MECHANICAL STRAIN AND MICROTUBULE-ASSOCIATED SIGNALING IN THE LUNG ENDOTHELIUM

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SUMMARY
This study demonstrates that pathological cyclic stretch (CS) alters microtubule (MT) stability and induces MT reorientation. MT stabilization inhibits stress fiber formation and delays cell alignment in response to pathological CS. MT-associated GEF-H1 knockdown attenuates stretch-induced rearrangement of actin skeleton and controls stretch-dependent MT dynamics in pulmonary endothelium (EC). GEF-H1 depletion and MT stabilization attenuate ventilator-induced lung injury in vivo.

INTRODUCTION
Reorganization of the EC cytoskeleton, which is composed of actin filaments, MT, and intermediate filaments, leads to alteration in cell shape and provides a structural basis for an increase in vascular permeability, implicated in the pathogenesis of many diseases including asthma, sepsis, and acute lung injury (ALI). MT depolymerization in pulmonary EC results in increased myosin light chain (MLC) and MLC phosphatase (MYPT) phosphorylation, cell contraction, and EC barrier dysfunction [1]. These effects are linked to the activation of small GTPase Rho, and can be attenuated by cell pretreatment with MT stabilizer taxol. Guanine nucleotide exchange factor H1 (GEF-H1) is a Rho-specific GEF localized on MT [2]. Our studies show that attenuation of Rho activity reduces lung vascular leak and promotes barrier recovery in the in vitro and in vivo models of ALI [3,4]. In the current study using in vitro and in vivo models of ventilator-induced lung injury (VILI) we evaluated the involvement of MT-associated Rho-specific GEF-H1 in the development of lung vascular dysfunction induced by mechanical stimulation.

METHODS
Cell culture under cyclic stretch (CS): CS experiments were performed using an FX-4000T Flexcell Tension Plus system (Flexcell, Hillsborough, NC). After 72 hrs of culture, human pulmonary artery endothelial cells (HPAEC) were exposed to high magnitude (18% linear elongation, sinusoidal wave, 25 cycles/min) cyclic stretch to recapitulate the mechanical stresses experienced by the alveolar endothelium at high tidal volume mechanical ventilation.

Knockdown of GEF-H1: To reduce the content of endogenous GEF-H1 cells were treated with pre-designed standard purity siRNA sets were ordered from (Dharmacon, Lafayette, CO).

Mechanical ventilation protocol: C57BL/6J mice were anesthetized and placed on mechanical ventilator (Harvard Apparatus, Boston, MA). Mice were given a single dose of intratracheal thrombin-derived activating peptide (TRAP6) (1.5 x 10-5 mol/kg) followed by 4 hours of mechanical ventilation with high tidal volume (30 ml/kg, HTV) ventilation. In experiments with taxol, mice were injected with taxol (3.75 x 10-7 mol/kg, i/v) prior to TRAP6 instillation and mechanical ventilation. Measurements of cell count and protein concentration were performed in bronchoalveolar lavage fluid (BAL).

RESULTS AND DISCUSSION
To study the involvement of MT in cytoskeletal remodeling and cell orientation in response to CS EC were pretreated with vehicle or taxol followed by pathological (18%) CS exposure for 30 min. CS induced rapid cell reorientation accompanied by formation of stress fibers. Stabilization of MT by taxol prior to CS stimulation significantly decreased stress fiber formation and attenuated CS-induced cell orientation (Figure 1A). MT stabilization also suppressed CS-induced MYPT1 and MLC phosphorylation during acute phase of CS (Figure 1B). These results demonstrate the essential role of MT in CS-mediated cytoskeletal rearrangement and intracellular signaling.

Analysis of MT structure shows that under static conditions, MT organized into faint uniformly distributed lattice network (Figure 2A). Acute CS induced alignment of MT perpendicular to the main distension vector, and this rearrangement was accompanied by MT depolymerization and significant reduction in a number of assembled MT. CS also decreased amount of acetylated/stable MT during acute phase of CS, as detected by western blot analysis. Stabilization of MT with taxol completely restored levels of acetylated tubulin in CS-subjected EC (Figure 2B).

We next tested the involvement of GEF-H1 in intracellular signaling and cytoskeletal remodeling in pulmonary EC exposed to pathologic CS using GEF-H1 knockdown by specific siRNA. GEF-H1 knockdown decreased CS-induced stress fiber formation and paracellular gap formation, prevented cell reorientation after 30 min of CS (Figure 3A). Biochemical analysis of Rho pathway activation showed that
GEF-H1 knockdown decreased phosphorylation of Rho downstream targets MYPT1 and MLC (Figure 3B) in response to acute 18% CS.

**Figure 3**: Role of GEF-H1 in CS-induced cytoskeletal rearrangement.

To test the role of GEF-H1 in the regulation of MT stability by mechanical forces, endogenous GEF-H1 was depleted and MT structure in control and stretched pulmonary EC was analyzed by immunofluorescence staining with tubulin antibody. GEF-H1 depletion inhibited MT re-orientation and prevented MT disassembly in EC exposed to 30-min CS (Figure 4A). GEF-H1 knockdown also significantly attenuated decline in the levels of acetylated tubulin induced by 30-min CS (Figure 4B). These findings support a critical role of GEF-H1 in CS-mediated regulation of MT organization.

**Figure 4**: Involvement of GEF-H1 in CS-mediated alterations of MT network.

To link *in vitro* results with Rho-dependent signal transduction *in vivo*, we used clinically-relevant two-hit model of lung injury induced by high tidal volume (HTV) mechanical ventilation and TRAP6, the thrombin-derived non-thrombogenic peptide. First, we investigated effects of MT stabilization by taxol on parameters of lung injury in the TRAP6/HTV model. Taxol administration significantly reduced TRAP6/HTV-induced increases in BAL cell count and protein concentration (Figure 5A). These data suggest a role MT and MT-mediated signaling in the development of VILI. In the following experiments we utilized *in vivo* TRAP6/HTV model to address a role of MT-associated GEF-H1-mediated signaling in lung injury induced by mechanical ventilation. Using siRNA approach, we performed *in vivo* knockdown of GEF-H1. HTV caused prominent increase in BAL cell count and protein concentration, TRAP6 instillation further promoted HTV-induced lung injury. GEF-H1 depletion significantly reduced HTV-induced elevation of cell count and protein content levels in BAL fluid from HTV- and TRAP6/HTV-subjected animals (Figure 5B).

**Figure 5**: Role of MT stabilization and GEF-H1 in the development of VILI.

**CONCLUSIONS**

This study describes a novel mechanism of stretch-induced activation of barrier disruptive Rho signaling by microtubule-associated Rho specific guanine nucleotide exchange factor GEF-H1. Our results suggest beneficial effects of stabilization of microtubule network and GEF-H1 targeting to MT that may be considered as future therapies for the treatment of ventilator induced lung injury.

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**REFERENCES**


