Stimulation of Rho signaling by pathologic mechanical stretch is a “second hit” to Rho-independent lung injury induced by IL-6

Anna A. Birukova, Yufeng Tian, Angelo Meliton, Alan Leff, Tinghuai Wu, and Konstantin G. Birukov

Lung Injury Center, Section of Pulmonary and Critical Medicine, Department of Medicine, University of Chicago, Chicago, Illinois

Submitted 2 September 2011; accepted in final form 20 January 2012

Birukova AA, Tian Y, Meliton A, Leff A, Wu T, Birukov KG. Stimulation of Rho signaling by pathologic mechanical stretch is a “second hit” to Rho-independent lung injury induced by IL-6. Am J Physiol Lung Cell Mol Physiol 302: L965–L975, 2012. First published February 17, 2012; doi:10.1152/ajplung.00292.2011.—Most patients with acute lung injury (ALI) and acute respiratory distress syndrome of septic and nonseptic nature require assisted ventilation with positive pressure, which at suboptimal range may further exacerbate lung dysfunction. Previous studies described enhancement of agonist-induced Rho GTPase signaling and endothelial cell (EC) permeability in EC cultures exposed to pathologically relevant cyclic stretch (CS) magnitudes. This study examined a role of pathologic CS in modulation of pulmonary EC permeability caused by IL-6, a cytokine increased in sepsis and acting in a Rho-independent manner. IL-6 increased EC permeability, which was associated with activation of Jak/signal transducers and activators of transcription, p38 MAP kinase, and NF-κB signaling and was augmented by EC exposure to 18% CS. Rho kinase inhibitor Y-27632 suppressed the synergistic effect of 18% CS on IL-6-induced EC monolayer disruption but did not alter the IL-6 effects on static EC culture. 18% CS also increased IL-6-induced ICAM-1 expression by pulmonary EC and neutrophil adhesion, which was attenuated by Y-27632. Intratracheal IL-6 administration in C57BL/6J mice increased protein content and cell count in bronchoalveolar lavage fluid. These changes were augmented by high tidal volume mechanical ventilation (HTV; 30 ml/kg, 4 h). Intravenous injection of Y-27632 suppressed IL-6/HTV-induced lung injury. In conclusion, this study proposes a novel mechanism contributing to a two-hit model of ALI: in addition to synergistic effects on Rho-dependent endothelial hyper-permeability triggered by thrombin, TNFα, LPS, or other agonists, ventilator-induced lung injury-relevant CS may also exacerbate Rho-independent mechanisms of EC permeability induced by other inflammatory mediators such as IL-6 via mechanisms involving Rho activity.

We (2) have previously described enhancement of agonist-induced endothelial cell (EC) barrier disruption by VILI-relevant magnitude of cyclic stretch (CS). Further studies identified a Rho-dependent mechanism of synergistic effects by pathologic CS and thrombin on the lung EC barrier disruption (4, 8) and demonstrated a beneficial effect of inhibition of Rho signaling on HTV-induced pulmonary vascular permeability and barrier recovery in the in vitro and in vivo models of VILI (8, 29).

Interleukin-6 (IL-6) is a well-recognized inflammatory mediator upregulated in ALI/acute respiratory distress syndrome patients and in the animal models of septic and ventilator-induced lung injury (35, 36). The IL-6 receptor system is distinct from the Toll-like receptor family and consists of two polypeptide chains: an 80-kDa IL-6 receptor (IL-6R) and a 130-kDa signal transducer (gp130). IL-6R also exists in the transmembrane and a soluble form (23). The soluble IL-6R can form a stimulatory complex with IL-6, which associates with the gp130 coreceptor on the EC membrane surface and triggers activation of JAK-Jak/signal transducers and activators of transcription (Stat)-mediated transcription, phosphatidylinositol 3-kinase (PI3-kinase)/AKT cascade, and MAP kinase pathways (19). The signaling pathways activated in pulmonary endothelium by IL-6 are not clearly understood, and the precise mechanisms of IL-6-mediated EC barrier dysfunction remain to be elucidated. Unlike other mediators of ALI such as lipopolysaccharide (LPS), thrombin, and TNFα, IL-6 has not been shown to directly activate Rho signaling. In the current study, we utilized in vitro and in vivo models of VILI to test the interplay between Rho-dependent and Rho-independent mechanisms of EC permeability and lung barrier dysfunction induced by IL-6 and pathologic mechanical stimulation.

Address for reprint requests and other correspondence: K. Birukov, Lung Injury Center, Section of Pulmonary and Critical Medicine, Dept. of Medicine, Univ. of Chicago, 5841 S. Maryland Ave, Office N-611 Chicago, IL 60637 (e-mail: kbirukov@medicine.bsd.uchicago.edu).
MATERIALS AND METHODS

Cell culture and reagents. Human pulmonary artery endothelial cells (HPAEC) and cell culture basal medium with growth supplements were obtained from Lonza (Allendale, NJ), cultured according to the manufacturer’s protocol, and used at passages 5–8. Mouse and human IL-6 and human IL-6 soluble receptor were obtained from R&D Systems (Minneapolis, MN). Di-phospho-myosin light chain (MLC), phospho-heat shock protein (HSP)27, phospho-Stat3, and IkBα antibodies were from Cell Signaling (Beverly, MA); ICAM-1 antibody was from BD Transduction Laboratories (San Diego, CA); phospho-myosin-associated phosphatase type 1 (MYPT1) antibody was purchased from Millipore (Billerica, MA); and Jak 1 inhibitor and Y-27632 were from EDM (La Jolla, CA). All reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Unless specified, biochemical reagents were obtained from Sigma (St. Louis, MO).

Cell culture under CS. CS experiments were performed using FX-4000T Flexcell Tension Plus system (Flexcell International, McKeesport, PA) equipped with a 25-mm BioFlex loading station, as previously described (2). In brief, untreated EC or cells after siRNA transfection were exposed to high magnitude CS (18% distension, sinusoidal wave, and 25 cycles/min) to recapitulate a HTV mechanical ventilation regimen. At 2 h, plates were treated with vehicle or IL-6 followed with continuous exposure to CS. Control BioFlex plates with static EC culture were placed in the same cell culture incubator and processed similarly to CS-preconditioned cells. At the end of experiment, cell lysates were collected for Western blot analysis, or CS-exposed endothelial monolayers were fixed with 3.7% formaldehyde and subjected to immunofluorescence staining as previously described (7).

Small interfering RNA transfection. To reduce the content of endogenous RhoA, cells were treated with gene-specific small interfering (si)RNA duplexes. Predesigned standard purity siRNA sets (Homo sapiens) were ordered from Dharmacon (Lafayette, CO), and transfection of EC with siRNA was performed as previously described (30). After 48 h of transfection, cells were used for experiments or harvested for Western blot verification of specific protein depletion. Nontargeting, nontargeting siRNA (Dharmacon, Lafayette, CO) was used as a control treatment.

Immunofluorescence. Endothelial monolayers plated on glass coverslips were subjected to double immunofluorescence staining with appropriate antibody, as described previously (7). Texas Red phalloidin was used to visualize F-actin. After being immunostained, slides were analyzed using a Nikon video imaging system (Nikon Insectech, Tokyo, Japan). Images were processed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) software.

Immunoblotting. After stimulation, cells were lysed, and protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies as previously described (2).

Measurement of transendothelial electrical resistance. The endothelial monolayer barrier properties were evaluated by the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) that allows measurements of transendothelial electrical resistance (TER) in real time, which reflects agonist-induced EC permeability changes (8).

Neutrophil migration and adhesion assays. Neutrophil chemotaxis was measured in a 96-well chemotaxis chamber (Neuroprobe, Gaithersburg, MD) as described previously (27). Briefly, freshly isolated neutrophils were placed in a 96-well chemotaxis chamber and incubated with 200 μl of preconditioned culture media that were collected from static EC cultures or from pulmonary EC grown on BioFlex and exposed to 4-h CS at 18% amplitude with or without 2-h pretreatment with IL-6 soluble receptor (SR; 40 ng/ml/10 ng/ml). Preliminary experiments have established the number of cells (4 × 10^4 cells) used allow the optimal cell migration without clogging the pores of Transwell filter of the upper chamber. Data were expressed as percentage of cell migration. Polymorphonuclear leukocyte (PMN) adhesion to the CS-preconditioned EC was assessed at the end of 2-h CS session by addition of the neutrophils freshly isolated from healthy donors to the EC monolayers grown in the sixwell BioFlex plates right after CS experiment. Neutrophil adhesion on HPAEC was assessed as described previously (28). Neutrophil adhesion data were expressed as a percentage of adhesion for all treated groups.

Cytokine analysis. Concentrations of IL-8, keratinocyte-derived chemokine, and macrophage inflammatory protein-1α in control and treated bronchoalveolar lavage (BAL) fluid or cell conditioned medium samples were measured using an ELISA kit available from R&D Systems according to manufacturer’s instructions.

Mechanical ventilation protocol. All animal care and treatment procedures were approved by the University of Chicago Institutional Animal Care and Use Committee and were handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were randomized to concurrently receive sterile saline solution or IL-6 (5 mg/kg it, 16 h) followed by 4 h of mechanical ventilation with HTV (30 ml/kg) as previously described (6). Spontaneously breathing animals served as controls. In experiments with Rho inhibitor, mice were injected with Y-27632 (2 mg/kg iv) before IL-6 instillation. Bacterial LPS (0.63 mg/kg; Escherichia coli O55: B5) was injected in mice intratracheally, and parameters of lung injury were measured at 16 h after LPS challenge. BAL was performed using 1 ml of sterile Hank’s balanced salt buffer and measurements of cell count and protein concentration were conducted as previously described (15). Measurements of Evans blue were performed as described elsewhere (6).

Statistical analysis. Results are expressed as means ± SD. Experimental samples were compared with controls by unpaired Student’s t-test. For multiple-group comparisons, a one-way ANOVA and post hoc multiple comparison tests were used. P < 0.05 was considered statistically significant.

RESULTS

IL-6 increases permeability and activates signaling in human pulmonary EC. Effects of IL-6 on pulmonary EC permeability were monitored by measurements of TER. Treatment with either IL-6 or its SR alone did not significantly change basal resistance, while a combination of IL-6 and SR caused TER decline in a dose-dependent manner (data not shown), which reached maximal levels after 5 h of treatment (Fig. 1A). All following in vitro experiments were performed using IL-6 and SR at concentrations of 40 and 10 ng/ml, respectively.

IL-6-induced EC permeability changes were associated with time-dependent phosphorylation of the regulatory molecule Stat3, which reflects activation of the canonical IL-6-mediated Jak/Stat pathway. IL-6 also induced degradation of the NF-κB complex inhibitory subunit IκBα and phosphorylation of p38 MAPK downstream target HSP27 (Fig. 1B). EC treatment with IL-6 over the duration of experiment did not change total Stat, p38 MAPK, and HSP27 protein levels (data not shown). These events indicate an activation of an inflammatory NF-κB cascade and p38 stress MAP kinase signaling by IL-6. To evaluate the role of Jak/Stat cascade in IL-6-induced EC barrier dysfunction, HPAEC were pretreated with a pharmacologic Jak inhibitor followed by analysis of EC barrier properties after IL-6 challenge. TER measurements demonstrate that Jak inhibition attenuated IL-6-induced hyperpermeability (Fig. 2A). Morphologic analysis of pulmonary endothelium revealed dis-
studies, we investigated the role of Rho signaling in IL-6-induced EC permeability. Activation of Rho signaling was evaluated by analysis of site-specific phosphorylation of Rho-kinase substrate, myosin-binding subunit of MYPT1, and MLC. In contrast to Rho-activating agonist thrombin, IL-6 treatment induced barely detectable increases in MYPT1 and MLC phosphorylation (Fig. 3A). Total MYPT1 and MLC protein levels in IL-6 treated EC did not change (data not shown).

Potential involvement of the Rho pathway in EC barrier dysfunction induced by IL-6 was further examined in experiments with HPAEC pretreatment with pharmacological Rho inhibitor Y-27632. Inhibition of Rho did not significantly affect IL-6-induced permeability (Fig. 3B, left). In contrast, Y-27632 was effective against thrombin-induced Rho-dependent permeability (Fig. 3B, right). The effects of Rho inhibition on IL-6-induced EC cytoskeletal remodeling were examined by immunofluorescence staining and visualization of actin cytoskeleton in pulmonary EC after 5 h of IL-6 treatment. Pretreatment of HPAEC with Rho inhibitor Y-27632 did not affect IL-6-induced formation of paracellular gaps, which suggests a Rho-independent mechanism of IL-6-induced permeability (Fig. 3C).

Pathologic mechanical stimulation promotes IL-6-induced barrier disruption in lung endothelium. To assess the effects of mechanical stimulation on development of IL-6-induced EC barrier dysfunction, we utilized a previously characterized model of ECs subjected to pathologically relevant levels of CS (4). Human pulmonary EC grown to confluence on Flexcell plates and preconditioned at 18% CS for 2 h were treated with vehicle or IL-6 for 1 h with continuous CS. Activation of intracellular signaling by IL-6 was evaluated by increased phosphorylation of signaling proteins. CS preconditioning further increased activation of p38 MAPK and NF-κB pathways induced by IL-6, as detected by increased phosphorylation of HSP27 and enhanced IκBα degradation, respectively. Of note, Stat phosphorylation was not significantly affected by CS preconditioning. In contrast to static conditions, MLC phosphorylation was significantly elevated in CS-preconditioned EC upon stimulation with IL-6 (Fig. 4A). CS exposure over the duration of experiment did not change total protein levels of Jak/Stat, p38 MAPK, IL-6, and MYPT (data not shown).

Recent reports (5, 13) suggest involvement of Rho signaling in the development of lung injury induced by mechanical ventilation at HTV. We next tested involvement of the Rho pathway in exacerbation of IL-6-induced EC barrier disruption by mechanical forces. HPAEC preconditioned at 18% CS (1.5 h) were pretreated with Rho kinase inhibitor Y-27632 for 30 min and then stimulated with IL-6 under continuous CS. Y-27632 completely inhibited CS/IL-6-induced MLC phosphorylation in EC exposed to IL-6 and 18% CS and markedly increased activation of p38 MAPK and NF-κB pathways. CS/IL-6 stimulation of EC cytoskeletal remodeling were examined by immunofluorescence staining and visualization of actin cytoskeleton in pulmonary EC after 5 h of IL-6 treatment. Pretreatment of HPAEC with Rho inhibitor Y-27632 did not affect IL-6-induced formation of paracellular gaps, which suggests a Rho-independent mechanism of IL-6-induced permeability (Fig. 3C).

IL6-induced EC barrier compromise is Rho independent. Recent reports, including our works, show the role of the Rho pathway in the increased lung vascular endothelial permeability induced by inflammatory agonists LPS (33), transforming growth factor-β (3, 9), and TNFα (21). In the following
induced by combined treatment with IL-6 and 18% CS. In contrast, IL-6- and CS-induced Stat phosphorylation was not inhibited by Rho knockdown (Fig. 4C).

The role of Rho signaling on EC barrier disruption and cytoskeletal remodeling induced by IL-6 and 18% CS was examined in the next experiments. Pulmonary EC were preconditioned at 18% CS for 1.5 h followed by Y-27632 pre-treatment for 30 min and stimulation with IL-6 for 5 h under continuing CS. CS further promoted IL-6-induced gap formation (Fig. 4D), compared with static EC culture (Fig. 2B). Addition of Y-27632 after 1.5 h of CS did not affect orientation of CS-preconditioned EC (data not shown) but markedly attenuated gap formation induced by 18% CS and IL-6 (Fig. 4D).

The next experiments attempted to recapitulate more a clinically relevant scenario of EC exposure to 18% CS after IL-6 challenge, which relates to mechanical ventilation of diseased lungs. Cell monolayers pretreated with IL-6 for 1 h were next exposed to 18% CS exposure for 2 h. Similarly to the CS preconditioning model described above, application of CS after IL-6 exposure did not affect activation of Jak/Stat but did increase IL-6-induced activation of p38 MAPK and NF-κB pathways and stimulated Rho signaling and MLC phosphorylation.
lation (Fig. 5A). Rho kinase inhibition by Y-27632 (30-min pretreatment before CS exposure) suppressed HSP-27 phosphorylation and IκBα degradation and completely blocked MLC phosphorylation in response to IL-6 and 18% CS but was without effect on Stat3 phosphorylation (Fig. 5B). Next, we performed analysis of cytoskeletal remodeling in agonist-stimulated stretched EC. In agreement with our previous findings (2, 6), pretreatment with Y-27632 before CS exposure inhibited CS-induced cell orientation (data not shown). Importantly, Y-27632 attenuated the potentiating effect of 18% CS on IL-6-induced paracellular gap formation (Fig. 5C).

Eighteen percent of CS promotes vascular inflammatory responses induced by IL-6. To further assess the role of 18% CS in the modulation of pulmonary EC inflammatory effects induced by IL-6 treatment, confluent EC monolayers were pretreated with IL-6 (2 h) followed by 18% CS exposure (4 h) and analysis of PMN adhesion. The optimal time (4 h of CS exposure) for detection of PMN adhesion was determined in preliminary studies. In static conditions, IL-6 modestly increased PMN adhesion (by 40%), which was further elevated by EC exposure to 18% CS. Interestingly, CS alone caused twofold increase in PMN adhesion to CS-preconditioned EC. Combination of IL-6 and 18% CS further increased PMN adhesion by 45% compared with 18% CS treatment alone (Fig. 6A). Other experiments examined effects of IL-6, 18% CS and their combination on activation of ICAM-1 surface expression in EC. In contrast to LPS stimulation, IL-6 treatment of static EC cultures (5 h) caused only modest increase in ICAM-1 expression detected by Western blot (Fig. 6B, top). Similarly, 18% CS exposure alone (4 h) did not induce significant elevation of ICAM-1 expression, whereas combined treatment with IL-6 and 18% CS caused a synergistic effect on the increase in ICAM-1 expression, which was partially suppressed by Rho kinase inhibition (Fig. 6B, bottom).

To further test the role of the Rho pathway in CS-mediated enhancement of IL-6 inflammatory response, HPAEC were stimulated with IL-6 (1.5 h), treated with Y-27632 or vehicle (30 min), and exposed to 18% CS (4 h). Interestingly, IL-8 production measured by ELISA assay (Fig. 6C) and neutrophil migration in the presence of culture media from control and treated EC (Fig. 6D) was not significantly affected by IL-6 treatment, and Y-27632 pretreatment did not alter IL-6 effects. In contrast, 18% CS preconditioning caused a 2.4-fold increase in IL-8 production and enhanced neutrophil migration, compared with static EC culture. Inhibition of Rho kinase significantly attenuated effects of 18% CS. These data suggest that in our model IL-6-induced inflammatory response is mediated at least in part via ICAM-1-mediated neutrophil adhesion and does not depend on release of chemoattractant IL-8, whereas
pathologic CS affects both pathways of endothelial activation leading to development of inflammation.

Pathological mechanical ventilation enhances IL-6-induced lung injury in vivo. We next evaluated the results from pulmonary EC models in the murine two-hit model of lung injury induced by HTV mechanical ventilation and IL-6. Optimal IL-6 concentration for in vivo use was determined in the initial set of experiments (data not shown). Intratracheal instillation of 1.5, 5, or 15 mg/kg of mouse IL-6 with or without IL-6 soluble receptor (ratio 4:1) was performed for 4, 8, and 16 h, and analysis of protein content and phosphorylation with specific antibodies. Rho protein depletion was confirmed by Western blot. D: immunofluorescence staining of CS-preconditioned (2 h), IL-6/SR challenged (2 h, with continuing CS) EC with or without prior Y-27632 pretreatment (30 min) was performed with Texas Red-conjugated phalloidin to detect actin filaments. Black arrows indicate the main direction of CS vector. Shown are representative results of 3–5 independent experiments.

Fig. 4. Effects of stretch preconditioning on IL-6-induced EC barrier dysfunction: role of Rho. A and B: HPAEC grown on Flexcell plates were subjected to 18% cyclic stretch (CS) for 2 h followed by IL-6/SR (40–10 ng/ml, 1 h) stimulation (A) with or without Y-27632 (2 μM, 30 min) pretreatment (B). Levels of HSP27, Stat3, MLC, MYPT1 phosphorylation, and IκBα degradation in the total cell lysates were determined by Western blot analysis. C: human pulmonary EC were transfected with Rho-specific or nonspecific siRNA. After 48 h of transfection cells were subjected to 18% CS for 2 h followed by combined IL-6 and SR treatment for 1 h and analysis of protein content and phosphorylation with specific antibodies. Rho protein depletion was confirmed by Western blot. D: immunofluorescence staining of CS-preconditioned (2 h), IL-6/SR challenged (2 h, with continuing CS) EC with or without prior Y-27632 pretreatment (30 min) was performed with Texas Red-conjugated phalloidin to detect actin filaments. Black arrows indicate the main direction of CS vector. Shown are representative results of 3–5 independent experiments.
treatment with Y-27632 also significantly reduced lung tissue myeloperoxidase activity (Fig. 7D) and lung vascular leakage detected by Evans blue accumulation in the lung parenchyma (Fig. 7E) in HTV + IL-6 stimulated mice. Western blot analysis of lung tissue samples revealed attenuation of IL-6- and HTV-induced IκBα degradation and upregulated ICAM-1 expression in Y-27632-treated animals (Fig. 7F).

Finally, analysis of inflammatory cytokines in BAL fluid in this two-hit model of ALI demonstrated that combined treatment with HTV and IL-6 induced production of keratinocyte-derived chemokine and macrophage inflammatory protein-1α, while Rho kinase inhibition by Y-27632 suppressed production of these cytokines (Fig. 7G).

**DISCUSSION**

This study characterized for the first time the two-hit model of ALI induced by IL-6 and HTV mechanical ventilation. The results demonstrate that inflammatory and barrier disruptive pathways triggered by pathologic stretch and IL-6 are initiated independently but employ both common and independent mechanisms. Precise signaling mechanisms activated by IL-6 remain to be elucidated. Rapid ERK, p38 MAPK, and JNK phosphorylation induced within 5 min has been demonstrated after IL-6 injection and led to PMN infiltration and cytokine production in the muscle (24). Our results extend these findings and demonstrate that treatment of static pulmonary EC cultures with IL-6 caused activation of Stat3 but also stimulated p38 MAPK-HSP27 signaling and IκBα degradation leading to activation of the NF-κB pathway, increased ICAM-1 expression and PMN adhesion, and increased EC permeability.

Our data also show that in addition to activation of barrier-disruptive inflammatory signaling, IL-6 increased EC permeability via stimulation of VE-cadherin dissociation from adherens junctions. Because VE-cadherin internalization is induced by its serine/threonine or tyrosine phosphorylation (10, 20), and ligation of IL-6 with its receptor gp130 has been shown to activate PI3-kinase and Src kinase family member, Fer (40, 42), it is possible that IL-6-induced VE-cadherin dissociation from adherens junctions may be mediated by gp130-Fer/PI3-
kinase mechanism, although analysis of this mechanism was not the focus of this study. Both the IL-6-induced EC permeability and Stat3, NF-κB, and p38 signaling was abolished by Jak inhibition suggesting that potential IL-6-induced activation of Src is downstream of Jak kinase.

The results of this study show synergistic effects of pathologic CS on IL-6-induced inflammatory signaling in pulmonary EC and suggest a role for Rho signaling in CS-induced enhancement of IL-6 barrier disruptive and proinflammatory effects. Activation of Rho leading to increased MLC phosphorylation and actomyosin contraction is well-recognized mechanism of increased vascular endothelial permeability. In agreement with previous reports, 18% CS increased MLC phosphorylation in vehicle-treated and IL-6 stimulated EC. However, in addition to MLC phosphorylation, 18% CS also increased activation of p38, NF-κB, but not Stat pathways in the IL-6-stimulated EC monolayers. This synergistic effect was dependent on Rho activity, inhibited by Y-27632, and resulted in exacerbation of IL-6-induced EC monolayer disruption, lung barrier dysfunction, and inflammation. Although cross talk between p38 MAPK, NF-κB, and Rho signaling has been reported (17, 25), the precise mechanisms and the hierarchy of these interactions remain to be investigated. Altogether, these signaling events contribute to IL-6- and CS-induced EC barrier disruption. Thus convergence of CS and IL-6 signaling pathways on downstream targets that can be additionally regulated by Rho provides a mechanistic basis for inflammatory and barrier disruptive signal amplification observed in two-hit models of ALI.

With the use of two protocols of pulmonary EC challenge with IL-6 and CS in vitro, one with 18% CS preconditioning before IL-6 treatment, and another with IL-6 pretreatment followed by 18% CS, similar synergistic effects on gap formation and barrier disruptive signaling were observed. In both protocols, inhibition of Rho or Rho kinase significantly attenuated EC barrier dysfunction in the two-hit ALI model. These results support the notion that Rho signaling is a synergistic mechanism of IL-6 and CS barrier disruptive and inflammatory
IL-6 is clearly elevated in acute respiratory distress syndrome patients and correlates with severity of outcomes (1). However, despite well-documented involvement of IL-6 in the mechanisms of VILI-associated inflammation and EC barrier dysfunction, also shown in this study, the idea of IL-6 blockage as a therapeutic option for VILI treatment should be considered with caution. Injection of IL-6 blocking antibodies in mice after ventilator induced lung injury significantly increased rates of BAL albumin flux (38). Surprisingly, IL-6 overexpression even protected mice against hyperoxic lung injury by regulating Bax and reducing apoptosis through a PI-3 kinase pathway (22). In turn, IL-6 generated by mast cells improved survival during sepsis by enhancing neutrophil killing of bacteria (32) and reduced DNA damage during hypoxia (37). IL-6 from a hematopoietic cell source limited alveolar barrier disruption potentially by reducing neutrophil contact with the endothelium (38). These data suggest that such paradoxical effects of IL-6 on modulation of ALI appear to be dependent on the particular cell type producing IL-6 and etiology of ALI.

In conclusion, this study demonstrates a synergistic mechanism of IL-6 and pathological mechanical stimulation of pulmonary vascular endothelium leading to activation of inflam-

Fig. 7. Role of Rho in the development of lung injury in IL-6/HTV two-hit in vivo model. Mice were subjected to intravenous injection with vehicle or IL-6 (5 mg/kg it) with or without Y-27632 (2 mg/kg iv) instillation followed by mechanical ventilation at high tidal volume (HTV; 30 ml/kg, 4 h). Control animals were allowed to breathe spontaneously. LPS-treated mice (0.63 mg/kg it) served as positive controls. A–C: measurements of protein concentration (A), total cell count (B), and differential PMN count (C) were performed in bronchoalveolar lavage (BAL) fluid taken from control and experimental animals. Data are expressed as means ± SD of 4 independent experiments; n = 6–10 per condition; *P < 0.05. D: myeloperoxidase (MPO) activity was determined in control and treated lung tissue samples. MPO data are expressed as %control ± SD of 4 independent experiments; n = 6–10 per condition; *P < 0.05. E: Evans blue dye (30 ml/kg iv) was injected 2 h before termination of the experiment. Lung vascular permeability was assessed by Evans blue accumulation in the lung tissue. Quantitative analysis of Evans blue labeled albumin extravasation was performed by spectrophotometric analysis of Evans blue extracted from the lung tissue samples; n = 4 per condition; *P < 0.05. F: expression levels of ICAM-1 and ICAM-1 were determined in control and treated lung tissue samples. Equal protein loading in Western blot experiments was confirmed by determination of β-tubulin content in tissue homogenates. Rearranged lanes from the same blot are outlined by vertical dotted line. G: analysis of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-1α (MIP)-1α levels was performed in BAL samples from control and treated mice by ELISA. Data are expressed as means ± SD of 4 independent experiments; *P < 0.05.
matory signaling and EC barrier dysfunction. Our results show that parallel activation of Rho-independent mechanisms by circulating IL-6 and Rho-dependent mechanisms by suboptimal mechanical ventilation lead to further exacerbation of ALI. Thus, in addition to reduction of ALI induced by Rho-activating mediators such as thrombin, the beneficial effect of Rho downregulation shown in the IL-6/HTV model suggests that inhibition of Rho may be a promising strategy for general treatment of VILI conditions.

ACKNOWLEDGMENTS

We thank Katherine Higginbotham for proofreading the manuscript.

REFERENCES


31. Singleton PA, Chatchavalvanich S, Fu P, Xing J, Birukova AA, Fortuna JA, Klihanov AM, Garcia JG, Birukov KG. Akt-mediated transactivation of the S1P1 receptor in caveolin-enriched microdomains


