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highlighted topics

Cellular Responses to Mechanical Stress
Invited Review: Plasma membrane stress failure in alveolar epithelial cells

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Vlahakis, Nicholas E., and Rolf D. Hubmayr. Invited Review: Plasma membrane stress failure in alveolar epithelial cells. *J Appl Physiol* 89: 2490–2496, 2000.—In this review, we examine the hypothesis that plasma membrane stress failure is a central event in the pathophysiology of injury from alveolar overdistension. This hypothesis leads us to consider alveolar micromechanics and specifically the mechanical interactions between lung matrix and alveolar epithelial cell cytoskeleton and plasma membrane. We then explore events that are central to the regulation of plasma membrane tension and detail the lipid-trafficking responses of in vitro deformed and/or injured cells. We conclude with a reference to upregulation of stress-responsive genes after membrane injury and resealing.

lipid trafficking; mechanical stress; lung injury; cell deformation

THIS REVIEW IS MOTIVATED by a clinical problem, namely ventilator-induced lung injury. Although there are several excellent recent reviews of this topic (12, 13, 21, 44), we wish to examine it from a slightly different perspective, that of alveolar epithelial wounding. In doing so we will extract and integrate information from what might seem to be very different fields of investigation: classic lung mechanics, lung morphometry, cytoskeletal mechanics, lipid translocation between cell compartments, membrane biology, and proinflammatory signal transduction. Our reasons for probing the literature of these diverse fields become immediately apparent if we consider a few seminal questions: How do lung parenchyma and its cellular constituents deform during breathing? To what extent do epithelial cells resist the shape change that must accompany a deformation of the matrix (basement membrane) to which they adhere? What is the stress distribution between cytoskeleton and the plasma membrane during such a shape change? Is there a basement-membrane strain threshold beyond which the plasma membrane breaks? If so, what happens to a cell with a plasma membrane break? Although many answers to these questions are incomplete, they nevertheless provide a useful framework for thinking about the biology of lung overdistension injury.

EVIDENCE OF EPITHELIAL CELL PLASMA MEMBRANE INJURY IN PHYSICALLY STRESSED LUNGS

As pointed out by West in his review on pulmonary capillary stress failure (52), the blood gas barrier is extremely thin and fails when it is exposed to high transmural pressures. The spectrum of injury as defined with electron microscopy on perfusion-fixed tissue specimens includes endothelial and epithelial plasma membrane blebs, transcellular and intercellular gaps, and overt breaks of the basement membrane (13, 14, 51). These lesions have been described in small as well as large animals after vascular perfusion with high pressures or inflation to volumes exceeding total lung capacity (22). Remarkably, these lesions cannot

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be demonstrated unless the lungs are fixed under the experimental conditions that produced them. This observation suggests that the cytopathological changes that accompany overdistension injury of the lungs are rapidly reversible. The light microscopic appearance of injured lungs is dominated by the consequences of the loss of blood gas barrier integrity, namely hemorrhage, edema, and the influx of inflammatory cells. The accumulation of proteinaceous edema in alveolar spaces causes profound changes in pulmonary mechanics and gas exchange that are the clinical signatures of the syndrome "acute lung injury." These downstream effects of epithelial and endothelial deformation injury have received comparatively more attention in lung research than the cell mechanical determinants of the injury themselves (54). The latter is the focus of our review.

DEFORMATION OF THE ACINUS DURING MECHANICAL VENTILATION

Whereas there is general agreement that the surface area for gas exchange increases with lung volume, there is less certainty about how alveoli deform to accomplish this. The principal reasons for this uncertainty are the shortcomings of the available imaging methods either in terms of insufficient spatial resolution or artifacts from tissue fixation. Most students of the topic view the alveolus as a spherical structure of wetted parenchyma that is covered with a continuous film of surface active material, i.e., surfactant (3). Some believe that the aqueous lining of the alveoli is discontinuous (20); others consider alveolar walls to be supported by foamlike bubble structures (37). To the first approximation, parenchymal strain (defined here as the fractional length change of a line element) should scale with tidal volume to the 1/3 power and produce a surface area change of tidal volume to the 2/3 power. Accordingly, the strain of a lung region, the volume of which doubles during mechanical ventilation, ought to be ~0.25. Actual measurements of lung deformation patterns in mechanically ventilated dogs have yielded regional strain values that are generally consistent with this prediction (36). However, these observations do not mean that alveolar walls are stretched by 25% on every breath. Indeed, the prevailing consensus is that alveolar walls themselves carry little stress during normal breathing and that they simply "unfold" rather than undergo an elastic deformation (3, 28, 45). This view is supported by morphometric data from perfusion-fixed rat and rabbit lungs on which the Wilson-Bachofen model of alveolar micromechanics is based (53). Accordingly, surface tension, as opposed to alveolar wall tissue, counterbalances the hoop stress at the alveolar entrance rings. The latter are formed by helical fibers that support the alveolar ducts. Only at lung volumes approaching total lung capacity are alveolar walls thought to come under tension and their basement membranes (the walls' principal stress-bearing elements) considered strained. Recent estimates place basement membrane strains of rat lungs inflated to total lung capacity in the vicinity of 0.25 (45).

IN VITRO SYSTEMS TO INVESTIGATE ALVEOLAR EPITHELIAL DEFORMATION

Although estimates of alveolar wall strain have been calculated, the precise strain that is borne by the constituents of the alveolar wall, and in particular the epithelial cells, is unknown. It is hypothesized that type I cells that cover >95% of the alveolar surface area might be exposed to a greater strain, whereas type II cells that reside in the alveolar corners might be relatively protected (45). To investigate directly deformation effects and responses in alveolar epithelium, investigators have used reduced, in vitro strain systems. The reader is directed to recent excellent reviews on this topic (25, 26). Suffice it to say there are numerous devices being used that simulate basement membrane deformation by utilizing a deformable substratum on which epithelial cells are grown (7). It is worth noting that injury responses resulting from substratum stretching vary in magnitude depending on the nature (frequency, time, and magnitude) of deformation used (46). It is also important to highlight briefly the cell culture systems used for study of deformation-alveolar epithelial cell responses. It would seem pertinent to investigate type I epithelial cell responses; however, a primary culture system is not established or readily useable. As a result, biologists and physicists have resorted to using two representative cell systems, namely *1*) primary rat type II epithelial cell cultures at various stages of differentiation (33) and *2*) human continuous cell lines including A549 (48), H441 (8), and MLE-12 (50). Pros and cons exist in each system (32, 35); however, when used to answer the appropriate biological question, either model would seem suitable.

RESPONSE OF ALVEOLAR EPITHELIAL CELLS TO MATRIX DEFORMATION

In attempting to understand the response of epithelial cells to basement membrane deformation, one must consider the mechanical properties of the cell's cytoskeleton, plasma membrane, and the interaction between the two components. Like all adherent cells, alveolar epithelial cells interact with extracellular matrix, i.e., the alveolar basement membrane, through transmembrane adhesion receptors such as integrins. These receptors transmit forces from the surrounding matrix to the cytoskeleton via focal adhesion complexes (FACs). FACs are complex protein structures that play a pivotal role in the bidirectional signaling of integrin receptors (9). A discussion of integrin-mediated signaling is beyond the scope of this review. However, the reader is reminded that any and all cell deformation responses might involve the integrin-FAC complex and that this complex is dynamic and not merely an inert glue that "spot welds" cells to the alveolar basement membrane.

As the acinus expands during large tidal breaths, the matrix of alveolar epithelial cells is deformed. In a lung

that is injured and therefore unable to expand uniformly, some regions are likely to undergo large deformations during positive-pressure ventilation. When the basement membrane is strained, adherent epithelial cells must change shape. To the extent to which alveolar epithelial cells maintain a constant volume and stay in intimate contact with the matrix, their surface (plasma membrane)-to-volume ratio must increase during such a deformation. Four hypothetical plasma membrane deformation responses, all of which have been observed in experimental systems, are shown in Fig. 1: unfolding of excess plasma membrane (*A*), elastic deformation (i.e., stretch) of the plasma membrane (*B*), translocation of lipids from intracellular stores to the plasma membrane (*C*), and plasma membrane stress failure (*D*). Any or all of these responses might be active at any one time, and the relative contribution of each differs depending on the type and magnitude of deformation imposed and the cell system studied (1, 26, 27, 46).

It is generally believed that the plasma membrane of living cells carries little load in the undeformed or unstimulated state. The evidence for this statement rests on transmembrane pressure measurements with patch-clamp micropipettes and on force-displacement measurements of plasma membrane lipid tethers (10, 34, 38, 39). As discussed in a recent review by Stamenovic and Wang (40), the so-called cortical membrane models depict the cell as an elastic shell under sustained tension that is balanced by the pressurized cytoplasm and by traction at extracellular adhesions. These authors argue on the basis of cytoskeletal morphology and strain hardening observed during magnetic twisting cytometry that a tensegrity structure captures cell mechanical behavior better than a prestressed cortical shell. The low plasma membrane tension found in both adherent and nonadherent cells provides additional support for this view.

Many cells possess surface ruffles and invaginations that serve as a plasma membrane "reservoir" (34). The excess plasma membrane can be readily recruited in response to a deforming stress. Solsona and colleagues (39) were able to "inflate" patch-clamped mast cells to approximately four times their initial volumes by applying a hydrostatic pressure of 5–15 cmH_2O. Concomitant capacitance measurements (an index of plasma membrane volume) suggested that the increase in cell diameter and apparent cell surface area were primarily the result of plasma membrane unfolding as opposed to elastic membrane expansion. At a physiological temperature, plasma membrane lipids exist in a liquid state, and the lipid bilayer offers little resistance to shape change. However, the plasma membrane resists lateral expansion, and most membranes fail when their tension rises above ~4–6 dyn/cm. It follows from these observations that a typical plasma membrane can sustain strains between only 2 and 3% (in the plane of the membrane) before it breaks.

The length-tension behavior of lipid tethers formed by pulling with an optical trap on a plasma membrane-associated bead is consistent with the interpretation of capacitance measurements. An initial 5–10 pN force is required to lift the lipid bilayer off the subcortical cytoskeleton to form a tether (34). This force reflects both plasma membrane tension and the osmotic pressure gradient between the protein-poor tether and the protein-rich plasma membrane that is still in contact with the cytoskeleton (38). Once formed, the tether can be elongated by variable amounts without further increases in tether force. This observation has been attributed to recruitment (lipid flow) of excess plasma membrane into the tether (34). When the plasma membrane reservoir is exhausted, however, tether tension increases abruptly. Although much of this work has been conducted in fibroblasts and not in epithelial cells, there are some other observations on lipid tether mechanics that might give insights into the mechanisms of plasma membrane injury associated with alveolar overdistension. The volume of lipid that can be recruited into the tether (i.e., the size of the plasma

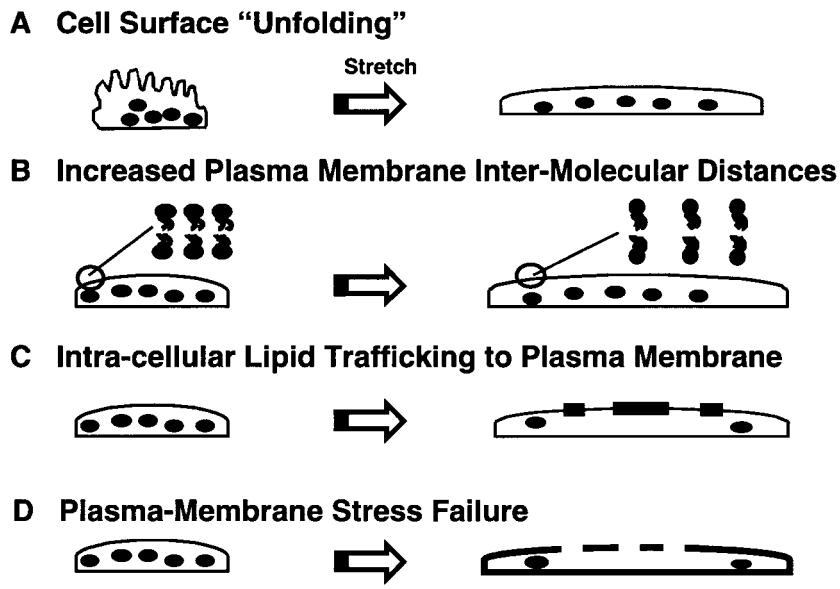

Fig. 1. Deformation responses of alveolar epithelial cells. This schematic highlights four hypothesized responses of alveolar epithelial cells to basement membrane deformation. Cell surface unfolding (*A*), increased plasma membrane intermolecular distances (*B*), and intracellular lipid trafficking to the plasma membrane (*C*) serve to facilitate deformation-induced changes in plasma membrane surface area and ultimately prevent membrane rupture. In contrast, plasma membrane stress failure (*D*) represents the short-term failure of cytoprotective mechanisms. There is evidence in both lung and nonlung cells that plasma membrane breaks are nonlethal and that they are resealed by site-directed exocytosis.

membrane reservoir) increases over time and with each subsequent tether formation. This suggests that cells can call on mechanosensitive lipid trafficking responses to add to the plasma membrane reservoir and thereby prevent the plasma membrane from becoming stress bearing during deformation. Studies on osmotically challenged molluscan neurons also support the notion that cell surface area regulation is achieved by the production of excess membrane available for surface area regulation (10, 19). Increases in membrane capacitance and cell imaging techniques revealed the formation of vacuole-like dilatations. These plasma membrane-associated lipid membrane invaginations formed when neurons were returned to isotonic conditions after hypotonic cell swelling. Additionally, these vacuolelike dilatations were recruited to the cell surface with reswelling or vacuolized with maintenance of isotonic conditions. In contrast to membrane wound resealing (see PLASMA MEMBRANE STRESS FAILURE), calcium influx and intracellular calcium release are not requirements for this exocytic response (19). These findings support a membrane-tension hypothesis that proposes that high plasma membrane tensions directly recruit additional membrane from mechanosensitive stores to the cell surface, whereas low tensions favor endocytosis of surface membrane (10).

Vlahakis and colleagues (49) tested this hypothesis in alveolar epithelial cells that were grown on matrix-coated malleable membranes and deformed ex vivo. For this purpose, the plasma membrane of alveolar epithelial cells was loaded with BODIPY (Molecular Probes) lipid analogs, which are fluorophores with concentration-dependent spectral shifts. Specifically, when BODIPY lipid labels are excited with blue light, their red-to-green fluorescence intensity decreases with a decrease in the molar concentration of the label. Figure 2 shows histograms of the red-to-green fluorescence intensity ratios of plasma membrane pixels of an alveolar epithelial cell labeled with BODIPY-sphingomyelin before and during a 25% substratum strain. This lipid label is largely confined to the outer leaflet of the plasma membrane bilayer, and the shift in fluorescence intensity spectra from red toward green as seen in the deformed state indicates that the plasma membrane label concentration had fallen during deformation. This observation is consistent with dilution of the BODIPY label with unlabeled molecules, reflecting deformation-induced lipid trafficking from intracellular lipid stores to the plasma membrane. This trafficking response occurred in the setting of an ~35% increase in apparent cell surface area, thus suggesting that deformation-induced lipid trafficking is an adaptive phenomenon of alveolar epithelial cells induced by basement membrane deformation to facilitate increases in cell surface area.

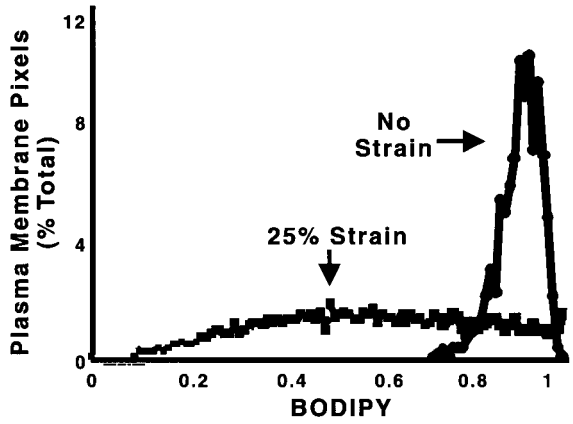

Fig. 2. Deformation-induced lipid trafficking to the plasma membrane of alveolar epithelial cells. A frequency-distribution graph of BODIPY-sphingomyelin concentration (red-to-green ratio shown in *x*-axis) in the plasma membrane of a single cell before and 45 s into a 25% basement membrane strain. The shift to the left with strain represents a decrease in the molar density of BODIPY within the plasma membrane. This finding suggests that unlabeled lipid traffics to the plasma membrane of alveolar epithelial in response to deformation.

PLASMA MEMBRANE STRESS FAILURE

As previously mentioned, epithelial cell injury in the lung has been investigated primarily either from a purely morphological standpoint or with a focus on the resultant downstream inflammatory protein response. Lesser attention has been paid to the mechanisms of plasma membrane wounding and repair in the lung as a result of basement membrane deformation. With the use of fibroblasts and sea urchin eggs as cell models, plasma membrane disruptions have been induced by many different mechanisms, including needle micropuncture, electroporation, syringing, and contracting collagen matrices (1, 17, 27). It is apparent in these systems that breaks in the plasma membrane are of varying size (3 nm to 100 μm) depending on the wounding mechanism, are rapidly resealed (10–120 s) at the sites of injury, and require influx of extracellular calcium (5, 42).

Plasma membrane disruptions have been demonstrated by a number of mechanisms, including laser confocal microscopy, electron microscopy, and the uptake of high-molecular-weight fluorescent dextran (FITC-Dx) (27, 42, 52). When the plasma membrane is disrupted, FITC-Dx molecules are able to enter the cell down a concentration gradient and thus homogenously label the cell interior. This labeling pattern is in contrast to the granular intracellular pattern of uptake when cells are able to pinocytose the dextran over longer time periods. Cells that reseal their membranes retain FITC-Dx and remain attached to the substratum and are viable in culture or lift off the substratum and eventually die. Those cells that do not reseal their membranes will not retain FITC-Dx and lift off the cell substratum and lyse. With the use of FITC-Dx of varying molecular size, estimates of deformation type and amplitude-dependent variations in plasma membrane break size have been made (11, 24).

By using various techniques, including the activity-dependent cell fluorescent dye method (2, 4), it has been shown that intracellular lipid stores are utilized to repair plasma membrane disruptions. In this

method, FM1–43, for example, is used to fluorescently label intracellular lipid vesicles before deformation. This styryl dye partitions reversibly into the outer leaflet of membranes and when endocytosed remains in the luminal leaflet of vesicles. In addition, it has the unique property of a 50-fold decrease in fluorescent intensity when partitioning from the membrane to an aqueous solution, such as with exocytosis to the plasma membrane. By using this technique, an exocytic lipid trafficking response has also been demonstrated in alveolar epithelial cells (both A549 and primary rat type II cells) after a lung-relevant deforming force, namely, basement membrane deformation (47). Exocytosis of FM1–43 was found to be strain amplitude dependent and was inhibited by ATP depletion, suggesting that the response in alveolar epithelium is energy dependent. Cells strained by a similar amplitude were also found to have plasma membrane wounds as manifested by FITC-Dx uptake. Strikingly, however, despite the large increases in cell surface area (~35%), the number of injured cells was small (<3%). In addition, the plasma membrane breaks in these cells were found to be reversible and nonlethal. These findings support not only the concept that deformation-induced lipid trafficking in alveolar epithelial cells serves to regulate cell surface area but also that it might prevent and repair deformation-induced plasma membrane breaks.

MECHANISMS OF DEFORMATION-INDUCED LIPID TRAFFICKING

In order for vesicles to fuse with the plasma membrane, they must be recruited and move to the cell surface. Although passive diffusion might explain a portion of vesicular translocation to the plasma membrane, active transport involving cytoskeletal proteins appears to be important. Molecular motors, namely, kinesin, dynein, and myosin, are needed to power the cytoskeletal "tracks" that facilitate membrane movement to the cell surface. "Long-range" vesicle transport seems to occur along polarized microtubules and "short-range" transport along actin filaments (23). This model of transport suggests that separate pools of vesicles might exist and that their recruitment to the cell surface could be time dependent. In sea urchin eggs, Bi and colleagues (5, 6) demonstrated slow and fast phases of vesicle recruitment to sites of cell wounding that were dependent on cytoskeletal motor proteins. The slow phase of vesicle recruitment could be specifically inhibited with SUK-4 antikinesin antibody; butanedione monoxime (a myosin ATPase inhibitor) and an inhibitory peptide to Ca^{2+}/calmodulin-dependent protein kinase were shown to inhibit both the slow and fast phases of recruitment. Ca^{2+}/calmodulin kinase is hypothesized to facilitate wound repair by the release of vesicles from actin-binding sites through phosphorylation of synapsin I (41). The findings from repeated cell-wounding experiments have also strengthened the concept of separate recruitable membrane stores (43). In these experiments, the phenomenon of facilitated membrane resealing was demonstrated; that is, repair of a second wound was more rapid when compared with the first wounding event. This process is Ca^{2+} and protein kinase C (PKC) dependent. By blocking Golgi production of new vesicles with brefeldin A, facilitation is suggested to be a result of PKC activation (from calcium influx from a primary wounding event) and stimulation of Golgi vesicular production that can be recruited to the cell surface for more rapid membrane resealing. The initial wound event is thought to draw on vesicles participating in endocytic trafficking pathways. Blocking the function of proteins involved in the SNARE vesicle docking and fusion process through the use of botulinum neurotoxins and tetanus toxin has also demonstrated the importance of plasma membrane-directed vesicular repair mechanisms in wound healing (5, 41, 43).

Miyake and McNeil (29) have highlighted the role of the subcortical actin cytoskeleton, a site where vesicle accumulation occurs before exocytosis at a membrane disruption. Calcium, through proteins such as gelsolin, has been demonstrated to solate the subcortical actin barrier and as such would allow recruited vesicles to fuse with the cell surface (30). Evidence also exists that cells possess mechanoprotective mechanisms. These might act by adaptation of their subcortical cytoskeleton, thus increasing membrane stabilization and/or the release of growth factors. Forces applied to integrin receptors result in a calcium- and PKC-dependent actin gelation by recruitment of actin-binding protein 280 into cortical adhesion complexes. This actin assembly in turn was found to decrease the stretch sensitivity of calcium-permeable stretch-activated ion channels and to protect the cells from death as measured by propidium iodide uptake (15). Plasma membrane breaks not only serve as a mechanism for transducing intracellular processes but might also serve as a means to release cytoprotective growth factors important for cell proliferation and tissue remodeling and reinforcement. This role for deformation-induced plasma membrane breaks has been coined the "wound-hormone" hypothesis. Basic fibroblast growth factor (bFGF), which has been localized in vivo in developing and adult lungs (18), was found to be released from wounded endothelial cells (27, 31) both in vitro and in vivo. It is proposed that release of bFGF occurs through the induced membrane breaks because bFGF lacks the required signal sequence for exocytic-driven release from cells. This finding provides evidence that injured cells might possess autocrine mechanisms that are utilized for cytoprotection.

EFFECTS OF PLASMA MEMBRANE DISRUPTION ON STRESS RESPONSIVE GENES

In a recent review, Dos Santos and Slutsky (12) have detailed mechanoreceptive mechanisms leading to proinflammatory signaling by lung cells. Among these, the authors emphasized both regulated (i.e., receptor-mediated signaling pathways) and unregulated excitation of stress-responsive genes. Among the latter they

pointed to work by McNeil's group (16), who showed in a series of elegant studies that the calcium influx accompanying plasma membrane disruptions is associated with translocation of the nuclear transcription factor nuclear factor-κB and upregulation of stress-responsive genes. These, in turn, could represent upstream events leading to the production and release of chemokines such as tumor necrosis factor-α and interleukin-8, which can be readily recovered from alveolar spaces of patients.

CONCLUDING REMARKS

We have examined the syndrome ventilator-induced lung injury from the perspective of alveolar epithelial mechanics. In doing so we have speculated how the deformation of the lung matrix could impose injurious stress on the plasma membranes of alveolar epithelial cells. We have drawn on observations from many other cell systems that have been studied in vitro. Therefore, the sequence of events that we have outlined must be viewed as a hypothesis, not a proof. However, we are intrigued by the large number of observations on patients and experimental animals that can be reconciled with the proposed mechanisms. Finally, these mechanisms, if proven relevant, suggest novel therapeutic targets, such as membrane traffic, in the pharmacoprotection from ventilator-associated lung injury.

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