Variable stretch pattern enhances surfactant secretion in alveolar type II cells in culture

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Arold SP, Bartolák-Suki E, Suki B. Variable stretch pattern enhances surfactant secretion in alveolar type II cells in culture. Am J Physiol Lung Cell Mol Physiol 296: L574–L581, 2009. First published January 9, 2009; doi:10.1152/ajplung.90454.2008.—Secretion of pulmonary surfactant that maintains low surface tension within the lung is primarily mediated by mechanical stretching of alveolar epithelial type II (AEII) cells. We have shown that guinea pigs ventilated with random variations in frequency and tidal volume had significantly larger pools of surfactant in the lung than animals ventilated in a monotonous manner. Here, we test the hypothesis that variable stretch patterns imparted on the AEII cells results in enhanced surfactant secretion. AEII cells isolated from rat lungs were exposed to equibiaxial strains of 12.5, 25, or 50% change in surface area (∆SA) at 3 cycles/min for 15, 30, or 60 min. 3H-labeled phosphatidylcholine release and cell viability were measured 60 min following the onset of stretch. Whereas secretion increased following 15-min stretch at 50% ∆SA and 30-min stretch at 12.5% ∆SA, 60 min of cyclic stretch diminished surfactant secretion regardless of strain. When cells were stretched using a variable strain profile in which the amplitude of each stretch was randomly pulled from a uniform distribution, surfactant secretion was enhanced both at 25 and 50% mean ∆SA with no additional cell injury. Furthermore, at 50% mean ∆SA, there was an optimum level of variability that maximized secretion implying that mechanotransduction in these cells exhibits a phenomenon similar to stochastic resonance. These results suggest that application of variable stretch may enhance surfactant secretion, possibly reducing the risk of ventilator-induced lung injury. Variable stretch-induced mecanotransduction may also have implications for other areas of mechanobiology.

PULMONARY SURFACANT, A COMPLEX mixture of phospholipids and apoproteins, lines the air-liquid interface throughout the lung, lowering surface tension and contributing to alveolar stability (35, 36). Alveolar epithelial type II (AEII) cells are responsible for the synthesis, secretion, and, to some extent, reuptake and recycling of surfactant within the alveolar cavity (11, 18, 23, 38). Whereas surfactant secretion by AEII cells can be induced by a host of pharmacological agents, the primary stimulus for secretion in vivo appears to be mechanical stretch. Wirtz and Dobbs (38) were the first to demonstrate that mechanical strain induces surfactant secretion in isolated AEII cells by showing that a single stretch resulted in a transient increase in intracellular calcium followed by a sustained elevation in surfactant secretion. Whereas mechanotransduction plays a vital role in surfactant homeostasis, durations of excessive stretch applied to AEII cells in both whole lung preparations (37) and in culture results in significant cellular injury with loss of barrier function (8, 34). Such injury, termed volutrauma, is thought to contribute to the development of ventilator-induced lung injury (VILI) during mechanical ventilation of human subjects suffering from acute respiratory distress syndrome (13, 14). Consequently, numerous studies have sought to optimize ventilation strategy so as to maintain adequate oxygenation while minimizing the deleterious effects of stretch on the epithelium (13, 14, 37).

One ventilation strategy in particular, variable ventilation, has shown significant promise in improving gas exchange while simultaneously reducing lung injury during mechanical ventilation (1, 2, 4, 24, 32). In this ventilation mode, tidal volume (VT) and frequency are randomly varied on a cycle-by-cycle basis as opposed to the continuous delivery of monotonous or varying VT and frequency currently used by conventional ventilation modes. More interestingly, we found that healthy guinea pigs that underwent variable ventilation experienced a near doubling in the amount of pulmonary surfactant present in bronchoalveolar lavage samples compared with animals that received monotonous ventilation (2). We hypothesized that the observed increase in lung surfactant was directly due to the variable stretch patterns imparted by the variations in VT on the AEII cells.

The purpose of this study was to directly test this hypothesis in cell culture. To this end, we cultured primary rat AEII cells on elastic membranes, stretched them with monotonous and variable stretch patterns, and quantified surfactant secretion and uptake as well as cell viability with each stretching modality. We found that variable stretch patterns enhanced surfactant secretion in AEII cells without any increase in the prevalence of injury.

METHODS

Cell culture. AEII cells were isolated from Sprague-Dawley rats using a technique adapted from Dobbs et al. (12) under a protocol approved by the Animal Care and Use Committee of Boston University. Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg body wt ip), the trachea was cannulated, an abdominal aortomy was performed, and the pulmonary artery was perfused to remove blood from the vasculature. The lungs were then excised and lavaged eight times with phosphate-buffered saline supplemented with 50 μg/ml gentamicin and 0.01 g/ml glucose (Sigma, St. Louis, MO). Following lavage, the lungs were instilled with 8 ml of elastase solution (5 U/ml; Sigma) and incubated for 30 min with two additional 8-ml elastase solutions added after 10 and 20 min. Following digestion, the lungs were finely minced in the presence of deoxyribonuclease (Sigma), and the digestion was halted with the addition of 10 ml of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10%
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FBS (Sigma). The sample was then filtered through progressively finer meshes, and the resulting supernatant was incubated for 1 h on plastic Petri dishes precoated with rat IgG (Sigma) for differential adhesion. The nonadherent cells were removed from the dish by panning. During optimization of the culture technique, purity of the isolated cells was verified by immunocytochemistry. About 95% of the cells stained positively for E-cadherin as well as for surfactant protein B (SP-B), confirming the AEII phenotype. The negative staining for von Willebrand factor, desmin, or v-smooth muscle actin indicated that the cultures contained negligible amounts of endothelial cells, smooth muscle cells, or myofibroblasts, respectively. Additionally, the macrophage marker ED1 and the fibroblast markers fibroblast surface protein and prolyl-4-hydroxylase showed no staining, suggesting little to no macrophages or fibroblasts in our culture. Furthermore, the AEII cells are morphologically quite distinct from the above cell types, and visual inspection with a light microscope indicated that indeed a vast majority of these cells were type II epithelial cells due to their small, round, cuboidal shape and the surfactant lamellar bodies that are distinctly visible in both light and fluorescent images.

AEII cells were then resuspended in DMEM supplemented with 10% FBS, 8 mM l-glutamine, 100 U/ml penicillin, and 50 μg/ml gentamicin (Sigma). Cells were seeded at 10^5 cells/cm² on BioFlex elastic membranes (Flexcell International, Hillsborough, NC) precoated with 50 μg/ml human fibronectin (Roche Diagnostics). Cell seeding was limited to a 0.90-cm² area central portion of each membrane using flexiPERM inserts (Sigma). The yield of AEII cells from each rat was in the range of 30 × 10⁶ to 40 × 10⁶ cells with >95% viability per isolation. All cell experiments started 24 h following isolation.

Stretch protocol. Cells were stretched using a device described in detail elsewhere (3). Briefly, six-well BioFlex culture plates were mounted on a sliding platform situated such that each well was located directly above a lubricated indenter post. A computer-controlled linear actuator controlled the vertical displacement of the platform containing the plates, and the downward displacement of each membrane over the indenter post resulted in a radial expansion of the elastic membrane. Thus cells cultured on this surface received an equibiaxial change in surface area (ΔSA). The device fits into a temperature- and CO₂-controlled cell culture incubator allowing the cells to remain in a physiological environment over the course of the experiments.

Stretching cells started after a period of 24 h of incubation following isolation using half-sinusoidal waves at a rate of 3 cycles/min. Cell populations designated to receive monotonic stretching were randomized to receive a constant amplitude (A₀) half-sinusoidal stretching with ΔSA of 12.5, 25, or 50%. These populations were then further randomized (n = 6 wells per group) to one of four groups: 1) unstretched control group, 2) 15-min stretch, 3) 30-min stretch, or 4) 60-min stretch. As shown in Fig. 1A, the appropriate measurement (see below) was taken 60 min following the onset of mechanical stretching. For example, a group of cells stretched for 15 min would be left unstretched for another 45 min for a total of 60 min, whereas in the case of cells stretched for 60 min, the measurements were taken immediately following the completion of stretching.

Cells receiving variable stretch pattern were stretched at three mean ΔSA with A₀ of 12.5, 25, or 50%. A single cycle of δSA had an A = A₀ + η, where variability was introduced through the term η randomly drawn on a cycle-by-cycle basis from a zero-mean uniform distribution between the limits of ± half-width (W) (Fig. 1B). Stretching period was 60 min at a rate inversely proportional to A on a cycle-by-cycle basis while maintaining a mean rate of 3 cycles/min. For each A₀, there were five additional groups (n = 5 wells) characterized by different values of W: 0, ±2.5, ±5, ±10, or ±15% variability. For example, at A₀ = 50% with W = ±15%, each stretch had an equal probability of having any value between 35 and 65% ΔSA. The W = ±15% group was eliminated from the A₀ = 12.5% group because this amount of variability would require stretches below 0.

Figure 1C shows the five different distributions for cells stretched at A₀ = 50% mean ΔSA.

Two additional groups of cells were isolated as described above. One group was seeded on 12 plastic tissue culture dishes (n = 4), and, to validate our phosphatidylcholine (PC) secretion protocol, cells were incubated in media containing 0, 10, or 100 nM TPA (Sigma) (n = 3), a known agonist for surfactant secretion. Following this incubation time, the media and cells were analyzed to determine surfactant secretion. Five other wells (n = 5) were seeded on flexible membranes and washed, and surfactant secretion was determined immediately.
following wash as well as 15, 30, and 60 min after the wash to characterize the time course of unstimulated PC secretion.

**Phospholipid secretion.** Immediately following seeding, \(^3\)H-labeled choline chloride (GE Healthcare-Amersham Biosciences, Piscataway, NJ) was added to the media to a final concentration of 1 \(\mu\)Ci/ml, and cells were incubated for 24 h under standard cell culture conditions (100% humidity, 37°C, and 5% CO\(_2\)). Immediately before stretching, the cells were washed three times, and the media was replaced with serum-free DMEM. Following the 60-min stretching, the media was removed, the cells were scraped, and the lipids from both the media and the cells were isolated according to the Folch partition (28). The percentage of \(^3\)H-labeled PC secreted was obtained as the detections per minute of the media normalized by the total detections per minute of the cells and the media. Surfactant secretion under various conditions was then characterized by the percentage release of PC.

**PC internalization.** Cells were placed in serum-free DMEM containing \(^3\)H-labeled PC (GE Healthcare-Amersham Biosciences) at a concentration of 1 \(\mu\)Ci/ml. The cells were then immediately stretched for the proscribed protocol, and, following the 60-min stretch, the cells were scraped, and the lipids from the cells were isolated according to the Folch partition. The uptake of PC was calculated as the ratio of detections per minute in the stretched cells normalized to the detections per minute in unstretched control cells.

**Cellular injury determination.** Before stretching, the cells were washed three times, and the media was replaced with serum-free DMEM containing 0.23 \(\mu\)M ethidium homodimer-1 and 0.12 \(\mu\)M calcine acetoxymethyl (AM) (LIVE/DEAD; Invitrogen). Ethidium homodimer-1 is nonmembrane-permeable and enters cells through a compromised plasma membrane and fluoresces bright red (529/617 nm) on binding to nucleic acids but is excluded from cells with an intact plasma membrane. Calcine AM is membrane-permeable and well-retained within live cells resulting in a bright green fluorescence (494/517 nm). After stretching, the media was removed, and the cells were examined using an upright fluorescent microscope (Nikon Eclipse 80i) with a \(\times20\) objective, and the images were captured and stored with a digital camera (CoolSNAP HQ; Photometrics, Tucson, AZ). Green and red images were taken from five random locations within each well, and the ratio of the injured cells to total, live, and injured cells was calculated.

**Statistical analysis.** All data are presented as means \(\pm\) SD for each treatment group unless otherwise specified. Each image was treated as a separate sample, and data were analyzed by one-way ANOVA followed by Bonferroni tests (SigmaStat; Jandel Scientific, San Rafael, CA). Differences between groups were considered statistically significant when \(P<0.05\).

**RESULTS**

**Baseline secretion and response to secretagogue.** The baseline secretion rate as a function of time in AEII cells not exposed to mechanical stretch or chemical stimuli is shown in Fig. 2A. At time 0, when the media from the wells was taken immediately following three washings, there was already 0.7% PC secretion, which is likely due to residual \(^3\)H-labeled choline left after the wash and not entirely eliminated during the Folch partition. At 15 min, PC secretion increased quickly to \(\sim 2\%\) and continued to further increase at 30 and 60 min. However, the shape of the percent PC vs. time curve suggests that the secretion rate was slowed at the later time points. The increase in PC secretion was significant at all time points compared with the measurement at time 0. The 3 h of exposure of AEII cells to 10 nM concentration of the surfactant secretagogue TPA resulted in a 50% increase in PC secretion compared with sham-incubated cells (\(P = 0.065\)). When the TPA concentration was raised to 100 nM, PC secretion increased fivefold (\(P < 0.001\)), demonstrating a dose-dependent relationship between TPA and PC secretion (Fig. 2A).

**Monotonous stretching of AEII cells.** The time course and stretch amplitude dependence of PC secretion are summarized in Fig. 3. The total PC secretion in cells exposed to 15 min of stretch at \(A_0\) of 12.5, 25, or 50% \(\Delta S\) are shown in Fig. 3A. At the two lower values of \(A_0\), there was no significant change in PC secretion; however, at \(A_0 = 50\%\), PC secretion increased significantly to \(>6\%\) compared with the unstretched control cells, which demonstrated \(\sim 4\%\) PC secretion. The secretion pattern dramatically changed when cells were stretched at the same amplitudes but for 30 min (Fig. 3B). In this case, at \(A_0 = 12.5\%\), there was a statistically significant increase in secretion with respect to unstretched controls with no change at \(A_0 = 25\%\). Surprisingly, however, at \(A_0 = 50\%\), there was a statistically significant decline in secretion to a value nearly half of the control in contrast to the significant increase in secretion when the same amplitude was applied for only 15 min (Fig. 3A). When AEII cells were stretched for 60 min, no group demonstrated any increase in PC secretion compared with the unstretched cells (Fig. 3C). Furthermore, the groups stretched
at 25 and 50% showed statistically significant declines down to 3.0 and 2.6%, respectively.

The effects of stretch duration and amplitude on $^3$H-labeled PC uptake are summarized in Table 1. Values are normalized to unstretched control cells. Stretch had no significant effect on PC uptake at any time or any level of strain, suggesting that surfactant reuptake by the AEII cells did not play a significant role in how stretching influenced surfactant secretion in our experiments. When the internalization values for stretched cells, regardless of duration or magnitude of stretch, were pooled and compared with unstretched control cells (1.00 ± 0.48%), there was a mild decline in internalization (0.74 ± 0.51%) vs. unstretched controls (1.00 ± 0.48).

at $A_0 = 50\%$ ΔSA at 3 cycles/min (middle) and at 12 cycles/min for 60 min. The cells in the top are those stained green and are living, whereas those stained red (bottom) are dead or contain a compromised plasma membrane. In the unstretched cells, there was essentially a monolayer with very few or no dead or injured cells per image. After 60 min of stretching with $A_0 = 50\%$ at 3 cycles/min, minor disruption of the monolayer could be seen, and there were injured cells that, in general, were located around the periphery of the breaks. Also, there were a greater number of cells that had a round up shape and slightly out of the plane of focus. In the case of 60 min of

Table 1. $^3$H-labeled phosphatidylcholine internalization as a function of stretch amplitude ($A_0$) and duration of stretch

<table>
<thead>
<tr>
<th>$A_0/Duration$</th>
<th>0 Min</th>
<th>15 Min</th>
<th>30 Min</th>
<th>60 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5% 0 Min</td>
<td>0.71 ± 0.51</td>
<td>1.28 ± 0.11</td>
<td>0.87 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>25% 1.00 ± 0.48</td>
<td>0.72 ± 0.50</td>
<td>0.49 ± 0.23</td>
<td>0.72 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>50% 0.88 ± 0.69</td>
<td>0.51 ± 0.17</td>
<td>0.78 ± 0.55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD normalized to unstretched controls. There were no significant changes in phosphatidylcholine (PC) internalization for any $A_0$ or duration. Pooled internalization data for all stretched measurements demonstrated a significant decrease ($P = 0.036$) in internalization (0.74 ± 0.51) vs. unstretched controls (1.00 ± 0.48).

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Representative examples of the LIVE/DEAD assay are shown in Fig. 4A for unstretched (left) cells or cells stretched at 25 and 50% showed statistically significant declines down to 3.0 and 2.6%, respectively.

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stretch with \( A_0 = 50\% \) at 12 cycles/min, the effects were significantly more dramatic. There were obvious breaks in the living monolayer and a number of cells rounded up with a large majority of the cells exhibiting compromised plasma membranes and hence likely dead. This increased stretch rate was used to verify that this assay is capable of reliably determining strain-induced cell death.

The quantified results from the LIVE/DEAD assay are represented in Fig. 4B as the percentage of injured/dead cells for each stretch amplitude as a function of the duration of stretch at 3 cycles/min. The dotted line represents the average percentage of dead cells for the unstretched control samples (1.1 \( \pm \) 0.77\%). Regardless of the duration of stretch, cells stretched at \( A_0 = 12.5\% \) demonstrated no significant deviation from the unstretched cells. At both \( A_0 = 25 \) and 50\% \( \Delta S_A \), there was a statistically significant increase (\( P < 0.05 \)) in the percentage of injured/dead cells to approximately 2.1 and 2.6\%, respectively. There was no difference in injury between cells stretched at 25 and 50\% amplitude. The total cell count for each treatment demonstrated no significant differences, or even trends, between groups indicating that there was little cell detachment and the breaks in the monolayer were likely a consequence of rounding up or covering a smaller surface area.

**Variable stretching of AEII cells.** Figure 5 shows the PC secretion for the cells exposed to mean stretch \( A_0 \) of 12.5 and 25\% \( \Delta S_A \). After 60 min of constant amplitude cyclic stretch at \( A_0 = 12.5\% \), there was an \( \approx 2.5\% \) secretion in these cells. Adding variability (\( W = \pm 2.5, \pm 5, \) or \( \pm 10\% \)) at this mean level of strain had no additional effect on phospholipid secretion. However, at \( A_0 = 25\% \) mean \( \Delta S_A \), adding variability had no effect on PC secretion until the amount of variability reached \( W = \pm 15\% \) of the mean strain at which point secretion nearly doubled from approximately 2\% to 4\%.

Superimposing variability to a mean \( \Delta S_A \) with \( A_0 = 50\% \) is shown in Fig. 6. In cells stretched for 60 min adding \( W = \pm 2.5\% \) variability had no effect compared with monotonous stretch. However, increasing the variability to \( W = \pm 5\% \) suddenly increased PC secretion statistically significantly from \( \approx 1.2\% \) with no variability up to 2.2\%. Further increasing variability to \( W = \pm 10\% \) served to slightly decrease the final PC secretion to \( \approx 2\% \), whereas \( W = \pm 15\% \) caused PC secretion to decrease to \( \approx 1.8\% \), which was no longer significantly different from the \( W = 0 \) case.

To further investigate the dynamic nature of variability-induced PC secretion, an additional experiment was carried out in which another group of cells were stretched using \( A_0 = 50\% \) at each level of variability; however, now the experiment was halted at 30 min followed by an immediate assay of PC secretion. The results were qualitatively similar to those obtained after 60 min of stretch (Fig. 6). The addition of \( W = \pm 2.5\% \) variability resulted in no change in surfactant secretion, whereas increasing variability to \( W = \pm 5.0\% \) somewhat (although not significantly) increased PC secretion. \( W = \pm 10.0\% \) resulted in a further significant increase in secretion (\( P < 0.05 \)), whereas at \( \pm 15\% \) variability there was a slight decline in secretion compared with the peak.

Based on the low cell injury results obtained at \( A_0 = 12.5\% \), injury was not measured during variable stretching with \( A_0 = 12.5\% \). Table 2 summarizes the percentage of injured/dead cells for groups stretched at both \( A_0 = 25\% \) and 50\% as a function of variability. Note that at 0\% variability, the numbers are slightly different from those in Fig. 4B, which is due to the fact that the controls for the variability studies are different from the controls used in the amplitude/duration study. Nevertheless, there were no significant differences in cell injury/death due to the addition of any variability in stretch.

**Table 2. Dead-to-live ratio as a function of mean amplitude \( (A_0) \) of %surfaces area change and %variability half-width \( (W) \)**

<table>
<thead>
<tr>
<th>( A_0/W )</th>
<th>( \pm 0% )</th>
<th>( \pm 2.5% )</th>
<th>( \pm 5% )</th>
<th>( \pm 10% )</th>
<th>( \pm 15% )</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>1.60( \pm )0.45%</td>
<td>1.43( \pm )0.50%</td>
<td>1.81( \pm )0.73%</td>
<td>1.63( \pm )1.12%</td>
<td>1.67( \pm )1.13%</td>
</tr>
<tr>
<td>50%</td>
<td>3.15( \pm )2.35%</td>
<td>2.43( \pm )1.62%</td>
<td>2.57( \pm )1.62%</td>
<td>3.68( \pm )2.82%</td>
<td>2.70( \pm )2.43%</td>
</tr>
</tbody>
</table>

Values are means \( \pm \)SD. There were no significant changes in injured/dead-to-live ratio at any levels of variability for both \( A_0 = 25\% \) and \( A_0 = 50\% \). Dead-to-live ratio was higher for all \( A_0 = 50\% \) groups pooled together vs. the same at \( A_0 = 25\% \) (\( P < 0.05 \)).
DISCUSSION

The primary goal of this study was to test our hypothesis that variable stretch patterns directly applied to AEII cells augment surfactant secretion in these cells. To accomplish this goal, we first characterized the secretory response of AEII cells in terms of cyclic stretching over a range of durations and magnitudes. To this end, we utilized the changes in $^3$H-labeled PC in rat AEII cells in culture as a surrogate of surfactant release or uptake. The main findings of the study are fivefold. 1) Not only do short-term cyclic stretching at high amplitude induce PC release in AEII cells, as previously demonstrated (38), but also moderate duration stretch (15 min) even at low amplitude can result in enhanced surfactant secretion. 2) Extended stretch (60 min), regardless of amplitude, had an inhibitory effect on PC secretion. 3) PC uptake was independent of duration and strain amplitude. 4) At high and medium levels of mean strain amplitude, 50 and 25% ΔSA, respectively, adding moderate levels of variability to strain amplitude on a cycle-by-cycle basis significantly increased PC secretion. 5) Finally, the variability-induced PC secretion was not accompanied by additional cell injury and at high mean strain amplitude, the variability could be “tuned” to optimize surfactant secretion.

Before interpreting the results, we first discuss the limitations of the study.

In some instances where we observed increased PC secretion, Western blot analyses were also conducted for SP-B on media samples to verify that the secreted PC was pulmonary surfactant and not an artifact of cellular injury or membrane origin. The SP-B profiles qualitatively matched the PC patterns (data not shown). These data, in conjunction with the observation that PC secretion was decoupled from cellular injury (Fig. 3) and the percentage of dead cells (Fig. 4), suggest that the changes in PC secretion were almost certainly representative of changes in regulation of surfactant secretion and not a result of cellular injury.

The primary drawback to this study likely lies in the frequencies and stretch magnitudes used. It has been estimated that in rats, an excursion to total lung capacity results in an ∼37% with normal breathing resulting in an ∼12% ΔSA at 60–90 cycles/min (33). First, it is unknown what stretch magnitudes the lung may experience during ventilation in the setting of lung injury. With significant atelectasis and flooding, it is likely that portions of the lung could receive volumes far in excess of what would be seen during normal breathing. Consequently, localized over distention beyond even total lung capacity could easily be experienced, and hence our stretch magnitudes of 25 and 50% do not seem unreasonable. As far as stretch rate compared with breathing frequency is concerned, other studies investigating surfactant release have used a single slow mechanical distention or cyclic stretch with a very low stretch frequency similar to ours (3 cycles/min) (17). We also demonstrated that at higher stretching rates there was significant cell injury and/or death, and hence surfactant secretion under such conditions has little application to a physiological system in vivo. The 3 cycles/min allow us both to study surfactant secretion in viable cell culture and compare our data with that in the literature.

Additionally, our study differed in two more important aspects from what these cells experience in vivo. First, these cells were cultured on fibronectin-coated elastic membranes, whereas a collagen and/or laminin coating would perhaps more likely represent the mechanical and biochemical properties of the basement membrane in the lung. Fibronectin was used primarily because this coating has been widely used in the literature due to the difficulties associated with AEII cells in culture adhering to more relevant substrates. Second, our experiments involved single AEII cell monolcure and neglected the impact of type I epithelial cells. Patel et al. (26) demonstrated that AEII cells cultured in the presence of type I cells exhibited an enhanced response to stretch, in terms of surfactant secretion, compared with AEII cells alone. Although a coculture of both cell types would perhaps better approximate the in vivo conditions, we believe that such a coculture would still have its own limitations, and in this study our aim was to characterize the response of AEII cells with as little additional confounding conditions as possible.

Single and monotonous stretching of AEII cells. Since the work of Wirtz and Dobbs (38), it has been assumed that mechanotransduction of strain plays an integral role in the secretion of surfactant by AEII cells. Indeed, a number of investigators have confirmed and elaborated on this observation. Most of these studies used a single stretch or an extended static stretch in cell culture, and these are not representative of what is occurring in vivo in the lung (18, 29, 30). Our data in Fig. 3 suggest that the dynamics of surfactant secretion in response to cyclic stretch, a more physiological mechanical condition than the single and static stretch, may be more complex than previously thought. To date, the only other investigation that looked at the effects of cyclic strain on surfactant secretion was a study conducted by Edwards et al. (17) whereby they exposed cultured AEII cells to 30 min of strain at 50% ΔSA at 3 cycles/min. Our results demonstrate some similarities and also some striking differences. Edwards et al. (17) reported no detectable change in PC secretion after 30 min of stretch followed by an additional 30-min incubation, whereas we found a significant decline in PC secretion (Fig. 3). When they extended the incubation time out to 90 min, they found a slight but significant increase in PC secretion, and when that time was further extended to 3.5 h, they saw an even more significant increase in PC secretion. Thus, taken together with our data in Fig. 4, it is clear that the dynamics of cyclic stretch-induced surfactant secretion are substantially more complex than following a single stretch reported by Wirtz and Dobbs (38), and this issue warrants further investigation.

The downregulation of PC secretion by stretch may appear contradictory to previous experimental results. However, when considering the AEII cells within an intact lung, the response of these cells to extended periods of high-amplitude strain is less contradictory. In the normal lung, the epithelium probably does not experience 50% strain (33), at least not repetitively. Indeed, intermittent deep inspirations are thought to result in surfactant release (16). The situation is quite different in the diseased lung. For example, in the case of highly heterogeneous lung disease such as adult lung injury, the aerated or nondependent regions of the lung likely undergo excessive ventilation resulting in lung expansion to or beyond total lung capacity (25). This results in repetitive, high-magnitude strains being imparted to the lung tissue and directly experienced by the epithelium. To the extent that data obtained in cell culture can be extrapolated to the lung in vivo, our results suggest that...
extended repetitive stretch at high amplitudes in fact down-regulates surfactant secretion (Fig. 3C).

Regarding the mechanism of downregulation of secretion, we note the following. In individual groups, reuptake was not substantially affected by stretch (Table 1); however, when all stretched groups were pooled and compared with unstretched controls, uptake was significantly lowered. Thus a reduction in uptake would, in fact, increase the apparent PC secretion detected by our method. Another possibility is related to phenotypic changes. Edwards (16) has shown that stretch may promote differentiation of AEII cells to the type I phenotype. However, this is unlikely over the course of 60 min in this study. Furthermore, the amount of cell death did not depend on how long the cells were stretched, and cell injury/death remained relatively low (<3%) even at the highest stretch magnitude (Fig. 4B). This level of cell death is significantly lower than we found when we stretched the cells at a higher frequency and those seen by Tschumperlin and Margulies (34) when they stretched cells in a similar manner. In an elegant study, Gajic and colleagues (19) demonstrated that some cells exposed to injurious deformations as a result of injurious ventilation modes do experience plasma membrane stress failure. They found, however, that most cells were capable of resealing their plasma defect without undergoing necrosis. It is thus possible that the low level of cell injury (Fig. 4B) in this study was a result of the slow rate of stretch (3 cycles/min), which might have provided sufficient time for the plasma membrane to repair between peak stretches. Although this phenomenon is beyond the scope of our current study, it does not seem to explain the downregulation of PC secretion, which also merits further investigation.

Variable stretching of AEII cells. The most interesting finding of this study is the observation that variable stretch enhances surfactant secretion by AEII cells. From the systems point of view, the mechanism behind this result is related to the highly nonlinear nature of the secretory response of these cells to strain. In any nonlinear system, the addition of variations or “noise” to the input signal in the region where the input-output relation is nonlinear will result in a different mean output than if there were no noise present (15). The complexity of the data in Fig. 3 certainly suggests that stretch-induced PC secretion is a highly nonlinear and time-dependent phenomenon. Furthermore, our results in Fig. 6 are similar to a phenomenon known as stochastic resonance whereby the output of a nonlinear system with a weak input can be amplified with the addition of noise to the input signal (15). Such a phenomenon has been shown to occur in a number of biological systems ranging from ion channels to tactile sensation (5, 6, 9, 10, 27) including the recruitment in the lung (31). Any nonlinearities along the mechanotransduction pathways could result in such an amplification effect. For example, the mechanical properties of both the cell membrane and the cytoskeleton, in particular the actin network, are nonlinear. Bezrukov and Vodyanoy (5, 6) demonstrated that noise-enhanced signal transduction in voltage-dependent ion channels is present in the cell membrane. Thus, since Ca2+ channels in the cell membrane are sensitive to stretch, they may play a role in the case of variable stretch by allowing a higher net influx of Ca2+ into the cell during the higher amplitude strains. Another possibility is that variable stretch simply produces a net increase in the average diameter of the fusion pores allowing the surfactant to flow more freely from the lamellar bodies to the extracellular space. Although the exact mechanism behind noise-enhanced surfactant secretion as a result of variable stretch remains unclear, the results presented here support our hypothesis that variable stretch pattern delivered to the epithelium leads to enhanced surfactant release by AEII cells.

Patients suffering from acute lung injury frequently require mechanical ventilation and exhibit significant impairments in pulmonary surfactant content and function leading to degradation in general lung function (20, 22). Thus, mechanism aside, the present findings could have implications in the design of strategies to ventilate patients in the setting of acute respiratory distress syndrome. It has been well-documented in animal studies (1, 2, 4, 24, 32), and more appreciably in a recent human trial (7), that variable ventilation improves gas exchange through what has been thought to primarily be a recruitment-based mechanism. We suggest that, in addition to improving recruitment of lung units, the variable stretch patterns applied to the alveolar epithelium promotes surfactant release. Indeed, our findings that variability increases PC secretion by AEII cells (Figs. 5 and 6) are qualitatively similar to that seen in our whole animal study (2) despite the differences in ventilation frequency and stretch rate suggesting that the mechanism is likely related to variability in amplitude and not rate. This “endogenous surfactant therapy” could lower surface tensions within the lung and would likely mitigate lung injury resulting from airway and/or alveolar collapse and reexpansion.

Before concluding, we note that a broad range of cell types are highly sensitive to their mechanical environment and the mechanotransduction of physiological forces play a dominating role in many basic regulatory cell functions (21). In the laboratory, the mechanism of stretch-induced phenomena is studied using either constant or monotonous cyclic stretches; however, cells in the body are exposed to irregularly varying mechanical stimuli. It is likely that the response of other cellular systems that experience and transduce mechanical signals is also substantially altered with the incorporation of physiological levels of randomness into the stretching protocol. This has been overlooked in cell and tissue culture studies and can have significant implications for the design of future experiments involving mechanotransduction. Our novel findings could thus have far-reaching implications for the understanding how signaling works in vivo and hence biology and medicine.

Summary. Here, we have shown that, whereas short durations of stretch may induce surfactant secretion, longer durations of stretch have the opposite effect and actually inhibit surfactant secretion below that of even the unstretched condition. These results may help explain the development of VILI. Moreover, we have found that the addition of random variability in the magnitude of strain ameliorates this effect. This finding may have direct implications for the design of ventilation strategies and therapies during lung disease. Finally, adding variability to cell and tissue stretching protocols may have implications for general mechanobiology and may call into question experimental results that do not take into account the “natural variability” of a physiological system.
REFERENCES


