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Measurement of stretch-induced loss of alveolar epithelial barrier integrity with a novel in vitro method

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Cavanaugh, Kenneth J., Jr. and Susan S. Margulies. Measurement of stretch-induced loss of alveolar epithelial barrier integrity with a novel in vitro method. Am J Physiol Cell Physiol 283: C1801–C1808, 2002. First published August 14, 2002; 10.1152/ajpcell.00341.2002.—Mechanical ventilation with high tidal volumes has been shown to contribute to the formation or worsening of interstitial and alveolar edema. Previously we showed that application of large bi-axial deformations in vitro perturbs the concentration and distribution of functional tight junction proteins in alveolar epithelial cells. Using a novel method, we determined that applied epithelial strain increases paracellular permeability in a dose- and rate-dependent manner. Primary rat alveolar epithelial cells were subjected to 12%, 25%, or 37% change in surface area (ΔSA) cyclic equibiaxial stretch for 1 h. Cells were also stretched noncyclically at 25% ΔSA for 1 h. During the experimental period, a fluorescently tagged ouabain derivative was added to the apical fluid. Evidence of binding indicated functional failure of the paracellular transport barrier. The percentage of field area stained was quantified from microscopic images. There was no significant evidence of basolateral fluorescent staining at 12% ΔSA or at 25% ΔSA applied cyclically or statically. However, cyclic stretch at 37% ΔSA resulted in significantly more staining than in unstretched cells (P < 0.0001) or those stretched at either 12% (P < 0.0001) or 25% cyclic (P < 0.0005) or static (P < 0.05) ΔSA. These results suggest that large cyclic tidal volumes may increase paracellular permeability, potentially resulting in alveolar flooding.

injury; lung; transport

Patients suffering from acute or chronic lung injury, or who experience poor pulmonary gas exchange, are able to receive respiratory assistance through controlled mechanical ventilation. However, ventilation with excessive regional lung volumes or high pressures can result in the development of ventilator-induced lung injury (VILI) (14). VILI occurs in ~5–15% of ventilated patients and carries an associated mortality rate of 34–60% (14, 20, 25), representing a significant health risk for patients requiring ventilation (20, 25). VILI is commonly marked by the formation or worsening of alveolar air leaks, interstitial and pulmonary edema, and alveolar cell dysfunction, indicative of decreased endothelial and epithelial barrier function (9).

Previously, investigators demonstrated a strong qualitative link between lung inflation volume and pulmonary barrier function in whole lung and animal models. In an open-chest preparation, the number of pulmonary endothelial and epithelial breaks increased at higher lung volumes, regardless of the transmural pressure of the alveolar wall (13). When animals were ventilated with high airway pressures but normal tidal volumes, lungs were uninjured, as determined by wet-to-dry ratio and albumin permeability (8). Conversely, ventilation with high tidal volumes, with or without high airway pressure, resulted in increased wet-to-dry ratios and permeability to albumin in these studies, demonstrating that large tidal volumes impair barrier properties of previously uninjured lungs.

To quantify the extent of barrier dysfunction due to “volutrauma,” investigators have measured transport of large and small solutes across the epithelium, often expressing the results as an equivalent pore radius, a measure that increases as the epithelium allows larger molecules to pass more easily. Many of these findings indicate that high static inflation volumes adversely affect the solute and fluid balance that is required for proper pulmonary function (10–12, 15). However, none of these studies examined lungs experiencing cyclic changes in volume, which is the most clinically relevant mode of ventilation. Also, these studies examined lobar or whole lung inflations, which are inherently heterogeneous, and did not examine the effects of lung inflation at the local, or cellular, level.

The cellular structures that provide the majority of resistance to paracellular transport are the gasketlike tight junctions (TJ) of the alveolar epithelium located...
at the apical end of the intercellular space between adjacent epithelial cells. Intact, they minimize paracellular transport, allowing the cells to regulate transport via transcellular pathways (16). Previously, we demonstrated (5) that application of high magnitudes of cyclic mechanical stretch decreases the concentration of functional TJ proteins in cultured alveolar epithelial cells and alters their distribution around the cell membrane. Given the importance of the TJ in maintaining the alveolar epithelial paracellular transport barrier, we hypothesized that these stretch-induced cytoskeletal changes are associated with an increase in epithelial paracellular permeability.

In this article, we test this hypothesis by examining the alveolar epithelial paracellular permeability response to changes in the magnitude and mode of stretch. The common measures of epithelial permeability, such as transepithelial electrical resistance and transport of labeled tracers (3, 6, 18), require the cells to be cultured onto a permeable substrate. Furthermore, stretching of cultured cells usually involves the use of elastic and flexible substrates. Hereafter, the lack of cell culture substrates that are permeable, elastic, and biocompatible hindered the measurement of stretch-induced alterations in paracellular permeability in cultured cells. In this article we present a new method to assess paracellular permeability useful for alveolar epithelial cells cultured onto an impermeable substrate. Changes in paracellular permeability of cultured alveolar epithelial cells were evaluated after application of stretch at various magnitudes under both cyclic and static stretch modes. At a given magnitude, stretch mode did not affect paracellular permeability in these cells. Paracellular permeability increased after 1 h of cyclic stretch at 37% change in surface area (ΔSA), consistent with our previously reported structural data. Together, these results indicate that sustained cycling at physiological magnitudes of alveolar strain may impair epithelial barrier function, increasing the likelihood of pulmonary edema formation or enhancement.

**Materials and Methods**

**Method rationale.** The technique used for the assessment of paracellular permeability described in this article is made possible by the polarity that alveolar epithelial cells display and by the extremely low paracellular permeability that confluent monolayers of type I cells demonstrate, as indicated by their high transepithelial electrical resistance of ~2,000 Ω·cm² (6). These cells possess functional Na⁺-K⁺-ATPases only on the basolateral surfaces of the plasma membrane (26). The Na⁺-K⁺-ATPases are inhibited by the compound ouabain, which has a high affinity for the extracellular domain of this transmembrane protein in all cell types. In designing this study, we hypothesized that paracellular permeability will increase when cells are stretched above a certain threshold. Thus, if ouabain is present only in the fluid surrounding the apical surface of a confluent monolayer of type I-like cells, stretched cells will allow a greater amount of ouabain transport through the TJ than unstretched cells and will therefore demonstrate a greater extent of ouabain-ATPase binding. To detect the appearance of ouabain in the basolateral space and to measure the extent of binding, we used a derivative of ouabain bound to the fluorescent dye BODIPY-FL that possesses the same specificity of binding as unconjugated ouabain.

**Cell culture protocol.** The protocol for lung cell isolation was approved by the University of Pennsylvania Institutional Animal Care and Use Committee and is based on the method of Dobbs and coworkers (7). Alveolar type II cells were isolated from healthy male Sprague-Dawley rats (180–200 g). The rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). The trachea was cannulated, the lungs were mechanically ventilated, an abdominal aortotomy was performed to exsanguinate the animal, and excess blood was removed via pulmonary arterial perfusion. The lungs were then excised and instilled with a elastase solution (3 U/ml; Worthington Biochemical, Lakewood, NJ) and minced in the presence of deoxyribonuclease (Sigma, St. Louis, MO) with a tissue chopper (Sorvall, Newtown, CT). The elastase reaction was stopped with fetal bovine serum (Life Technologies, Rockville, MD). Cells were filtered through progressively finer Nitex mesh (Crosswire Cloth, Bellmawr, NJ), and placed on an IgG-coated culture dish (3 mg/5 ml Hanks HCl). After a 1-h incubation at 37°C, gentle panning isolated type II cells from the macrophages and contaminating cells preferentially adhered to the culture dish. Ultimately, cells were spun down and resuspended in minimum essential medium (MEM) with Earle’s salts and supplemented with 10% fetal bovine serum, 25 μg/ml gentamicin, and 0.25 μg/ml amphotericin B (Life Technologies). Cell purity of this isolation procedure is >95%, as determined by phosphate 3R staining of adherent cells (17).

All cells were seeded at a density of 1 × 10⁶ cells/cm² onto fibronectin-coated (10 μg/cm²; Boehringer Mannheim Biochemicals, Indianapolis, IN) flexible Silastic membranes (Specially Manufacturing, Suginaw, MI) mounted in custom-made wells. The cells were cultured in MEM supplemented as above for 5 days. It was reported previously that rat alveolar type II cells differentiate into type I cells (which cover ~90% of the alveolar surface in vivo) after 5 days under similar culture conditions (4). The medium was replaced daily. By the fifth day of culture, the cells had formed a confluent monolayer and displayed a phenotype consistent with that observed for cultured type I cells. We showed previously (19) that >95% of identically cultured cells stain positively for RTI-40, a type I cell-specific surface antigen. After 5 days, the cells were washed with dye-free Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) and subjected to the stretch protocol described in Stretch application. Unstretched wells served as controls in all experiments.

Four isolations were performed for each stretch magnitude. Two wells per isolation were stretched at each magnitude, resulting in a total of eight wells for each group. The unstretched control group also consisted of two wells per isolation.

**Stretch application.** Wells were mounted on a custom-built cell-stretching device capable of applying equibiaxial strain to the samples at a precise user-defined magnitude and frequency, as previously described (22). Cells were stretched cyclically at 15 cycles/min for 1 h at 12%, 25%, or 37% ΔSA. These changes in surface area approximately correspond to strain changes of the alveolar epithelium in vivo at 70%, 90%, and 100% total lung capacity (TLC), respectively (23). An additional set of wells was stretched statically (0 cycles/min) for 1 h at 25% ΔSA. As a positive control, a separate population of unstretched cells was scraped once with a rubber policeman. During the 1-h stretch...
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/deg K
C to minimize endocytosis of the dye but not impair
cellular homeostasis.

Under identical conditions, we reported previously (22) that cyclic mechanical stretch of similarly cultured cells produces death because of membrane rupture in 2–9% of the monolayer. To determine whether BODIPY-ouabain was preferentially binding to dead cells, two wells from each of three isolations stretched at 37% ΔSA were also incubated with 230 nM ethidium homodimer-1 (Molecular Probes), which specifically stains cells whose plasma membranes have been compromised. Unstretched double-stained wells from each isolation served as unstretched controls.

After stretch, the cells were washed three times with DMEM before the apical BODIPY-ouabain and ethidium homodimer-1. The wells were then examined with the protocol described in Microscopic examination.

Microscopic examination. Wells were mounted onto a Nikon TE-300 inverted epifluorescence microscope and examined at ×20 magnification. Two fields from each well were selected randomly, and two images of each field with a phase objective or a green (BODIPY-ouabain fluorescence) emission filter were captured and stored with Metamorph imaging software (Universal Imaging, West Chester, PA) and a microscope-mounted Hamamatsu camera and controller. Identical image acquisition times were used for all images of each type acquired.

After all images were captured, we wanted to distinguish whether the observed green fluorescence was due to endocytosis of BODIPY-ouabain into the cell or whether BODIPY-ouabain had in fact bound to the basolateral surface of the plasma membrane. Anti-BODIPY antibody (Molecular Probes) was added in a 1:40 dilution to one of the two wells from each group in every isolation. All wells were then kept overnight at 4°C to inhibit nonspecific endocytosis (21, 24), and images were obtained again as above on the following day. Anti-BODIPY, when bound with BODIPY-ouabain, quenches the fluorescent signal of the BODIPY-ouabain bound to Na+-K+-ATPase pumps. Given the large size of the anti-BODIPY molecule (radius ~15–20 Å) and the low incubation temperature, it was expected that anti-BODIPY would not be internalized by the cells. To rule out the possibility that loss of BODIPY-ouabain fluorescence overnight could be attributed to dye photobleaching and not antibody quenching, the other well from each group was incubated overnight without the antibody for comparison. If both wells remained brightly fluorescent, the BODIPY-ouabain had been internalized. If both became dark, the BODIPY-ouabain had been photobleached. However, if wells treated with anti-BODIPY demonstrated reduced fluorescence compared with those without antibody treatment, we would conclude that the BODIPY-ouabain was bound in the extracellular domain.

Additional experimentiation. The influence of specific endocytic pathways on cell staining was determined by treating one unstretched well and one well stretched at 37% ΔSA from each of three isolations with 5 μM phenylarsine oxide (PAO; Sigma), an inhibitor of receptor-mediated endocytosis. These wells were treated during the entire 1-h experimental period with PAO, BODIPY-ouabain, and ethidium homodimer-1 and examined as described in Microscopic examination. One
untreated, unstretched well and one untreated well stretched at 37% ΔSA from each isolation served as untreated controls. Treatment of alveolar epithelial cells with PAO using a similar concentration and duration has been shown to decrease endocytotic activity without adversely affecting cellular viability (2). In a limited manner, cells were also treated with a higher concentration of PAO (10 μM), but these results were no different than those at 5 μM (data not shown) significantly more BODIPY-ouabain staining in the stretched, PAO-treated group compared with the stretched, untreated group would indicate that receptor-mediated endocytosis was involved in stretch-induced BODIPY-ouabain binding.

Two additional wells from each of three isolations were treated with 0.1% Triton X-100 (TX; Sigma) for 10 min to permeabilize the cells. After this time, the cells were washed and bathed in BODIPY-ouabain and ethidium homodimer-1, during which one of these wells was then stretched for 1 h at 37% ΔSA and the other remained unstretched. One untreated, unstretched well and one untreated well stretched at 37% ΔSA from each isolation served as untreated controls. Wells were then examined microscopically as described in Microscopic examination. These experiments served as a positive control for BODIPY-ouabain transport to the cell membrane due to nonspecific membrane permeabilization.

The contribution of specific stretch-induced endocytosis to increased BODIPY-ouabain staining was also investigated. Six wells from each of two cell isolations were stretched for 1 h at 37% ΔSA. Half of the wells were treated with BODIPY-ouabain for 1 h during stretch, and the remainder were treated for 1 h after completion of the stretch stimulus. Three wells from each isolation served as unstretched controls. Wells were then examined microscopically and analyzed as above. Significantly more staining in the wells stained during stretch would indicate that the stretch-induced increase in BODIPY-ouabain staining is the result of transient and specific stretch-induced uptake.

Data analysis. With Metamorph, a fluorescence intensity threshold was applied to each fluorescence image to examine the signal above background fluorescence levels. Background fluorescence levels were determined for each isolation by selecting a large region from each unstretched fluorescence image and calculating the maximum pixel intensity in each of these regions. This intensity was used as the threshold for all stretched and unstretched images from that isolation. Occasional regions that contained non-cell-specific autofluorescence were not included. For each image, the pixels possessing intensities above the threshold and below saturation were counted and expressed as a percentage of total field area. Average intensity of these pixels was also calculated for each field of stretched cells. Stained area and intensity measurements from unstretched and stretched wells were compared with the Tukey test for significant differences across multiple groups (28). Results are expressed as means ± SE.

RESULTS

Extent of BODIPY-ouabain stained area. Both stretched and unstretched cells displayed at least a small degree of BODIPY-ouabain staining (Fig. 1). Cells stretched cyclically for 1 h at 12% ΔSA (2.4 ± 0.6% of field area stained) or 25% ΔSA (4.2 ± 0.9%) did not experience significantly greater BODIPY-ouabain staining than unstretched, untreated cells (2.1 ± 0.3%). In contrast, cyclic stretch at 37% ΔSA increased the area of BODIPY-ouabain staining to 16.8 ± 4.7% of
the field. This increase was significant compared with unstretched cells and with all other stretched populations (Fig. 2). BODIPY-ouabain staining was not preferentially colocalized with ethidium homodimer-1 binding, indicating that plasma membrane rupture was not a main factor by which BODIPY-ouabain was able to bind to cells (Fig. 3). Previously, we showed (5) that this stretch regimen alters distribution and expression of the functional TJ protein occludin in similarly cultured cells. Together, these results indicate that stretch magnitude is a determinant of the extent of paracellular barrier dysfunction in cultured alveolar epithelial cells. To determine whether this increase in fluorescence could be due to an increase in visible cellular surface area, we measured the surface areas of individual cells before stretch application and compared them to the areas of the same cells measured after 1 h of 37% ΔSA cyclic stretch. Paired analysis of the measured areas did not reveal a significant change in cell surface area (data not shown).

Static stretch at 25% ΔSA (7.5 ± 1.7%) did not result in increased fluorescence staining compared with unstretched cells or those stretched cyclically at 25% ΔSA (Fig. 2). Thus stretch mode did not affect paracellular permeability at this magnitude during this study.

BODIPY-ouabain staining in scraped monolayers was confined to cells immediately bordering the scraped region (Fig. 1, left). No staining was observed in the denuded region or in areas where the monolayer remained confluent. As in stretched cells, this staining did not colocalize with ethidium homodimer-1 staining (Fig. 3), indicating that BODIPY-ouabain binding in cells near the scraped regions was not due to plasma membrane rupture.

Treatment of cells with unconjugated BODIPY dye during 1 h of cyclic stretch at 37% ΔSA did not result in significant cellular staining (7.3 ± 2.9% staining compared with 5.8 ± 3.1% staining of unstretched controls; n = 8 wells for both groups), indicating that BODIPY-ouabain binding to the cells was not merely the result of nonspecific interactions between the BODIPY-ouabain and the cells (Fig. 2). Furthermore, overnight treatment of cells at 4°C with an anti-BODIPY antibody after stretch significantly decreased the observed stained region from 16.8 ± 4.8% to 1.2 ± 0.5% of the field (Fig. 4). This decrease is not merely the result of dye photobleaching or other factors that would cause a nonspecific loss of fluorescence overnight, because cells incubated overnight without anti-BODIPY showed no significant loss of fluorescent signal (15.3 ± 2.3% of region stained). Because fluorescence was quenched to 1.2 ± 0.5%, we believe that the BODIPY-ouabain was not internalized and that the fluorescent regions measured after stretch were caused by BODIPY-ouabain that was bound to the cell exterior.

PAO treatment did not affect BODIPY-ouabain staining in either stretched or unstretched cells (Fig. 5), indicating that receptor-mediated endocytosis was not involved in BODIPY-ouabain binding. Cellular vi-
ability was not affected by PAO treatment, as determined by ethidium homodimer-1 staining. Treatment of cells with TX produced an increase in ethidium homodimer-1 staining, confirming that the cells had been permeabilized (Fig. 6). The amount of BODIPY-ouabain fluorescence in unstretched cells increased from 0.3 ± 0.1% to 46.3 ± 4.6% after TX treatment. Stretched cells also experienced a significant increase in BODIPY-ouabain fluorescence after TX treatment, from 14.2 ± 0.8% to 62.4 ± 4.5%. The increase in fluorescence after TX treatment in unstretched cells is significantly greater than in stretched and untreated cells (Fig. 6), indicating that stretch-induced BODIPY-ouabain staining occurs via a pathway different from that which occurs after permeabilization of the cells. This difference in binding pathways is also evident in the significant difference between BODIPY-ouabain staining in unstretched, TX-treated cells and stretched, TX-treated cells (Fig. 6).

Stretch-induced BODIPY-ouabain staining did not differ in cells stained during (16.1 ± 1.3%) or after (17.4 ± 2.3%) stretch. This provides further evidence that the observed increase in staining is not due to stretch-induced endocytotic pathways specific to BODIPY-ouabain.

Intensity of BODIPY-ouabain staining. Average intensity of suprathreshold pixels in stretched cell fields did not differ significantly from those in unstretched control wells at 12% (108.0 ± 4.3% of average intensity of controls), 25% cyclic ΔSA (103.9 ± 6.6%), and 25% static ΔSA (121.0 ± 5.5%; Fig. 7). However, at 37% ΔSA, intensity was significantly higher than in controls (130.7 ± 5.9%; *P < 0.01). This result suggests that this magnitude of stretch increases the amount as well as the extent of BODIPY-ouabain binding to the basolateral surface.

DISCUSSION

Our findings support the hypothesis that moderate static and cyclic inflation volumes do not adversely affect paracellular barrier function. The BODIPY-ouabain staining in unstretched, untreated cells was low, averaging 2.1% of the microscopic field areas examined. Cyclic stretch for 1 h at 12% or 25% ΔSA did not result in an increase in fluorescence. One hour of static stretch at 25% ΔSA did not produce a significantly different staining intensity compared with control levels or with staining after cyclic stretch at 25% ΔSA. Thus we conclude that paracellular permeability did not increase in cells stretched at these magnitudes, which correspond roughly to inflations to lung volumes averaging 70% and 90% TLC (23). In contrast, 1 h of cyclic inflations to 100% TLC (23) (37% ΔSA) resulted in significantly increased paracellular transport (16.8% of the field stained), compared with all other experimental groups.
Given that mechanical stimuli can induce endocytosis in other kinds of epithelia (1), we performed specific studies to rule out this pathway as a significant mechanism responsible for the observed increase in fluorescence after stretch. First, PAO treatment did not affect dye binding in stretched cells (Fig. 5), ruling out the contribution of receptor-mediated endocytosis to the process of BODIPY-ouabain binding. Second, staining cells after stretch instead of during stretch did not affect the extent of BODIPY-ouabain binding, ruling out the contribution of specific stretch-induced endocytosis. Third, stretch studies with BODIPY-ouabain administered at 37°C were followed by treatment with anti-BODIPY at 4°C. This temperature has been reported to block endocytosis in similar alveolar epithelial cells (21, 24), restricting the quenching action of anti-BODIPY to externally bound BODIPY-ouabain only. The treatment quenched the fluorescent staining from 16.8% to 1.2% of the field area (Fig. 4). Thus we conclude that most of the BODIPY-ouabain is bound extracellularly, ruling out nonspecific endocytotic pathways. Fourth, our TX experiments, which demonstrated significantly higher fluorescence after cell permeabilization compared with stretch-induced fluorescence levels (Fig. 6), disprove the hypothesis that stretch-induced increases in plasma membrane permeability, rather than TJ permeability, are primarily responsible for the findings presented in Fig. 2. Fifth, we show that stretch did not increase the amount of cell staining with unconjugated BODIPY (Fig. 2), confirming that the stretch-induced increases in fluorescence were specific to increased numbers of BODIPY-ouabain-bound sites rather than nonspecific stretch-induced increases in intracellular dye concentration. Finally, BODIPY-ouabain fluorescence was demonstrated in unstretched and intact cells near a scraped monolayer region (Fig. 1), suggesting that any type of TJ disruption, not merely mechanical stretch, can result in increased BODIPY-ouabain staining.

Our data also reveal additional information regarding BODIPY-ouabain binding in these cells. The fact that anti-BODIPY is apparently able to diffuse through the TJ and quench BODIPY-ouabain fluorescence provides evidence that barrier injury in this model is irreversible over the 24-h experimental period. The absence of a strong correlation between regions of strong BODIPY-ouabain and ethidium homodimer-1 binding indicate that plasma membrane rupture does not by itself produce cellular BODIPY-ouabain binding. Also, because treatment with unconjugated BODIPY did not produce a significant difference in staining between cells stretched at 37% ΔSA and unstretched cells, we can conclude that the binding of BODIPY-ouabain to the cell exterior was specific to the Na⁺-K⁺-ATPase sites. The average intensity of the stained regions also increased at 37% ΔSA, indicating that a higher amount of BODIPY-ouabain was bound...
in these regions than in the stained regions of monolayers stretched at lower magnitudes.

Our cyclic stretch data from the current study indicate the possibility of a stretch magnitude threshold between 25% and 37% ΔSA above which barrier integrity suffers. This stretch magnitude corresponds to the average strain experienced by the alveolar epithelium between 90% and 100% TLC and is the same threshold magnitude previously discovered for TJ structural disruption in cells cultured under identical conditions (5). Both studies provide evidence that high, yet physiologically relevant, epithelial strains can cause alveolar dysfunction and injury in an in vitro preparation.

Similar threshold-dependent, stretch-induced TJ injury at high lung inflation volumes has been observed in animal preparations using tracers of similar size to BODIPY-ouabain (radius ~20 Å). Egan et al. (10, 12) observed the effects of static inflation on whole sheep and rabbit lungs, noting that inflation below 100% TLC did not produce an increase in paracellular permeability to tracers of 14- to 34-Å radius (11, 12). Inflation volumes above 100% TLC removed all resistance to paracellular transport of these molecules, indicative of a complete loss of epithelial barrier function (11, 12). These injury thresholds determined in whole lung studies agree with our structural (5) and functional (current study) findings in vitro in a primary cell culture preparation.

Additional permeability assessment has been performed in animals with tracers of smaller size (radius <10 Å), which provide more sensitive detection of impaired barrier properties. Egan et al. (10, 12) observed that static inflation of dog and sheep lungs at 75–80% TLC produced an increase in paracellular permeability to tracers of small size. In contrast, Kim and Crandall (15) noted that static inflation of bullfrog lungs within the physiological range did not result in increased paracellular transport to these tracers and that only supraphysiological inflation caused increased solute and fluid transport across the alveolar barrier in these animals. Thus, there is some disagreement in the literature as to the extent of injury measured with small solutes at static inflation volumes below 100% TLC, but the results of Kim and Crandall support the current findings of a decrease in TJ integrity at or above 100% TLC.

In humans, repeated inflation of the whole lung to 80–85% TLC can occur in well-trained athletes during vigorous exercise with no apparent loss of pulmonary epithelial barrier function (27). These lung inflation volumes correspond to an alveolar epithelial change in surface area of <25% (23), a stretch magnitude that was found to be below the injury threshold in this study. Thus these in vivo observations are consistent with the results of the in vitro experiments presented here.

The main advantage provided by this in vitro experimental system is that the local physical environment of a monolayer of cultured cells can be controlled more readily than that of a whole lung. Furthermore, the relative contributions of the cellular and extracellular components of the monolayer and of the apical milieu can be investigated more effectively than in whole lungs. However, although our model of alveolar epithelial injury possesses type I-like pneumocytes that cover the vast majority of the alveolar epithelium, it is not fully representative of conditions in the in vivo alveolus. It does not contain macrophages or fibroblasts, and therefore the composition of the extracellular matrix and of the interstitial and luminal milieu may not be identical to that of the viable whole lung. Additionally, after 5 days in culture, the majority of type II cells differentiate to type I phenotype (19), resulting in a lack of surfactant production that may alter the surface tension milieu of the in vitro alveolar epithelium. Once the basic mechanisms of stretch-induced alveolar epithelial dysfunction are elucidated with new methods such as that presented here, a more representative in vitro model of the epithelium may be constructed to determine how these other constituents of the in vivo alveolus affect the susceptibility of alveolar epithelial cells to mechanical injury.

Using a novel technique for the assessment of paracellular permeability, we have demonstrated that the paracellular permeability of stretched alveolar epithelial cells in culture is dependent on the magnitude of the applied stretch. This study represents the first time that paracellular permeability has been assessed in cultured and mechanically stretched epithelial cells, and this method should prove useful to other transport physiologists, especially those interested in cellular injury. Although this study provides evidence that alveolar epithelial barrier integrity is altered at high magnitudes of applied strain, for a more complete description there is a need to supplement these findings with data from tracers of varying size and charge to determine critical cell deformations required to disrupt the epithelial barrier to transport of small charged solutes, large macromolecules, and other types of physiologically relevant compounds. After identifying the chemical pathways by which excessive stretch is transduced into an injurious cellular response, we may develop injury interventions to reduce the severity of stretch-induced alveolar epithelial injury and minimize the incidence and mortality of ventilator-induced lung injury.

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