MECHANOTRANSDUCTION IN THE LUNG: PATHOLOGIC PATHWAYS AND RHO-RAP1 CROSSTALK PARADIGM IN SEARCH FOR NOVEL PROTECTIVE STRATEGIES

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SUMMARY
Mechanical ventilation at high tidal volume (HTV) increased bronchoalveolar lavage (BAL) cell count and protein concentration reflecting ventilator-induced lung injury. In clinically-relevant two-hit model edemagenic agonist thrombin further promoted HTV-induced lung injury in mice. These effects were attenuated by iloprost administration. In vitro, pathological cyclic stretch (CS) and thrombin caused disruption of endothelial (EC) barrier manifested by increased paracellular permeability, stress fiber and gap formation, and Rho pathway activation. Iloprost treatment markedly inhibited thrombin-induced EC barrier compromise. Rap1 knockdown suppressed protective effects of iloprost in vitro and in vivo.

INTRODUCTION
Although ventilator support is an indispensable treatment for critically ill patients, ventilator-induced lung injury (VILI) with the associated multiorgan dysfunction may lead to significant morbidity and mortality and thus remains one of the most important problems in the management of patients in the intensive care unit. Increased levels of edemagenic agents and inflammatory cytokines such as thrombin, histamine, IL-8 also contribute to increased vascular leak observed in VILI patients[1]. Thus, combination of lung over-distention and increased levels of edemagenic agents may represent the real pathologic progression of VILI. Therefore, two-hit in vivo/in vitro models, which combine mechanical ventilation/stretch with pro-inflammatory agents stimulation provide vital information about mechanisms regulating lung permeability in VILI patients. Stable prostaglandin I$_2$ analog iloprost is currently used for treatment of pulmonary hypertension [2]. However, molecular mechanisms of pulmonary EC barrier protection by prostaglandins are not fully understood. This study investigated the involvement of small GTPase Rap1 in the EC barrier protection by iloprost in human pulmonary EC and in the murine models of ventilator-induced lung injury.

METHODS
Animal studies: C57BL/6J mice were placed on mechanical ventilator (Harvard Apparatus, Boston, MA) for 4 hours with high tidal volume (30 ml/kg, HTV) ventilation with or without TRAP6 (1.5 x 10$^{-5}$ mol/kg, i/t) instillation. Iloprost (2 µg/kg, i/v) was administered at three time points (0, 40, and 80 min) during mechanical ventilation. BAL was performed and protein concentration and cell count were determined. Cell culture under cyclic stretch: CS experiments were performed using FX-4000T Flexcell Tension Plus system. Human lung microvascular (HLMVEC) or macrovascular (HPAEC) endothelial cells were pretreated with iloprost (500 ng/ml) for 30 min followed by exposure to 18% elongation CS with or without thrombin (0.5 U/ml) challenge. At the end of experiment, cell lysates were collected for western blot analysis; or cells were used for immunofluorescence staining. Measurements of transendothelial electrical resistance (TER) across EC monolayers were performed using the electrical cell-substrate impedance sensing system (ECIS) (Applied Biophysics, Troy, NY). Depletion of Rap1: Pre-designed standard purity StealthTM siRNAs (Homo sapiens) sets were used, which possess the same sequences with mouse Rap1 corresponding segments (Invitrogen, Carlsbad, CA).

RESULTS AND DISCUSSION
To reproduce the two-hit model of lung injury, we used high tidal volume (HTV) mechanical ventilation and TRAP6, the thrombin-derived non-thrombogenic peptide ligand of PAR1 receptor. TRAP6 administration further promoted HTV-induced lung injury detected by increased BAL cell counts and protein concentration (Figure 1). Importantly, iloprost instillation suppressed lung injury in both, HTV and TRAP6/HTV models, as detected by measurements of BAL protein content and cell counts.

![Figure 1: Iloprost attenuates HTV and TRAP6-induced lung injury.](image)

![Figure 2: Protective effects of iloprost in two-hit model of lung EC barrier dysfunction.](image)
light chain (MLC) and MLC phosphatase (MYPT) phosphorylation (Figure 2B). Iloprost abolished these effects leading to preservation of monolayer integrity in CS- and thrombin-stimulated EC. Collectively, these results suggest the mechanism of the iloprost-induced reduction in actin stress fibers and paracellular gap formation in EC exposed to 18% CS and thrombin via iloprost-induced attenuation of Rho signaling.

To investigate whether another small GTPase, Rap1, is involved in iloprost-mediated protective effects against HTV-induced lung injury, endogenous Rap1 was depleted using siRNA approach. After 72 hours of transfection HTV model was performed with or without iloprost treatment. After four hours of ventilation, BAL and lung tissue were collected as described above. Knockdown of Rap1 did not affect elevation of BAL cell count and protein content caused by high tidal volume ventilation (Figure 3AB, left panels). However, Rap1 knockdown significantly attenuated protective effects of iloprost against HTV-induced increases in BAL cell count and protein concentration (Figure 3AB, right panels).

Figure 3: Effects of Rap1 depletion on protective effects by iloprost.

The role of Rap1 in the mediation of iloprost barrier-protective effect on the pulmonary endothelium has been further tested in vitro. Iloprost treatment not only attenuated thrombin-induced drop in resistance during acute phase, but also accelerated EC monolayer recovery phase after thrombin challenge in control cells. Knockdown of Rap1 did not affect acute phase of thrombin-induced permeability, but suppressed the phase of EC barrier restoration. More importantly, Rap1 inhibition markedly decreased protective effect of iloprost against thrombin-induced permeability during both acute and recovery phases of thrombin stimulation (Figure 4A). In addition, iloprost failed to inhibit Rho-dependent thrombin-induced phosphorylation of MYPT and MLC in Rap1-depleted cells (Figure 4B).

Figure 4: Effect of Rap1 depletion on iloprost-mediated protection against thrombin-induced EC barrier dysfunction.

Figure 5: Effect of Rap1 knockdown on iloprost-induced cytoskeletal remodeling and adherens junction integrity.

CONCLUSIONS

This study provides a new insight into mechanisms of pulmonary endothelial barrier regulation and demonstrates that iloprost attenuates the acute lung injury induced by pathologically relevant ventilation and TRAP-6 stimulation due to decreases of BAL protein contents and neutrophil accumulation. Our data show the role of Rap1 pathway in protective effects of iloprost against ventilator-induced lung injury and EC hyper-permeability. These pathways activated by iloprost lead to attenuation of vascular leak and leukocyte infiltration associated at least in part with inhibition of Rho signalin and may accelerate recovery of pulmonary endothelial barrier in the course of acute lung injury. These results support clinical data suggesting beneficial effects of prostacyclin analogs in the treatment of ALI/VILI.

ACKNOWLEDGEMENTS
NHLBI grants HL87823, HL76259, and HL58064

REFERENCES