolar environment in fresh intact mouse lungs, is a reasonable explanation of the empirical data obtained in this study. Although there is no firm proof that anatomically the daughter alveoli exist, there is also equally no firm evidence that they do not exist.

We are currently measuring the effects of further breathing cycles on alveolar mechanics to see what changes occur, as well as the effects of different ventilator settings representing values closer to tidal breathing.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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