Phosphoinositide 3-kinase, Src, and Akt modulate acute ventilation-induced vascular permeability increases in mouse lungs

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Miyahara T, Hamanaka K, Weber DS, Drake DA, Anghelescu M, Parker JC. Phosphoinositide 3-kinase, Src, and Akt modulate acute ventilation-induced vascular permeability increases in mouse lungs. Am J Physiol Lung Cell Mol Physiol 293: L11–L21, 2007. First published February 23, 2007; doi:10.1152/ajplung.00279.2005.—To determine the role of phosphoinositide 3-OH kinase (PI3K) pathways in the acute vascular permeability increase associated with ventilator-induced lung injury, we ventilated isolated perfused lungs and intact C57BL/6 mice with low and high peak inflation pressures (PIP). In isolated lungs, filtration coefficients (Kf) increased significantly after ventilation at 30 cmH2O (high PIP) for successive periods of 15, 30 (4.1-fold), and 50 (5.4-fold) min. Pretreatment with 50 μM of the PI3K inhibitor, LY-294002, or 20 μM PP2, a Src kinase inhibitor, significantly attenuated the increase in Kf, whereas 10 μM Akt inhibitor IV significantly augmented the increase in Kf. There were no significant differences in Kf or lung wet-to-dry weight (W/D) ratios between groups ventilated with 9 cmH2O PIP (low PIP), with or without inhibitor treatment. Total lung β-catenin was unchanged in any low PIP isolated lung group, but Akt inhibition during high PIP ventilation significantly decreased total β-catenin by 86%. Ventilation of intact mice with 55 cmH2O PIP for up to 60 min also increased lung vascular permeability, indicated by increases in lung lavage albumin concentration and lung W/D ratios. In these lungs, tyrosine phosphorylation of β-catenin and serine/threonine phosphorylation of Akt, glycogen synthase kinase 3β (GSK3β), and ERK1/2 increased significantly with peak effects at 60 min. Thus mechanical stress activation of PI3K and Src may increase lung vascular permeability through tyrosine phosphorylation, but simultaneous activation of the PI3K-Akt-GSK3β pathway tends to limit this permeability response, possibly by preserving cellular β-catenin.

capillary permeability; β-catenin; adherens junction

THE ARDS NETWORK STUDY has demonstrated the importance of protective ventilation strategies to prevent ventilator-induced lung injury (VILI) (10). Previous studies showing pharmacological reversal of the increases in pulmonary vascular permeability in response to high airway and vascular pressures indicate that an active endothelial response to mechanical strain controls permeability rather than a passive stress-induced failure of the capillary wall (43, 46, 70). Protective ventilation strategies using low tidal volumes were associated with a reduced inflammatory response and increased survival in acute respiratory distress syndrome (ARDS) patients. However, the signaling mechanisms of lung distention-induced increases in vascular permeability remained to be fully defined (20, 66, 76). Lung microvascular permeability increases rapidly after only 20 min of high peak inflation pressure (PIP) ventilation in isolated mouse lungs, whereas proinflammatory cytokine production increases at a much later time (74). In addition, inhibition of the rapidly responding cytosolic phospholipase A2 (cPLA2) pathway attenuated the permeability response to high PIP ventilation (75). These results support the concept of a rapid onset of capillary permeability resulting from tensile failure and signal transduction events in endothelial cells such as calcium entry and phosphorylation of junctional proteins rather than acute effects of proinflammatory cytokines (20, 45, 47, 74). Protein leak and edema may then augment inflammation and injury.

Phosphoinositide 3-kinases (PI3K) are enzymes that generate lipid second messenger molecules, resulting in the activation of multiple intracellular signaling cascades (22, 37, 38, 66). PI3Ks play an important role in cytoskeletal remodeling, metabolic control, and proinflammatory cytokine production (68). Lionetti et al. (30) found reduced histological evidence of lung injury after high volume ventilation in PI3K knockout mice after lung injury with saline lavage and 3 h of ischemia. Also, Uhlig et al. (67) showed that PI3K inhibition attenuated NF-κB activation and inflammatory cytokine gene activation during high PIP ventilation. However, in neither study were indexes of the acute vascular permeability response to high volume inflation measured or the functional effect of signal molecules downstream from PI3K on vascular permeability determined. PI3K activity generates phosphoinositides that anchor many kinases to cell membranes and can activate many pathways. Kinases that could affect vascular permeability include Src family kinases (SKF) and Akt (protein kinase B) (29, 68). Src and related nonreceptor tyrosine kinases have been implicated in the vascular permeability response to several agonists because an increased tyrosine phosphorylation of β-catenin causes a loss of β-catenin from adherens junctions (8, 14, 35, 77). Akt is also a major downstream signal molecule of PI3K, and its activation has been shown to induce various endothelial functions, including cell survival, migration, and nitric oxide production (17, 21, 26). The PI3K-Akt pathway has also been shown to phosphorylate and negatively regulate glycogen synthase kinase (GSK) 3β activity. Diphosphorylated GSK3β has increased kinase activity and can target β-catenin for degradation by increased serine phosphorylation.

In the present study, we investigated the contributions of the PI3K, SFKs, and Akt to the acute lung vascular permeability response to low and high PIP ventilation in isolated perfused mouse lungs using specific inhibitors of PI3K, SFKs, and Akt. Immunoblotting of pathway signal molecules in both isolated lungs and lungs of intact mice was used to measure protein

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quantities and their phosphorylation state. An intact animal preparation was used because the presence of whole blood and leukocytes in the lung increased endothelial phosphorylation of intracellular proteins by more than 10-fold and better represents in vivo effects of high pressure ventilation without additional insults such as surgical manipulation of the lung (73). Acute changes in vascular permeability were evaluated using the capillary filtration coefficients ($K_f$) in isolated perfused lungs and using bronchoalveolar lavage (BAL) fluid albumin concentrations in intact mice. Pulmonary edema formation was assessed using lung wet-to-dry weight (W/D) ratios. We measured the tyrosine and serine phosphorylation of β-catenin, a sentinel molecule for integrity of the adherens junction, and serine/threonine phosphorylation of ERK1/2, Akt, and GSK3β as an indicator of activity. Total β-catenin was also measured in isolated lungs, and its depletion during high PIP ventilation with the Akt inhibitor correlated with the increase in vascular permeability.

**MATERIALS AND METHODS**

**Isolated lung preparations.** C57BL/6 male mice ($n=39$) (Jackson Laboratory), weighing 18.3–27.3 g (22.1 ± 0.11 g), were anesthetized with an intraperitoneal injection of 100 mg/kg pentobarbital sodium. Both isolated and intact lung experimental protocols were approved by the University of South Alabama College of Medicine Institutional Animal Care and Use Committee. The trachea was cannulated, and the lungs were ventilated with a gas mixture of 20% $O_2$, 5% $CO_2$, and 75% $N_2$ by using a Harvard rodent ventilator (model 683; Harvard, South Natick, MA). The tidal volume was adjusted to obtain a PIP of 55 cmH$_2$O at a respiratory rate (RR) of 40 breaths/min. The chest was surrounded by surgical drapes to eliminate weight artifacts caused by air currents. The initial 1–2 ml of perfusate, which contained residual blood cells and plasma, were discarded and not recirculated. To exclude binding of LY-294002 to proteins in the perfusate, all lungs were perfused with 1% bovine serum albumin and 3% clinical grade dextran in Krebs-bicarbonate buffer by using a roller pump (Minipuls2; Gilson, Middleton, WI) at a constant flow rate of 0.75 ml/min in a recirculating system that had a system volume of 10 ml. Temperature was maintained at 37°C using a heating tape. The venous outflow was collected in a reservoir, the height of which could be adjusted to increase venous pressure. Pulmonary arterial, pulmonary venous, and airway pressures were measured by using Cobe pressure transducers (Lakewood, CO) that were zeroed at the midlum level, and pressures and lung weight were recorded on a Grass model 7D polygraph.

**Pulmonary vascular resistances.** Total pulmonary vascular resistance ($R_v$) and segmental pulmonary vascular resistance were calculated from the perfusate flow and the differences between pulmonary artery (Ppa), vein (Ppv), and double-occlusion capillary (Ppc) pressures as follows: $R_v = (Ppa – Ppv)/(Q/100 g)$, precapillary resistance ($R_c$) = $(Ppa – Ppc)/(Q/100 g)$, postcapillary resistance ($R_e$) = $(Ppc – Ppv)/(Q/100 g)$, where $Q$ is perfusate flow. All resistance was normalized to predicted lung weight as cmH$_2$O·1$^{-1}$·min$^{-1}$·100 g$^{-1}$.

**Capillary $K_f$, $K_t$.** (in ml·min$^{-1}$·cmH$_2$O$^{-1}$·100 g$^{-1}$) is a sensitive measurement of endothelial hydraulic conductivity in fully recruited lungs because filtration is dependent on the equivalent pore radius to the fourth power (62). After an isogravimetric state is attained, Ppv is increased by 6 cmH$_2$O for 20 min, and the change in capillary pressure is determined by double occlusion before and after the Ppv increase. $K_f$ was calculated as the rate of lung weight gain between 18 and 20 min divided by the change in Ppc. In those experiments where $K_f$ was markedly increased, a shorter period of Ppv elevation was used because rate of the weight gain rapidly became linear. All $K_f$ values were normalized to 100-g predicted lung weight on the basis of the ratio of lung weight to body weight according to PLW = (0.00452 ± 0.0003) BW, where PLW and BW are predicted lung weight and body weight, respectively (44).

**Isolated lung protocols.** In all experiments, the lungs were first perfused and ventilated for 30 min under isogravimetric state with 9 cmH$_2$O PIP with 2.5 cmH$_2$O positive end-expiratory pressure (PEEP) at a RR of 40 breaths/min, followed by vascular occlusion pressure and baseline $K_f$ measurements. The time course for Ppv and PIP used in all of the low and high PIP groups is shown in Fig. 1. Subsequent to the baseline $K_f$ measurement, lungs were ventilated with either 9 or 30 cmH$_2$O PIP and 2.5 cmH$_2$O PEEP for periods of 15, 30, and 50 min with measurements of the occlusion pressures and $K_f$ repeated after each ventilation period for a total of four $K_f$ measurements. In high PIP experiments, the lungs were randomly allocated to one of the following groups: group 1 (high PIP injury group; $n=8$), pretreated with 50 μl of DMSO vehicle only; group 2 (high PIP PI3K group; $n=5$), pretreated with 50 μM LY-294002, a PI3K inhibitor (Calbiochem); group 3 (high PIP Src group; $n=8$), pretreated with 20 μM PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine], a Src family kinase (SFK) inhibitor (Calbiochem); or group 4 (high PIP Akt group; $n=6$), pretreated with 10 μM Akt inhibitor IV, an Akt inhibitor (Calbiochem).

The low PIP control experiments were performed using the identical protocol as shown in Fig. 1 except that PIP was maintained at or below 9 cmH$_2$O for the duration of the experiments. These experiments were divided into one of the following groups: group 5 (low PIP control group; $n=3$), treated with 50 μl of DMSO only; group 6 (low PIP PI3K group; $n=3$), treated with 50 μM LY-294002; group 7 (low PIP Src group; $n=3$), treated with 20 μM SFK inhibitor, PP2; or group 8 (low PIP Akt group; $n=5$), treated with 10 μM Akt inhibitor IV.

**Intact mouse preparation.** Protein phosphorylation events were studied in intact mice because Yiming et al. (73) observed that the presence of blood leukocytes during lung distention increased the phosphorylation of endothelial proteins severalfold. Therefore, high PIP ventilation was performed in intact mice using a PIP of 55 cmH$_2$O that was previously determined to increase lung vascular permeability in these mice (76). A total of 15 C57BL/6 mice were anesthetized with an intraperitoneal injection of 65 mg/kg pentobarbital sodium. A tracheostomy was performed, and the cannula was inserted into the trachea. The mice were ventilated by use of a rodent ventilator (model 683, Harvard Apparatus). Anesthesia was maintained by intermittent intraperitoneal injection of pentobarbital sodium if necessary. Airway

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**Fig. 1.** Time course of peak inflation pressures (PIP) in high PIP (solid line) and low PIP (dashed line) protocols and venous pressures used in both protocols.

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pressure was measured using a Cobe pressure transducer, and electrocardiograph was monitored using a polygraph (model 7D, Grass).

**Intact mouse protocols.** Mice were divided into five groups of three each for each time point (n = 3) to determine the time course for phosphorylation of β-catenin, Akt, GSK3β, and ERK1/2. Mice were ventilated with 55 cmH₂O of PIP and 2.5 cmH₂O PEEP at a RR of 17 breaths/min for periods of 0, 15, 30, 60, or 120 min (76). The mice in the 0-min ventilation group were ventilated with 9 cmH₂O of PIP with 2.5 cmH₂O of PEEP at 120 breaths/min for a brief period to remove the lungs. The right lungs were lavaged for protein concentration measurements, and left lung lobes were quickly frozen in liquid N₂ for immunoblotting studies. In intact mice, tidal volume was adjusted as necessary to maintain a constant PIP throughout the experiment.

**BAL protein.** The right lung was lavaged four times with 0.5 ml of phosphate buffered saline. Albumin concentrations were measured with an ELISA (Bethyl Labs, Montgomery, TX) as previously described (76).

**Lung W/D ratio.** After the experiments, the left lung was tied at the left hilum and then removed. The left lung was weighed for wet weight and desiccated at 80°C for 1 wk before the dry weight was measured for the W/D ratio.

**Immunoprecipitation and Western blot analysis.** In isolated lung experiments, the right lung was frozen in liquid nitrogen. In intact animal experiments, lung lobes were excised and snap frozen by liquid nitrogen and stored at −70°C until analysis. For protein extraction, tissue samples were minced and sonicated, cells were lysed in ice-cold buffer at 4°C for 1 h, pH 7.4 (in mM: 50 HEPES, 5 EDTA, 100 NaCl), 1% Triton X-100, protease inhibitors (10 μg/ml aprotnin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and phosphatase inhibitors (in mM: 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate, 0.001 microcystin). Solubilized proteins were isolated using centrifugation (27,000 g for 15 min), and protein concentrations of the supernatant were determined using the Bradford assay. For Western analysis, samples were boiled in 1× SDS buffer and separated using SDS-PAGE and subsequently transferred to nitrocellulose membranes. Membranes were blocked at room temperature for 1 h in TBS containing 5% milk and 0.1% Tween 20. Following incubation with primary and secondary antibodies, proteins were detected by enhanced chemiluminescence. For immunoprecipitation, cell lysates were incubated with anti-β-catenin antibodies, and the immunocomplexes were collected with either A or G Plus-agarose beads for 3 h at 4°C. Following rinsing, samples were boiled in 1× SDS buffer before Western blotting procedures. Western blot analysis was then performed using an anti-phospho-tyrosine antibody. Band intensity was quantified using SigmaGen software. Each immunoblot bar graph represents a mean (±SE) of n = 3 separate experiments.

**Statistical analysis.** All values are expressed as means ± SE. A one- or two-way ANOVA followed by a Fishers least significant difference analysis was used where appropriate. Significant differences were determined where P < 0.05.

**RESULTS**

**Microvascular permeability.** Increases in Kᵣ are a sensitive indicator of increased vascular permeability in the fully recruited lung and allows separation of increased filtration due to increased permeability from increases in hydrostatic pressure (48). Figure 2 shows the effects of ventilation with low and high PIP on Kᵣ in isolated mouse lungs in groups treated with vehicle only or inhibitors of PI3K, Src, or Akt. In the low PIP groups, Kᵣ was increased significantly from baseline only in the low PIP Akt group (37%) but was not increased significantly among groups at any time period. In the high PIP injury group, Kᵣ increased significantly from baseline (0 min) by 4.1- and 5.4-fold at 65 or 135 min, respectively, whereas Kᵣ did not change significantly at 15 min. This Kᵣ increase was significantly attenuated compared with the high PIP injury group at 135 min by pretreatment with the PI3K inhibitor (2.8 times baseline) and the Src inhibitor (3.5 times baseline). In contrast, the Akt inhibitor significantly augmented (9.2 times baseline) the increased permeability at 135 min. The final Kᵣ at 135 min was significantly greater than both baseline values and low PIP Kᵣ at 135 min in all of the high PIP groups regardless of treatment, demonstrating that PI3K and Src inhibitors did not
completely prevent the increase in permeability. Pretreatment with PI3K and Src inhibitors also resulted in significant attenuation of the $K_t$ increase at 65 min compared with the high PIP Akt group and at 135 min compared with the high PIP injury and high PIP Akt groups. In addition, the $K_t$ of the high PIP Akt group was significantly higher at 135 min than that measured in the high PIP injury group. Thus increased activity of the PI3K/Src pathway tends to increase permeability, but activation of the PI3K/Akt pathway appears to oppose this increase.

The edema accumulation in the isolated lungs induced by ventilation is indicated by the lung W/D weight ratios summarized in Table 1. The final W/D weight ratios were significantly greater in all high PIP ventilation groups compared with the low PIP ventilation groups receiving the same treatment. Although there was a trend towards lower W/D weight ratios in the PI3K- and Src inhibitor-treated lungs, only the W/D weight ratios in the high PIP PI3K group and high PIP Akt group were significantly different from each other. The mean W/D weight ratios were 15% lower in the high PIP PI3K group and 9% lower in the high PIP Src group compared with the high PIP injury group, but these differences were not statistically significant. Pretreatment with the Akt inhibitor resulted in a 5% greater W/D ratio to the high PIP injury group, but this was also not significant. Since edema accumulation is a function of the $K_t$, hydrostatic pressure, and the time that venous pressure is increased, the reduced times for venous pressure elevation of as short as 5 min in the lungs with greatest injury reduced the edema accumulation in those groups. Weight gains during the high PIP ventilation periods were also variable between experiments. Thus the W/D weight ratio is a more variable and less specific indicator of vascular permeability than the $K_t$ measurements (62). However, the trends in W/D weight ratios support the concept that PI3K and Src inhibition reduced vascular injury and Akt inhibition augmented injury.

**Pulmonary hemodynamics.** Figure 3 summarizes the pulmonary vascular resistances in the low PIP and high PIP ventilation groups. There were no significant differences with time or treatment between any of the low PIP ventilation groups. Although there were no differences at baseline between high PIP groups, ventilation resulted in a significant increase from baseline at 135 min in $R_2$ in the high PIP injury and high PIP Akt groups. At 65 and 135 min, $R_1$ in the high PIP Akt group was significantly increased compared with that of the high PIP PI3K and high PIP Src groups.

**Indexes of lung injury in intact mice.** Vascular permeability and edema formation were evaluated in the lungs of intact mice using measurements of BAL albumin and lung W/D weight ratios, respectively. Figure 4A indicates the BAL albumin concentrations in groups of intact mice ventilated with a PIP of 55 cmH$_2$O for times up to 2 h and a group ventilated at low PIP (9 cmH$_2$O) for 2 h. BAL albumin concentrations increased progressively with time during ventilation with 55 cmH$_2$O PIP. BAL albumin concentrations at 30–120 min of ventilation were significantly higher than those in the baseline (0 min) and that of the 2 h low PIP ventilation group. The BAL albumin concentration at 120 min of high PIP ventilation was significantly greater than that of all other groups. Figure 4B shows the lung W/D weight ratios for the four time periods. W/D weight ratios after 30–120 min of ventilation were significantly higher than those at 0 min or after 2 h of low PIP ventilation.

**Time course for phosphorylation of lung proteins.** Phosphorylation of lung proteins potentially involved in vascular permeability were measured in intact mice because of the higher levels of protein phosphorylation observed after high volume distention in blood-perfused lungs compared with buffer-per-

### Table 1. Isolated mouse lung wet/dry weight ratios

<table>
<thead>
<tr>
<th>Group</th>
<th>W/D Ratio</th>
</tr>
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<tbody>
<tr>
<td>Low PIP control</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>Low PIP PI3K</td>
<td>6.0 ± 0.2</td>
</tr>
<tr>
<td>Low PIP Src</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>Low PIP Akt</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>High PIP injury</td>
<td>11.9 ± 0.5*</td>
</tr>
<tr>
<td>High PIP PI3K</td>
<td>10.3 ± 0.1*</td>
</tr>
<tr>
<td>High PIP Src</td>
<td>10.9 ± 0.5*</td>
</tr>
<tr>
<td>High PIP Akt</td>
<td>12.5 ± 1.1*#</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. low peak inflation pressures (PIP) group with same treatment. #P < 0.05 vs. high PIP phosphoinositide 3-kinase (PI3K) group.
fused lungs (73). These lungs will also only experience the insult of mechanical overdistention. Figure 5 demonstrates the time course of phosphorylation of β-catenin, Akt, ERK1/2, and GSK3β in the lungs of intact mice ventilated at high PIP. Total tyrosine phosphorylation of β-catenin (Fig. 5A) reached a maximum by 60 min (1.8-fold) and remained above basal levels for 120 min, whereas total β-catenin was unchanged at different ventilation times. Tyrosine phosphorylation of β-catenin was determined after immunoprecipitation of β-catenin. Phosphorylated Akt (Ser473; Fig. 5B) also increased significantly at 60 min, reaching a maximum by 30–60 min (1.8-fold) and decreased toward basal levels by 120 min. Phosphorylation of ERK1/2 (Thr202/Tyr204; Fig. 5C) was more rapid and intense (39.6-fold) than that of other proteins during high PIP ventilation and remained markedly elevated for more than 120 min. Total β-actin was used as a reference protein for phospho-Akt and phospho-ERK1/2 and did not change significantly with time of ventilation. Serine (Ser9) phosphorylation of GSK3β (Fig. 5D) was significantly increased (2.4-fold) after 15 min of high PIP ventilation and remained elevated for 2 h. Although mean densities of serine phosphorylation (Ser33, Ser37) of β-catenin varied by 24% from baseline values (Fig. 5E), these changes did not show statistical significance by ANOVA (P = 0.7).

Modulation of total β-catenin by kinase inhibitors in isolated lungs. The effects of the kinase inhibitors on the total β-catenin content divided by actin content in the isolated lungs ventilated with (Fig. 6A) low PIP of 9 cmH2O and (Fig. 6B) high PIP of 35 cmH2O are shown in Fig. 6. In Fig. 6A, there were no significant differences in the β-catenin/β-actin ratios between low PIP controls and groups treated with inhibitors of Akt, PI3K, or Src. Figure 6B compares the β-catenin/α-actin ratios in groups of mouse lungs with no ventilation, low PIP ventilation, high PIP ventilation, and high PIP ventilation after treatment with inhibitors of Akt and PI3K. There was a statistically lower β-catenin/α-actin ratio in the high PIP Akt inhibitor group compared with all other groups. The high PIP Akt ratio was only 14% that of the high PIP group, but there were no significant differences between the other groups. The β-catenin-to-actin density ratios were used to correct for loading differences. Although there was a trend towards increased β-catenin density after PI3K inhibition (146%) compared with the untreated high PIP ventilation groups, this increase was not statistically significant. Total cellular β-catenin reflects the cytoplasmic pool as well as the junctional pool, so a decrease indicated increased degradation of β-catenin.

Immunohistochemistry. Lung tissue sections from three experiments in each high PIP-isolated lung group were examined by immunohistochemistry. There was no evidence of cleaved caspase-3, an indicator of apoptosis in any of the groups (data not shown). We conclude that apoptosis did not contribute significantly to the permeability increases observed.

DISCUSSION

In the present study, \( K_f \) was used to evaluate vascular permeability in the isolated lung and the contribution of PI3K-activated pathways on the increase in permeability following ventilation with high PIP. \( K_f \) is a sensitive indicator of increased vascular permeability in the fully recruited lungs because its calculation normalizes for the filtration effects of pressure alone (18, 48, 63). However, \( K_f \) measurements could be affected by changes in surface area or interstitial pressures (58). The relatively large amount of edema gained by the high PIP-ventilated groups could have influenced these measurements. Excess edema in these lungs may have resulted from the constant rate of perfusion during high PIP ventilation because compression of alveolar capillaries at high airway pressures can increase Ppa during the airway pressure peaks (49). Lung W/D weight ratios in unventilated mice were previously found to average 4.47 (74), so the predicted lung weight increases ranged from 27% to 61% in the low PIP ventilation groups and 130% to 179% in the high PIP groups compared with unventilated lungs. Previous investigators have observed an increase in \( K_f \) after lung weight gains of ~50% (5, 19, 49). These increases are attributed to filling of the perivascular and peribronchial interstitium and initiation of alveolar flooding. The surface forces in partially flooded alveoli exert a high negative surface pressure for rapid filling due to the law of Laplace. As the radius of curvature decreases at the air-liquid interface, an increased negative pressure results in an all-or-nothing filling of alveoli (64). The weight gains in all of the high PIP ventilation groups exceeded the threshold for alveolar flooding.
PI3K, Src, and Akt Modulate Ventilator-Induced Lung Injury

A. Phosphorylated β-catenin and total β-catenin levels over time.

B. Phosphorylated Akt and total β-actin levels over time.

C. Phosphorylated ERK1/2 and total β-actin levels over time.

D. Phosphorylated GSK3β and total GSK3β levels over time.

E. Phosphorylated β-catenin and total β-catenin levels over time.
Alveolar edema could also compress alveolar vessels to reduce regional blood flow, vascular surface area, and $K_f$ (40), but high protein alveolar fluid such as occurs in permeability edemas had no significant effect on regional blood flow (41). In isolated lungs, Bhattacharya et al. (5) observed no decrease in total blood flow until lung weight reached 250% of baseline weight. Thus the absolute values of $K_f$ in the last measurements of the high PIP group should be interpreted with caution because they may be affected by mechanical and surface tension forces. However, the relative differences between groups should be valid because the W/D weight ratios differed by only 21% between the highest and lowest values in the high PIP group, whereas the last $K_f$ values differed by 330% between highest and lowest values. This suggests that the effects of excess edema were similar between groups and that the dramatic differences in $K_f$ measurements between the inhibitor and vehicle-treated groups represent a true affect of these signal pathways on the vascular permeability response.

The major new findings of the present study were that lung overdistention activates multiple PI3K-mediated pathways that oppose each other, and the balance between these pathways determines the net vascular permeability effect. Activation of the PI3K/Src pathway appears to significantly increase lung vascular permeability and edema formation after high PIP ventilation, whereas activation of the PI3K/Akt/GSK3β pathway appears to oppose the vascular permeability increase in isolated lungs. Permeability was evaluated using $K_f$, a sensitive indicator of increased permeability in fully recruited lungs, and the contributions to the permeability increase of PI3K, Src, and Akt were evaluated using specific inhibitors (48). $K_f$ was increased from baseline by 4.1- and 5.4-fold after the second and third ventilation periods with 30 cmH₂O PIP, respectively. Inhibition of PI3K and Src during high PIP ventilation significantly attenuated the $K_f$ increase, whereas inhibition of Akt augmented the increase. In these isolated lungs, the total amount of β-catenin was decreased in the high PIP Akt inhibitor group relative to other high PIP groups but not in low PIP controls. This suggests that lung distention activates an Akt-dependent pathway that tends to preserve cellular β-catenin content as well as permeability.

In intact mice, high PIP ventilation also increased lung vascular permeability and edema and induced phosphorylation of β-catenin, Akt, GSK3β, and ERK1/2. β-catenin had increases in tyrosine phosphorylation (1.8-fold) of known sites for Src phosphorylation and marginal phosphorylation increases in Ser33 and Ser37 (1.5-fold), the sites targeted by GSK3β that induce protein degradation. Akt phosphorylation was significantly increased (1.8-fold) at Ser473, an integrin-linked kinase (ILK) site induced by PI3K activation. GSK3β, in turn, had increased Ser9 phosphorylation (2.4-fold), the phosphorylation site for Akt that inactivates GSK3β activity. In addition, ERK1/2 phosphorylation increased by 40-fold at Thr202 and Tyr204.
PI3K is a pluripotent kinase involved in numerous signaling pathways that generate phosphoinositides that anchor kinases to the cell membrane to form signaling aggregates (29). Mechanical stretch can increase tyrosine and serine/threonine phosphorylation of cellular proteins by activation of receptor tyrosine kinases, G protein-coupled receptors, growth factor receptors, Ca\(^{2+}\) entry through stretch-activated cation channels, and deformation of cytoskeleton and integrins (1, 8). These pathways all activate PI3K, phospholipase C, and Src (56, 61). In spite of activating multiple pathways, blockade of nerns, and deformation of cytoskeleton and integrins (1, 8). receptors, Ca\(^{2+}\) phosphorylation of cellular proteins by activation of receptor to the cell membrane to form signaling aggregates (29). Me-
crease and caused a marked loss of the total phosphorylation of tyrosine kinases are activated by PI3K and appear to have a critical role in degrading the adherens junction, but Src may also be directly activated by cytoskeletal strain and, in turn, activate PI3K (57, 77). The SFKs were implicated in mechanical injury because the SFK inhibitor, PP2, attenuated the \(K_f\) increase in high PIP-ventilated mouse lungs. Tyrosine phosphorylation of \(\beta\)-catenin is known to block its adhesion to E-cadherin and VE-cadherin, reduce homophilic adhesion of cadherins, and degrade adherens junctions (55). At the same time, a PI3K-mediated increase in phosphorylation of Akt at the ILK site appears to limit degradation of the cytoplasmic \(\beta\)-catenin by phosphorylation and inactivation of GSK3\(\beta\) at the Akt phosphorylation site. Preservation of cellular \(\beta\)-catenin content may then have limited \(\beta\)-catenin displaced from the adherens junction complex and opposed the permeability increase. Phosphorylation of \(\beta\)-catenin by GSK3\(\beta\) at Ser33 and Ser37 and Thr47 targets \(\beta\)-catenin for ubiquitination and proteosomal degradation. In contrast, inhibition of GSK3\(\beta\) activity preserves the cytoplasmic \(\beta\)-catenin pool and facilitates its translocation to the nucleus to initiate transcription (11). Inhibition of Akt with the Akt IV inhibitor augmented the \(K_f\) increase and caused a marked loss of the total \(\beta\)-catenin pool in the high PIP-ventilated isolated mouse lungs, indicating the \(\beta\)-catenin sparing and permeability preservation function of Akt. Although mechanical activation of the PI3K/Akt/GSK3\(\beta\) pathway tends to preserve the total \(\beta\)-catenin pool and limit the increase in vascular permeability, the decreased permeability induced by PI3K inhibition indicates that other mechanically activated kinases impact Akt and GSK3\(\beta\) to preserve their vital functions.

PI3K has recently been implicated in VILI coupled with surfactant depletion and ischemia in studies by Lionetti et al. (30). They first injured the lungs of wild-type, PI3K\(^{-/-}\) knock-out, and PI3K\(^{-/-}\) kinase dead mice by repeated saline lavage, excised the lungs, and ventilated the lungs without perfusion with static low pressure inflation or ventilation with high (40 mL/kg) or low (7 mL/kg) tidal volumes for 3 h. In lungs of PI3K\(^{-/-}\) null mice, histological indexes of lung injury and phosphorylation of Akt and ERK1/2 were reduced compared with wild type, but indexes of apoptosis were increased. No differences in NF-\(\kappa\)B activation or cytokine production were observed between wild-type and PI3K\(^{-/-}\) null groups. The findings in the present study support their conclusion that PI3K inactivation reduces lung mechanical injury, but our data support a different mechanism than that proposed by Lionetti et al. The lungs in their study were subjected to multiple injuries besides lung distention due to saline lavage inactiva-
tion of surfactant followed by 3 h of ischemia. In addition, no specific indexes of vascular permeability were measured. In contrast, lungs in our preparations were continuously perfused, and we measured \(K_f\), a specific measure of vascular permeability. Lionetti et al. (30) concluded that suppression of downstream Akt activation in PI3K\(^{-/-}\) mice protected against VILI by increased apoptosis and favoring apoptosis over necrosis of lung cells. However, we observed no increase in apoptosis in our preparations during 2 h of ventilation without additional insults. Our mouse lungs were perfused and retained functional surfactant. Instead, we conclude that PI3K/Akt activation had a beneficial effect on permeability during high PIP ventilation by phosphorylating GSK3\(\beta\), which preserved the cellular pool of \(\beta\)-catenin and adherens junctions. The acute permeability increase appears to be initiated by simultaneous PI3K activation of the SFK. Tyrosine phosphorylation of \(\beta\)-catenin and its subsequent loss from the adherens junction could then mediate the acute permeability response by overriding the opposing PI3K/Akt/GSK3\(\beta\) pathway.

Inhibition of PI3K could also attenuate the increased permeability by inhibition of cytokine release and neutrophil recruitment during high PIP ventilation (67). Some studies show activation of NF-\(\kappa\)B by the PI3K-Akt pathway (2), whereas some studies found NF-\(\kappa\)B activation independent of Akt (53). Although we did not measure cytokine levels in the present study, previous studies suggest a minimal role of inflammatory cytokines in the immediate permeability increase due to high PIP ventilation (54, 74). PI3K activity is also crucial for both neutrophil recruitment and activation (52, 78). Although the perfusate buffer contained no blood in the present study, some neutrophils undoubtedly remain sequestered in the lung circulation during exanguination (36), and the lungs of the intact mice were blood perfused. Endothelial cell interaction with the resident neutrophils could augment endothelial activation and the vascular permeability response (6, 73).

Activation of PI3K by mechanical stretch is likely initiated by calcium entry through gadolinium-sensitive cation channels, which have previously been shown to activate PI3K and Akt in osteblasts during cyclical stretch (15). We have previ-
ously reported that the high PIP ventilation-induced increases in \(K_f\) in isolated rat lungs were attenuated using gadolinium chloride (GdCl\(_3\)), an inhibitor of stretch-activated cation channels (45, 50). Kuebler et al. (27) also observed that a left atrial pressure increase of 10 cmH\(_2\)O or higher in isolated rat lungs caused a gadolinium-sensitive calcium increase in venular endothelium cells in situ. Intracellular Ca\(^{2+}\) increases can affect endothelial permeability by calmodulin activation of myosin light chain kinase (MLCK). Inhibition of MLCK attenuated the permeability increase during VILI in the isolated rat lung, and a knockout of endothelium-specific MLCK attenuated VILI-associated injury and mortality in mice (43, 69). However, increased cytoskeletal retracted forces alone were unable to produce endothelial gap formation without a release of intracellular junction tethering, which further suggests the necessary role of \(\beta\)-catenin and associated proteins for permeability regulation (13).

Several investigators have reported an association of tyrosine phosphorylation of \(\beta\)-catenin with an increased vascular permeability (39, 65, 77). We have previously implicated an increased tyrosine phosphorylation in VILI. Inhibition of tyrosine kinase with genistein attenuated high PIP ventilation-
induced increases in $K_t$, whereas inhibition of protein tyrosine phosphatase with phenylarsine oxide greatly augmented the permeability increase in isolated perfused rat lungs (47). Mechanical strain markedly increases tyrosine phosphorylation of intracellular proteins in intact lungs and endothelial cells, and proteins associated with focal adhesions and adherens junctions are particularly affected (6, 72). Of particular relevance to VILI, Yiming et al. (73) observed that tyrosine phosphorylation of endothelial adhesion-related proteins in rat lungs after high volume ventilation was greatly augmented by the presence of neutrophils. Tyrosine phosphorylation of β-catenin by pp60c-src at Tyr654 was found to decrease β-catenin binding to E-cadherin by 92% and appears to be the site controlling E-cadherin function (55). Src phosphorylation of PLC and focal adhesion kinase also may contribute to the observed increase in vascular permeability (33). Liu et al. (32) subjected fetal lung cells to cyclical stretch and observed activation of Src, PLC, and PKC, which were attenuated by herbimycin A, a tyrosine kinase inhibitor. Tyrosine phosphorylation of β-catenin reduced epithelial and endothelial barrier properties (77). The increase in tyrosine phosphorylation of β-catenin in lungs observed after high PIP ventilation in the present study also correlated with the increase in vascular permeability.

The SFKs consists of some 10 nonreceptor tyrosine kinases, and some of these are associated with focal adhesion kinase and adherens junctions (8, 65). In cultured endothelial cells, Timsely et al. (65) inhibited gap formation and translocation of β-catenin from the cell junctions during neutrophil-mediated permeability increases using an SFK inhibitor. Naruse et al. (42) observed rapid activation of c-Src in human umbilical vein endothelial cells after uniaxial cyclical stretch, and morphological reorientation of these cells was blocked byantisense c-Src suppression. SFKs may also increase permeability by increasing cytoskeletal tension through stress fiber formation and myosin light chain phosphorylation in endothelial cells (14, 39) because activated pp60c-src directly phosphorylates nonspecific MLCK at Tyr464 and Tyr471 (7). SFKs may also be activated by various receptor-mediated pathways as well as cytoskeletal deformations (23). Although active Src degrades E-cadherin junctions and promotes cytosol accumulation of β-catenin, activated Src can also enhance β-catenin survival in cytosol, promote transcription, and facilitate the PI3K/Akt pathway (24). Activation of the PI3K/Akt pathway will also promote β-catenin survival in cytosol and transcription (37). Thus the effects of PP2 in attenuating high PIP-induced $K_t$ increases in the present study undoubtedly resulted in large part from inhibition of tyrosine phosphorylation of β-catenin.

PI3K and Akt activities are affected by many pathways that can both increase and decrease vascular permeability. Release of growth factors during high PIP ventilation is one mechanism for PI3K activation. Cyclic stretch increased expression of hepatocyte growth factor (HGF) at both the mRNA level and the protein level in human alveolar epithelial cells (71). HGF activated PI3K, increased junctional β-catenin, and enhanced lung endothelial barrier function (31). Our data suggest activation of this pathway may be operative because the Akt inhibitor increased $K_t$ significantly compared with high PIP alone. Inhibition of Akt would prevent GSK3β phosphorylation, which in turn would result in serine phosphorylation and degradation of β-catenin in the cytoplasmic pool (16). On the other hand, ventilation of rats with high tidal volumes for 2 h increased serum levels of VEGF (12), a mediator of increased vascular permeability that causes increased tyrosine phosphorylation of β-catenin and a significant loss of junctional VE-cadherin (25). VEGF administration caused phosphorylation of Src, PLC, and PI3K, but increased phosphorylation of myosin light chains, and catenins occurred through a pathway that did not involve Akt (8). Delayed and prolonged VEGF-induced hyperpermeability was associated with the loss of β-catenin staining at the sites of endothelial gap formation (14). Because PI3K inhibition decreased activation of ERK1/2 and markedly attenuated p38 activity in bovine lung microvascular endothelial cells (4), PI3K may also participate in the regulation of adherens junction function (37). This mechanism could be one of the protective effects of PI3K inhibition in the present study.

Inhibition of Akt during high PIP ventilation may also enhance lung injury through an increase in PLAC2 activity. We have previously demonstrated that high PIP ventilation increased cPLA2 activity in the lungs of intact mice, and inhibition of cPLA2 significantly reduced BAL albumin and protein concentrations (75). Akt activity was previously found to suppress MAP kinase and cPLA2 activity, so inhibition of Akt may enhance their activities (22, 28). Akt activity also has a well-established role in inhibiting apoptosis (59) and protects against oxidant-induced lung injury in mice exposed to 100% oxygen (34). In the present study, the high PIP ventilation period was too short to induce significant apoptosis. Immunostaining for caspase-3 showed no apoptosis in lung cells in any group, including the high PIP Akt group. Thus it is unlikely that the Akt inhibitor enhancement of the acute permeability increase was linked to induction of apoptosis.

Stretch-induced nitric oxide production in lung microvessels may also have contributed to the permeability effects of high PIP ventilation and the effects of inhibitors in the present study. Cyclic stretch of endothelial cells causes an upregulation of endothelial nitric oxide synthase (eNOS) activity (3). High volume ventilation of isolated lungs also activated eNOS and induced its expression in pulmonary endothelial cells in situ (26). In addition, blockade of nitric oxide production attenuated the increase in $K_t$ induced by high PIP ventilation in isolated perfused rabbit lungs (9, 12). In more selective studies, overexpression of eNOS protected transgenic mice against VILI. However, high volume ventilation increased inducible nitric oxide synthase (iNOS) activity and increased VILI, which was attenuated in iNOS knockout mice (51, 60). The PI3K/Akt pathway is also involved in the cyclical stretch-activated nitric oxide production in pulmonary endothelial cells (26). In the present study, inhibition of eNOS and nitric oxide production by the Akt inhibitor may have contributed to the increases in both $K_t$ and $R_t$ in the high PIP Akt group, but the overall effects of PI3K inhibition on nitric oxide relative to the lung permeability responses to high PIP ventilation are unknown. We conclude that many of the signal pathways mediated by PI3K during mechanical distention of the lung may have opposing effects on vascular permeability. Activation of SFK by PI3K may dislodge β-catenin from adherens junctions by tyrosine phosphorylation and increase vascular permeability, but tyrosine phosphorylation of focal adhesion kinase may also contribute. In contrast, simultaneous activation of the PI3K/Akt/GSK3β pathway may lead to preservation of the cytoplas-
mic β-catenin pool, which in turn may enhance junctional β-catenin and adherens junction stability by an unknown feedback mechanism or by other permeability-preserving pathways. Thus, Akt activation may enhance barrier protection as well as potentiate cell survival, but these effects are overwhelmed by propermeability pathways of PI3K activation, such as SFK-induced tyrosine phosphorylation, during high volume ventilation.

REFERENCES

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