Integrated Stress Response Mediates Epithelial Injury in Mechanical Ventilation

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Abstract

Ventilator-induced lung injury (VILI) is a severe complication of mechanical ventilation that can lead to acute respiratory distress syndrome. VILI is characterized by damage to the epithelial barrier with subsequent pulmonary edema and profound hypoxia. Available lung-protective ventilator strategies offer only a modest benefit in preventing VILI because they cannot impede alveolar overdistension and concomitant epithelial barrier dysfunction in the inflamed lung regions. There are currently no effective biochemical therapies to mitigate injury to the alveolar epithelium. We hypothesize that alveolar stretch activates the integrated stress response (ISR) pathway and that the chemical inhibition of this pathway mitigates alveolar barrier disruption during stretch and mechanical ventilation. Using our established rat primary type I-like alveolar epithelial cell monolayer stretch model and *in vivo* rat mechanical ventilation that mimics the alveolar overdistension seen in acute respiratory distress syndrome, we studied epithelial responses to mechanical stress. Our studies revealed that the ISR signaling pathway is a key modulator of epithelial permeability. We show that prolonged epithelial stretch and injurious mechanical ventilation activate the ISR, leading to increased alveolar permeability, cell death, and proinflammatory signaling. Chemical inhibition of protein kinase RNA-like

endoplasmic reticulum kinase, an upstream regulator of the pathway, resulted in decreased injury signaling and improved barrier function after prolonged cyclic stretch and injurious mechanical ventilation. Our results provide new evidence that therapeutic targeting of the ISR can mitigate VILI.

Keywords: ventilator-induced lung injury; integrated stress response; alveolar epithelium

Clinical Relevance

Alveolar epithelial barrier damage is central to the pathology of acute respiratory distress syndrome (ARDS). Despite lung-protective ventilation strategies, ventilator-induced lung injury contributes to ARDS by overdistending the alveolar epithelium in the inflamed lung regions. Our results show that the integrated stress response pathway is a key mediator of injury signals in epithelial cells in response to ventilator-induced lung injury. Targeted inhibition of the integrated stress response provides a new therapeutic approach to prevent ARDS.

Alveolar epithelial barrier injury is central to the pathology of acute respiratory distress syndrome (ARDS), which results in severe hypoxia and pulmonary edema formation (1). ARDS, which affects an estimated 190,000 patients in the United

States annually, usually presents as a complication of mechanical ventilation in sepsis that contributes to multiple organ failure syndrome (2). Human studies showed that low-tidal-volume mechanical ventilation improves survival

in ARDS (3), but imaging analysis revealed that even lung-protective ventilation can cause alveolar overdistension and injury in the inflamed regions of the lung (4), leading to alveolar barrier damage in some cases (5, 6). Despite extensive

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research, we do not have biochemical strategies to protect the alveolar–epithelial barrier.

The integrated stress response (ISR) is a highly conserved intracellular regulator of four common cell stressors in eukaryotes: the unfolded protein response (UPR), amino acid starvation, anemia (i.e., heme deficiency), and double-stranded DNA (e.g., viral infection). We focused our attention on the UPR because it is induced by hypoxia (7), a well-known factor associated with the pathogenesis of ARDS. The UPR has three cytosolic sensors: protein kinase RNA–like endoplasmic reticulum kinase (PERK), inositol-requiring protein-1 α (IRE1 α), and activating transcription factor 6 (ATF6). Because the PERK-activated response to unfolded protein stress regulates protein translation as part of the ISR signaling cascade, we focus on the downstream effects of PERK pathway-associated ISR in our studies. In this feedback pathway, others have shown that increased and aberrant (unfolded) protein load in the endoplasmic reticulum results in PERK activation and phosphorylation of the α -subunit of eukaryotic initiation factor-2 α (p-EIF2 α), producing a temporary cessation of EIF2 complex–mediated protein translation (8). As a result of a general decrease in protein translation, transcription factors such as activating transcription factor 4 (ATF4) and CCAAT/Enhancer-binding protein homologous protein (CHOP) are selectively translated to promote RNA transcription of growth arrest and DNA damage–inducible protein (GADD34), which then restores protein synthesis by dephosphorylation of EIF2 α when transiently activated (7). Continuous activation of the system by persistent UPR, however, results in cell death and activates the innate immune system (9). Epithelial CHOP-mediated signaling has been implicated in the pathology of lung diseases, including pulmonary fibrosis (10) and hyperoxia-induced lung injury (11), but its role in stretch-induced injury is unknown.

In this study we describe, for the first time, ISR activation in alveolar epithelial cells exposed to mechanical stretch *in vitro*, and extend our findings to an *in vivo* ventilator-induced lung injury (VILI) model to reveal mechanistic links of ISR to

VILI pathology and, most importantly, show that inhibition of the ISR pathway provides a new therapeutic target in ARDS.

Materials and Methods

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Sprague-Dawley rats ($n = 174$, 250–300 g) were purchased from Charles River Laboratories (Horsham, PA).

Alveolar Epithelial Monolayer Mechanical Stretch Protocol

Epithelial monolayers were prepared from freshly isolated rat type II pneumocytes ($n = 106$ rats) as previously described (12). In brief, isolated pneumocytes were cultured on fibronectin-coated silastic membranes. The cells transformed into type I–like alveolar epithelial cells (AEC-I) and formed monolayers, expressing only AEC-I surface markers (13). Monolayers were exposed to 25% stretch for 6 hours or used as unstretched controls. Time controls were stretched 1 or 12 hours. Additional monolayers were pretreated with PERK inhibitor GSK2606414 (PI; EMD Millipore, Danvers, MA) or its vehicle (0.01% DMSO). Tunicamycin-treated cells served as an ISR-positive benchmark. In gene-silencing studies, we pretreated monolayers

with PERK, ATF4, CHOP, or nontargeting siRNA (Dharmacon, Pittsburgh, PA). Culture media were analyzed using a multiplex bead assay (Millipore) for cytokines. Western blotting was performed on total cell protein using antibodies for phosphorylated (p)- and total (t)-EIF2 α and PERK (Cell Signaling, Danvers, MA); p- and t-IRE1 α , and XBP1 (Abcam, Cambridge, MA); ATF4 (Sigma-Aldrich, St. Louis, MO); and CHOP and β -actin (Santa Cruz Biotechnologies, Santa Cruz, CA) as previously described (14–16). Immunoprecipitation was performed for PERK (Santa Cruz) using the Pierce Classic IP kit (ThermoFisher Scientific, Waltham, MA; see online supplement).

Paracellular Permeability and Cell Death Studies

To detect stretch-induced permeability changes, cells were plated on biotin-containing fibronectin and permeability was detected when apical fluorescein isothiocyanate (FITC)-labeled streptavidin (ThermoFisher) increased binding to basal biotin. Cell death was assessed with ethidium homodimer-calcein-AM stain (ThermoFisher). Florescent light emission was captured with a Nikon TE300 microscope (Melville, NY) and quantified (see online supplement).

Table 1. Mechanical Stretch Induces Lung Injury–Specific Gene Transcription in AEC-I

Gene Ontology Category*	Percent of Significant Genes (%)	Representative Genes in Functional Groups	Fold Change (S versus NS)
Cation binding	18.75	<i>Rhobtb1</i>	0.51
Nuclear lumen	7.8	<i>Areg</i>	1.77
Cell death	7.57	<i>Gadd45</i>	0.35
Regulation of transcription	6.73	<i>Atf4</i>	1.94
		<i>Ddit3 (Chop)</i>	1.75
mRNA processing	1.92	<i>Ccl3 (Mip1a)</i>	3.9
		<i>Il1a</i>	2.55
		<i>Il1b</i>	1.97
Steroid metabolism	1.08	<i>Ptgs2 (Cox2)</i>	3.36
Protein kinase activity	0.96	<i>Map3k8</i>	2.68
Epithelial-to-mesenchymal transition	0.6	<i>Cldn18</i>	0.57
		<i>Cldn23</i>	0.5
		<i>Cldn1</i>	1.54

Definition of abbreviation: AEC-I, type I–like alveolar epithelial cells.

*Gene Ontology (GO) categories significantly enriched with genes after mechanical stretch (S) compared with unstretched (NS) controls. Representative genes of interest with fold change S versus NS in expression are shown. The complete gene list is provided in Table E1. Significance analysis was performed with the DESeq2 program package, adjusted P value < 0.05. GO functional grouping was performed with NIH DAVID. The complete GO analysis is shown in Table E2. $n = 5$ biological replicates/condition.

Figure 1. Mechanical stretch activates integrated stress response (ISR) signaling independently of the inositol-requiring protein-1α (IRE1α) pathway in primary alveolar type I-like epithelial (AEC-I) cells. AEC-I cells were cultured on silastic membranes coated with fibronectin to form monolayers. (A) Monolayers were subjected to biaxial stretch for 6 hours with a 25% surface change (S) and compared with unstretched controls (NS). Low-dose tunicamycin (TN, 1 μg/ml, 24 h treatment) was used as a positive control, and parameters were compared with DMSO-treated (0.01%), unstretched vehicle controls (VC). Mechanical stretch significantly increased the phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (p-PERK), which resulted in activation of ISR marked by eukaryotic initiation factor-2α phosphorylation (p-EIF2α) and the activation of activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein homologous protein (CHOP). Mechanical stretch did not affect phosphorylation of IRE1α (p-IRE1α) or activation of its downstream transcription factor, X-box binding protein 1 (XBP1). Quantified densities are shown in B–G. (H) Mechanical stretch activates the ISR in a time-dependent fashion in AEC-I monolayers. We detected time-dependent increases in p-EIF2α, ATF4, and CHOP after biaxial mechanical stretch with a 25% surface change for 1–12 hours. Maximum changes were observed at 6 hours. Quantified densities are shown in I–K. (L) Only a mild increase in cell death was detected at 6 hours with ethidium homodimer staining, but it increased to high levels by the 12-hour time point. We used total (t)-PERK, t-EIF2α, t-IRE1α, and β-actin as loading controls. Statistics: the Kruskal–Wallis test was performed for multiple-group comparison, and intergroup differences were analyzed using the Wilcoxon rank-sum test. $n = 6$ –8 biological monolayer replicates/condition; * represents a significant increase in protein phosphorylation (B, C, F, and I), protein expression (D, E, G, J, and K), and cell death percentage (L); $P < 0.05$. Data are presented as averaged values $\pm$ SEM.

PERK Inhibitor Treatment and Mechanical Ventilation Protocol

Rats were pretreated with 30 mg/kg PI (GSK Pharmaceuticals, Collegeville, PA) or its vehicle via gavage ($n = 32$). Subsequently, the animals received mechanical ventilation (4 h, 20 ml/kg tidal volume) via tracheostomy (Harvard Apparatus, Holliston, MA) or were allowed to breathe spontaneously. The rats were killed and samples were obtained for analysis (see online supplement). Bronchoalveolar lavage (BAL) samples were used for cell counts and protein content measurements. BAL and serum interleukin (IL)-18 were quantified by ELISA (Ray Biotech, Norcross, GA). Western blotting was performed on tissue protein for p-EIF2 α , ATF4, CHOP, and β -actin as explained above. Lung slides were prepared for immunofluorescence and hematoxylin-eosin staining. In a separate experiment ($n = 36$), endo/epithelial permeability was measured with FITC-labeled albumin (Sigma-Aldrich) extravasation (see online supplement).

RNA Analysis

We obtained global quantitative mRNA measures via QuantSeq (Lexogen, Vienna, Austria). Sequencing libraries were prepared from AEC-I total RNA. Reads were aligned with STAR (v.2.5.1b) to the reference *Rattus norvegicus* build 6, and the DESeq2 R package was used to measure the significance of differentially expressed genes between the stretched and unstretched samples (17). The data are available in the Gene Expression Omnibus under accession number GSE89024. *Il18* gene expression of PERK siRNA-treated and control cells was performed with TaqMan PCR (see online supplement).

Statistics

For comparative studies of densitometry, cytokine levels, quantitative PCR, and cell count, we used the Kruskal–Wallis test for multigroup comparisons, and analyzed intergroup differences using the Wilcoxon rank-sum test. For fluorescent image intensity analysis, we used ANOVA (JMP Software, Cary, NC). Results are presented as mean $\pm$ SEM. The significance level was $P < 0.05$.

Results

To identify genes and associated intracellular pathways that mediate injury

signaling in the alveolar epithelium, we used QuantSeq to compare the gene-expression profile of mechanically stretched AEC-I monolayers with that of unstretched controls. Cells were exposed to a biaxial cyclic (15/min) stretch for 6 hours with a 25% surface area change. This stretch magnitude corresponds to an 80% increase in total rat lung capacity, or 15 ml/kg positive-pressure mechanical ventilation (12). A Gene ontology enrichment analysis of the 924 differentially expressed genes (see Table E1 in the online supplement) showed that AEC-I cells respond to mechanical stretch primarily by regulating genes of cellular stress pathways (Tables 1 and E2). Among these genes were *Atf4* and *Chop* (aka DNA damage-inducible transcript 3, *Ddit3*), two transcription factors of the ISR pathway. The ISR pathway is a central integrator of cellular stress responses, and it has been implicated in the pathology of lung diseases (18). To validate the increase in mRNA expression that we observed in *Atf4* and *Chop*, we obtained results from previous microarray studies performed on stretched AEC-I (25% surface area change for 6 h) (19) and on mouse lungs ventilated with 10 ml/kg positive pressure and 2 cm H₂O positive end expiratory pressure (PEEP) for 8 hours (20). Both mechanical stretch and mechanical ventilation resulted in significantly increased mRNA expression of *Atf4* (2.4- and 1.6-fold, respectively) and *Chop* (5.0- and 1.8-fold, respectively) compared with untreated controls (adjusted P value < 0.05 ; statistical analysis was performed with ANOVA and Welch's two-sample t test, respectively). When Dombroski and colleagues (21) treated immortalized B cells with tunicamycin (4 μ g/ml for 18 h), a universal trigger of ISR, they found similar increases in the expression of *Atf4* and *Chop* genes (2.5- and 8.2-fold, respectively; adjusted P value < 0.05 via Bonferroni-corrected ANOVA). Consequently, we hypothesized that inhibition of ISR signaling would mitigate epithelial injury.

Cyclic Stretch Activates ISR Signaling

To study epithelial stress signaling in response to mechanical stretch, we first investigated the mechanism of ATF4 and CHOP activation in our *in vitro* cell stretch model. The phosphorylation of PERK and EIF2 α , as well as the expression of ATF4

Figure 2. PERK siRNA inhibition decreases stretch-induced epithelial monolayer permeability. (A) AEC-I monolayers were transfected with nontargeting (siRNA/NT) or PERK-specific (siRNA/PERK) siRNA (0.5 μ g, 24 h treatment) and subjected to cyclic mechanical stretch (25% surface change for 6 h). Unstretched monolayers served as controls. (B) PERK knockdown resulted in a significant decrease in t-PERK expression. (C) To measure monolayer permeability, cells were cultured on silastic membranes coated with biotinylated fibronectin. After the study, the membranes were stained with fluorescein isothiocyanate (FITC)-labeled streptavidin. Under unstretched conditions, the monolayer was impermeable to large molecules, which prevented streptavidin–biotin binding. Stretch resulted in cell–cell contact disruption and subsequent binding of streptavidin to the membrane-bound biotin, which was detected by fluorescent microscopy. PERK inhibition resulted in decreased monolayer permeability. For densitometry data analysis, the Kruskal–Wallis test was performed for multiple-group comparison, and intergroup differences were analyzed using the Wilcoxon rank-sum test. For permeability studies, we used one-way ANOVA with post hoc Dunnett's test, and for multiple conditions two-way ANOVA was performed with Tukey–Kramer *post hoc* analysis. * represents a significant change between siRNA/NT and siRNA/PERK conditions (B), and NS and S conditions (C); # represents a significant decrease between siRNA/NT and siRNA/PERK S conditions; $P < 0.05$; $n = 4$ biological monolayer replicates/condition. siRNA, small interfering RNA.

and CHOP, was compared between stretched and unstretched monolayers. We used tunicamycin (1 μ g/ml pretreatment for 24 h), a canonical activator of cell stress signals, as a positive control to benchmark the magnitude of ISR activation by mechanical stretch. We found that cyclic stretch resulted in increased PERK, EIF2 α phosphorylation, and subsequent ISR signaling, similar to what was seen with tunicamycin (Figures 1A–1E). To evaluate the role of PERK-independent stress signaling in our model, we measured IRE-1 α phosphorylation and the activation of its downstream transcription factor, factor X-box binding protein-1 (XBP1). Mechanical stretch did not increase IRE-1 α phosphorylation or XBP1 expression (Figures 1A, 1E, and 1F). We next assessed the time dependence of ISR signaling in response to stretch. We found that cyclic stretch activated ISR in a time-dependent manner by increasing p-EIF2 α , ATF4, and CHOP significantly by 6 hours (Figures 1H–1K). To assess cell death–mediated ISR activation, we stained monolayers with

ethidium homodimer. Substantial cell death was not detected before 12 hours (Figure 1L). These findings suggest that the PERK/p-EIF2 α /ATF4/CHOP cascade is a stretch-induced transmitter of mechanical stress signals in the epithelium.

PERK Inhibition Blunts ISR Activation and Improves Epithelial Barrier Function

To evaluate whether stretch-induced PERK activation affects epithelial monolayer barrier integrity, we treated monolayers with siRNA specific to *PERK* and compared the results with those obtained from the nontargeting siRNA treatment (Figures 2A and 2B). Monolayer integrity was studied via FITC-labeled streptavidin binding to biotin. An intact epithelial monolayer barrier excluded streptavidin from interacting with membrane-bound biotin, but stretch disrupted cell–cell contacts, allowing binding of the two molecules at the basal surface, resulting in quantifiable FITC emission. PERK inhibition improved monolayer dysfunction (Figure 2C). We

next tested whether PI, a specific chemical inhibitor of PERK phosphorylation, can be used to inhibit ISR activation. We observed that PI treatment decreased p-PERK, p-EIF2 α , ATF4, and CHOP (Figures 3A–3E), as well as stretch-induced monolayer permeability, confirming that PERK phosphorylation is critical for ISR-mediated permeability changes (Figure 3F). We also observed a decrease in stretch-induced cell death with PI treatment (Figure 3G), but cell death in untreated cells was low (Figure 1L). Taken together, our data provide new evidence that alveolar epithelial barrier function is modulated by PERK activation, and PI treatment can prevent stretch-induced monolayer barrier damage.

The ISR Regulates Epithelial Cytokine Release in an IL-18-Specific Manner

Given the interest in cytokines as a biomarker of injury, we assessed the contribution of ISR signaling to stretch-induced proinflammatory mediator release by measuring the level of 26 common

Figure 3. PERK phosphorylation inhibition reduces epithelial monolayer dysfunction. AEC-I monolayers were pretreated with 1 μ M PERK phosphorylation inhibitor GSK2606414 (PI) or its vehicle (0.01% DMSO) for 18 hours before experimentation to evaluate ISR-induced monolayer damage. (A–D) Levels of p-PERK, p-EIF2 α , ATF4, and CHOP were unaffected by PI treatment in unstretched monolayers (NS and NSPI). However, PI treatment (SPI) significantly reduced mechanical stretch (S)-induced ISR activation. Quantified densities are shown in B–E. (F) PI pretreatment significantly reduced monolayer permeability as measured by FITC-labeled streptavidin binding to biotin. (G) We detected decreased stretch-induced cell death in monolayers pretreated with PI. The same statistical tests were performed as described in Figure 2. * represents a significant difference between NS and S or SPI conditions; # represents a significant difference between S and SPI conditions; $P < 0.05$; $n = 6$ –8 biological monolayer replicates/condition.

Figure 4. ISR regulates epithelial cytokine levels in an IL-18-specific manner. (A–C) Increased IL-18, IL-1α, and macrophage inflammatory protein-1α (MIP-1α) levels were detected in the supernatant of stretch monolayers. PI treatment decreased IL-18 levels but did not affect IL-1α and MIP-1α. (D) We used siRNA to inhibit ISR signaling in stretched (S) and unstretched (NS) monolayers (0.5 μg siRNA, 24 h pretreatment). CHOP inhibition (siRNA/CHOP) decreased CHOP expression but did not affect ATF4. ATF4 inhibition (siRNA/ATF4) resulted in a partial reduction of both CHOP and ATF4. Transfection reagent (VC) and nontargeting siRNA (siRNA/NT) served as controls. Protein expression was normalized to β-actin and quantified by densitometry. Quantified densities are shown in E and F. (G) CHOP, ATF4, and PERK knockdown significantly decreased the IL-18 levels measured in the supernatant of stretched monolayers. Statistics: the Kruskal–Wallis test was used for multiple-group comparison, and intergroup differences were analyzed using the Wilcoxon rank-sum test. For cytokine studies, we used $n = 8$ –9 biological monolayer replicates/condition. For siRNA experiments, we used $n = 4$ –5 biological monolayer replicates/condition. In A–C, * represents a significant increase in cytokine levels NS versus S, and # represents a significant decrease between S and SPI conditions. In E and F, * represents a significant increase in protein expression in NS versus S conditions, # represents a significant decrease in ATF4 and CHOP protein expression compared with the siRNA/NT condition in unstretched monolayers, and \$ represents a significant decrease in ATF4 and CHOP expression compared with the siRNA/NT condition in stretched monolayers. In G, * represents a significant increase in IL-18 levels in S versus NS conditions, and # represents a significant decrease in IL-18 levels in siRNA/CHOP, siRNA/ATF4, and siRNA/PERK conditions versus siRNA/NT. The significance level was set at $P < 0.05$ for all experiments.

cytokines in the culture media in the presence and absence of PI. We found a generally low cytokine response to stretch, with modest elevations in IL-18, IL-1α, and

MIP-1α (Figures 4A–4C). PI treatment decreased IL-18 levels only (Figure 4A). To study the regulation of IL-18 by ISR, we treated stretched and unstretched cells with

CHOP, ATF4, and PERK siRNA (Figures 2A, 2B, and 4D–4F). ATF4 siRNA knockdown decreased expression of both ATF4 and CHOP protein, but CHOP siRNA treatment did not affect ATF4 protein levels, confirming that ATF4 is the activator of CHOP signaling in epithelial cells (Figures 4D–4F). IL-18 levels in cell culture media were compared with those observed with no siRNA and nontargeting siRNA treatments. We found that ISR inhibition reduced released IL-18 levels (Figure 4G), but PERK siRNA treatment did not affect *Il18* mRNA transcription (Figure E1). Based on these data, we concluded that ISR regulates the posttranscriptional modification of IL-18 in the alveolar epithelium.

ISR Activation in VILI

To establish the feasibility of translating our *in vitro* findings to *in vivo*, we used a rat model of VILI. Animals were pretreated via oral gavage with either 30 mg/kg PI or its vehicle for 4 hours before the start of the experiment. Rats were ventilated with 20 ml/kg tidal volume via tracheostomy for 4 hours without PEEP or a recruitment maneuver to induce injury. The lung tissue of ventilated animals was compared with that of spontaneously breathing controls. Immunofluorescence analysis of thin-cut sections showed increased EIF2α phosphorylation in response to mechanical ventilation (Figures 5A–5C) in multiple alveolar cell types, including type I alveolar epithelial cells labeled with occludin (Figure 5D). The tissue-homogenate results support our *in vitro* epithelial stretch findings, showing ISR activation via increased EIF2α phosphorylation and subsequent activation of ATF4 and CHOP after mechanical ventilation (Figures 6A–6D). PI treatment significantly decreased ISR activation in the lung tissue of ventilated animals compared with vehicle-treated ventilated rats (Figures 6A–6D), but PI had no effect on spontaneously breathing animals. These findings suggest that PERK inhibition *in vivo* can alter ISR signaling.

Oral PI Treatment Improves VILI

Acute lung injury is characterized by lung inflammation, pulmonary edema, and proinflammatory cytokine release. To test whether PI treatment *in vivo* reduces lung injury, we first measured its impact on the

Figure 5. Injurious mechanical ventilation increases EIF2 α phosphorylation *in vivo*. The fluorescent light emission of Alexa Fluor 488-labeled p-EIF2 α antibody was compared between lung tissues obtained from (A) spontaneously breathing and (B) mechanically ventilated (20 ml/kg tidal volume ventilation without positive end-expiratory pressure for 4 h via tracheostomy) rats. EIF2 α staining was present in multiple alveolar cell types, including AEC-I cells labeled with occludin. *Green*, Alexa Fluor 488-labeled p-EIF2 α ; *red*, Alexa Fluor 594-labeled occludin; *blue*, DAPI nuclear stain. *Scale bar*, 100 μ m. *Insets* show 100 μ m enlarged areas. (C) Quantified confocal images showed a significant p-EIF2 α increase in ventilated rats compared with spontaneously breathing controls. (D) Enlarged epithelial cells, with *arrows* pointing at intracellular p-EIF2 α staining. *Scale bar*, 10 μ m. Statistics: two-way ANOVA. * represents a significant increase C versus V conditions; $P < 0.05$; $n = 3$ biological replicates/condition and 5 images/biological replicates. C, control (spontaneously breathing); V, ventilated (mechanically).

BAL cell count. PI treatment significantly reduced VILI-mediated inflammatory cell recruitment to the alveolus, marked by a reduction of the BAL total, macrophage, and neutrophil cell counts to baseline levels compared with ventilated animals that only received vehicle (Table 2). It is important to mention that the PI treatment did not result in immunosuppression in spontaneously breathing animals. In addition, the VILI-induced pulmonary edema formation observed in vehicle-treated rats was significantly reduced in the PI-treated condition, as measured with the surrogate parameter of increased BAL total protein content (Figure 6E). PI pretreatment significantly reduced alveolocapillary permeability in the treatment group compared with vehicle-treated animals (Figure 6E). Our findings were confirmed by the FITC-labeled albumin extravasation index, a more sensitive and specific measurement of membrane integrity (Figure 6F). Lung-tissue-based analysis also showed decreased injury with PI treatment (Figures 6G and E2). To evaluate proinflammatory cytokine release, we measured IL-18 levels. Mechanical

ventilation increased IL-18 levels in the BAL (Figure 6H) and serum (Figure 6I). We detected a significant decrease of IL-18 in the BAL, but not the serum, of PI-treated animals. These results show that PI inhibition mitigates VILI. Furthermore, our combined *in vivo* and *in vitro* data provide evidence that ISR regulates both alveolar permeability and proinflammatory signaling in the lung.

Discussion

In this study, we observed increased PERK-mediated ISR signaling in alveolar epithelial cells in response to cyclic stretch and mechanical ventilation. The alveolar epithelium showed an ISR-dependent permeability increase, proinflammatory cytokine production, and cell death. Inhibition of PERK signaling mitigated lung injury signals *in vitro* and *in vivo*. This novel observation provides the first evidence that ISR regulates alveolar epithelial homeostasis in response to mechanical stimuli. Our experiments also show that ISR activation is time dependent and may affect cellular function

independently of cell death in the alveolar epithelium. In the absence of significant cell death, ISR transcription factor activation follows a bimodal pattern (22). With EIF2 α phosphorylation, ATF4 initially responds with increased expression and subsequently upregulates CHOP transcription (23). With continued EIF2 α activation, an adaptive transcription factor downregulation occurs to prevent premature cell death (24). In our model, the ISR signal peaks at 6 hours, with an adaptive decrease in ATF4 and CHOP by 12 hours in the presence of persistent EIF2 α activation. We observed increased epithelial barrier permeability without significant cell death after 6 hours of stretch exposure. Our laboratory has shown that early epithelial barrier dysfunction is caused by tight-junction protein dissociation (25). It is also known that ISR interacts with Ca^{2+} signaling (26) and members of the Rho kinase family of small-molecule guanylyl-triphosphates (27), both of which are critical modulators of tight-junction integrity and alveolar epithelial cell permeability in cyclic stretch (28, 29).

The UPR-responsive IRE1 α and ATF6 pathways are described in the literature as parallel mechanisms to PERK-mediated

Figure 6. Oral PI treatment reduces ISR activation and improves VILI. (A–D) Rats were treated with 30 mg/kg GSK2606414 compound (PI) or its vehicle (0.1% TWEEN 80 in 0.5% hydroxyethyl-methylcellulose) via oral gavage. Four hours later, the animals were anesthetized and mechanically ventilated (V) as previously described or allowed to breathe spontaneously (C). PI treatment (VPI) significantly reduced VILI-mediated phosphorylation of EIF2 α and the protein expression of ATF4 and CHOP but did not affect controls (CPI). (E) VILI increased the BAL total protein content, suggesting increased alveolocapillary permeability and pulmonary edema formation. PI-pretreated animals were protected from a VILI-induced permeability increase. (F) Alveolocapillary barrier dysfunction in VILI was confirmed by FITC-labeled albumin extravasation to the alveoli. VPI animals showed a decreased FITC-labeled albumin permeability index compared with V. (G) A composite lung injury score was calculated for all animals. PI pretreatment mitigated the lung injury score in ventilated animals. (H) VILI significantly increased proinflammatory cytokine IL-18 levels in the BAL. PI-pretreated animals exhibited reduced IL-18 BAL levels. (I) Serum IL-18 levels also increased in the serum of ventilated animals, but PI did not affect the levels. Statistics: the Kruskal–Wallis test was performed for multiple-group comparison, and intergroup differences were analyzed using the Wilcoxon rank-sum test. $n = 5$ – 9 animals/condition; * represents a significant increase in V versus C conditions, and # represents a significant decrease in VPI versus V conditions ($P < 0.05$).

ISR. They coregulate protein synthesis in response to cellular stress and have overlapping functions (30). Experiments with PERK-null mice suggest that IRE1 α and ATF6 can compensate for the loss of PERK. However, the same mice develop

an inflammatory phenotype with severe pancreas insufficiency, bone abnormalities, and premature mortality (31), demonstrating that the three pathways also have independent roles. This paradigm can be explained by the three pathways'

different sensitivities to various forms of endoplasmic reticulum stress (32, 33). In our cell-stretch model, we describe PERK-mediated ISR activation that is independent of the IRE1 α pathway. These findings suggest that PERK is a specific sensor of mechanical stretch and that this effect is independent of the UPR. Similarly, Mak and colleagues reported that mouse fibroblasts responded to mechanical force tension with PERK-induced apoptosis without IRE1 α or ATF6 signaling (34). Our *in vitro* findings are strengthened by the striking inhibition of inflammation and alveolocapillary permeability by PI in the rat VILI model. Based on these observations, we conclude that selective PERK inhibition can be used to manipulate cellular responses to stretch and thereby mitigate epithelial injury.

Our comprehensive cytokine profiling resulted in the identification of MIP-1 α , IL-1 α , and IL-18 as markers of epithelial injury. Only low levels of cytokines are released from the alveolar epithelium in response to a physiological magnitude of cell stretch, and they have not been shown

Table 2. PERK Inhibition Reduces Inflammation in Lung Injury

Condition	Total Cell Count ($\times 10^3$ cells/ml BAL)	Macrophage Cell Count ($\times 10^3$ cells/ml BAL)	Neutrophil Cell Count ($\times 10^3$ cells/ml BAL)
C	1.32 $\pm$ 0.18	0.56 $\pm$ 0.04	0.003 $\pm$ 0.001
CPI	1.44 $\pm$ 0.23	0.77 $\pm$ 0.17	0.003 $\pm$ 0.003
V	3.01 $\pm$ 0.13*	1.69 $\pm$ 0.23	0.48 $\pm$ 0.06*
VPI	1.75 $\pm$ 0.14*	1.28 $\pm$ 0.26	0.03 $\pm$ 0.009†

Definition of abbreviations: BAL, bronchoalveolar lavage; C, control, spontaneously breathing animals, pretreated with drug vehicle (0.1% TWEEN 80 in 0.5% hydroxyethyl-methylcellulose) 4 hours before they were killed; CPI, control animals pretreated with 30 mg/kg PERK inhibitor GSK2606414 4 hours before they were killed; PERK, protein kinase RNA-like endoplasmic reticulum kinase; V, vehicle-pretreated rats mechanically ventilated for 4 hours with 20 ml/kg tidal volume without positive end-expiratory pressure and a recruitment maneuver; VPI, mechanically ventilated rats as in condition V, pretreated with 30 mg/kg PI 4 hours before the start of ventilation.

Mechanical ventilation significantly increased the total, macrophage, and neutrophil cell count in the BAL fluid of mechanically ventilated rats. Pretreatment with PERK inhibitor significantly reduced the total BAL macrophage count and neutrophil accumulation in the alveolar space.

$P < 0.05$; $n = 8$ animals per condition.

* represents a significant change in cell count C versus V condition.

† represents a significant difference between V and VPI conditions.

Figure 7. Hypothetical role of ISR in the molecular pathology of VILI. Mechanical ventilation–induced overstretch of the alveolar epithelium and lung tissue results in PERK autophosphorylation and subsequent phosphorylation of EIF2 α . Concomitantly, p-EIF2 α activates the transcription factors ATF4 and CHOP downstream, resulting in proinjury signaling. ISR-mediated injury signals induce early epithelial permeability changes, proinflammatory cytokine release, and cell death, all of which contribute to barrier dysfunction and concomitant pulmonary edema formation.

to contribute to alveolar damage (35, 36). However, they may serve as mediators of injury signals. Epithelial-derived MIP-1 α is a strong neutrophil chemoattractant that has been shown to induce IL-1 and TNF- α secretion from recruited inflammatory cells (37). IL-1 α released from damaged epithelial cells resulted in fibroblast activation in a model of pulmonary fibrosis (38). Recently, Nowarski and colleagues reported that the deletion of IL-18 from colonic epithelial cells caused increased gut permeability and severe colitis (39), and we previously described IL-18 as a critical mediator of lung injury in patients with sepsis and ARDS (40). We further investigated the control of released IL-18 from the epithelium, and found that it is equally regulated by PERK, ATF4, and CHOP after transcription. In macrophages, which are the major source of the cytokine, IL-18 is produced in its pro form by the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)-containing inflammasomes and cleaves to its active (mature) form by Caspase (CASP)-1 (41). The NLRP3 inflammasome, which is most studied in response to proinflammatory signals, is sensitive to CHOP activation (42). In lung parenchymal cells, *Il18* mRNA

is constitutively expressed and RNA levels reflect pro-IL-18 protein levels (43). Recent data suggest that in alveolar epithelial cells, the cleavage of pro-IL-18 to mature IL-18 is independent of NLRP3 inflammasome or CASP-1 (36). In our study, we measured extracellular levels of mature IL-18. Our analysis cannot directly determine whether the observed changes in IL-18 in response to ISR inhibition were the result of decreased cytokine maturation or release, but data from Muñoz and colleagues suggest that secreted IL-18 levels correlate with intracellular expression of the mature cytokine (43). Our data shed light on a new mechanism by which IL-18 is controlled in the epithelium, and, more importantly, strengthen our hypothesis that epithelial cytokine levels are modulated upon stretch response. Although we did not investigate the direct benefit of reduced IL-18 levels for alveolar permeability, our combined *in vitro* and *in vivo* data suggest that IL-18 should be investigated further as a marker of alveolar injury.

Our study raises three questions about the cellular and molecular bases of ISR activation in VILI. First, how does PERK sense mechanical stretch? One possible

explanation is the interaction of PERK with cell-surface Rho-kinase Rac-1. Our laboratory has shown that cell stretch rapidly and directly activates Rac-1, which in turn modulates epithelial monolayer permeability (28). Second, is ISR activation cell specific in VILI? Our data show increased pEIF2 α staining in cell types other than epithelial cells (presumably endothelial cells and macrophages) of mechanically ventilated lung tissue (Figure 5). Stretch data obtained with macrophages and endothelial cells show inducible injury pathways, suggesting that ISR activation takes place in multiple cell types that may contribute to injury signaling (44, 45). Third, and most importantly, what are the biological and clinical implications of ISR inhibition in lung disease? In our hands, one dose of PI was sufficient to inhibit the ISR response without drug-related animal death or severe side effects. Our preliminary measurements confirm steady drug levels of 2 μ M in the lung tissue at the end of the 4 hours of mechanical ventilation (data not shown). These data suggest that a short interruption of the ISR system to modify acute lung disease is beneficial.

Our study is subject to limitations. In the absence of reliable mouse alveolar epithelial cell stretch models, we used rat AEC-I cells, which form strong monolayers. Currently there are no genetic-deletion models of ISR in rats; hence, we used siRNA and chemical inhibition in our *in vitro* studies. This approach is limited by the fact that we achieved only partial blockade of the pathway. Based on our experiments performed with rat AEC-I, we identified PERK as the primary activator of the ISR in response to mechanical stretch. However, as mentioned above, PERK gene-deleted mice have multiple anatomic abnormalities (31), which makes them unsuitable for VILI experiments. As an alternative, in our *in vivo* rat studies, we used PERK chemical inhibition. PI is an orally available, highly specific inhibitor of PERK that can be used to study the pharmacological action of PERK *in vivo*, but it remains unclear whether the drug affects other proinjury pathways. Effects on myosin light chain kinase 1 (MLCK1) are of particular concern because of its interaction with tight-junction proteins (46). At a 10 μ M concentration (significantly higher than the 2 μ M tissue concentrations used in our studies), PI inhibition of MLCK1

is only 15% (47). The low water solubility of PI precludes its intravenous use for ARDS rescue therapy, but its high oral availability makes it attractive to study as a preventative measure for VILI.

Overall, our findings suggest that the ISR pathway is activated in VILI and that selective inhibition of PERK reduces this activation, as evidenced by improved epithelial barrier function and reduced

inflammatory signaling (Figure 7). Further studies to understand the role of ISR signaling in the alveolar epithelium will uncover mechanisms of alveolar injury in ARDS. Because oral PERK inhibitors are available due to previous antitumor (48) and antineurodegenerative (18) effects shown in animal models, the development of selective inhibitors of PERK phosphorylation for preclinical testing in VILI and ARDS can be explored as a

therapeutic intervention to prevent ARDS. ■

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