Differential Regulation of Pulmonary Endothelial Monolayer Integrity by Varying Degrees of Cyclic Stretch

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Ventilator-induced lung injury is a life-threatening complication of mechanical ventilation at high-tidal volumes. Besides activation of proinflammatory cytokine production, excessive lung distension directly affects blood-gas barrier and lung vascular permeability. To investigate whether restoration of pulmonary endothelial cell (EC) monolayer integrity after agonist challenge is dependent on the magnitude of applied cyclic stretch (CS) and how these effects are linked to differential activation of small GTPases Rac and Rho, pulmonary ECs were subjected to physiologically (5% elongation) or pathologically (18% elongation) relevant levels of CS. Pathological CS enhanced thrombin-induced gap formation and delayed monolayer recovery, whereas physiological CS induced nearly complete EC recovery accompanied by peripheral redistribution of focal adhesions and cortactin after 50 minutes of thrombin. Consistent with differential effects on monolayer integrity, 18% CS enhanced thrombin-induced Rho activation, whereas 5% CS promoted Rac activation during the EC recovery phase. Rac inhibition dramatically attenuated restoration of monolayer integrity after thrombin challenge. Physiological CS preconditioning (5% CS, 24 hours) enhanced EC paracellular gap resolution after step-wise increase to 18% CS (30 minutes) and thrombin challenge. These results suggest a critical role for the CS amplitude and the balance between Rac and Rho in mechanochemical regulation of lung EC barrier. (*Am J Pathol* 2006, 168:1749–1761; DOI: 10.2353/ajpath.2006.050431)

Pathological lung overdilation is associated with mechanical ventilation at high-tidal volumes and compromises the blood-gas barrier, increases lung permeability, and may

culminate in ventilator-induced lung injury and pulmonary edema.^{1,2} Clinical studies suggest that vascular leak observed in ventilator-induced lung injury patients is associated with increased levels of edemagenic agents and inflammatory cytokines such as thrombin, histamine, tumor necrosis factor- α , interleukin-8, and interleukin-1.^{1,3–5} However, the significance of the interactions between the edemagenic agents and pathological mechanical distension of the lung tissue in progression of ventilator-induced lung injury-associated vascular leak and pulmonary edema has been only recently recognized. In this connection, the National Heart, Lung, and Blood Institute working group⁶ emphasized the importance of two-hit animal models that combine experimentally induced lung inflammation and mechanical ventilation at high-tidal volumes to more appropriately reflect common co-morbidities and risk factors present in patients with acute lung injury. Consistent with these findings, *in vitro* models of pulmonary cells exposed to pathophysiological regimen of mechanical stretch and edemagenic agonists may provide vital information about molecular mechanisms of mechanochemical regulation of lung endothelial or epithelial permeability.

Pulmonary endothelium forms a semiselective barrier for macromolecules and cell elements regulated by contractile and tethering forces generated by interaction of cytoskeletal elements and cell adhesions (focal contacts, tight junctions, and adherens junctions).^{7–11} Both edemagenic agents (thrombin, nocodazole) and barrier-protective agonists (sphingosine 1-phosphate, hepatocyte growth factor) exert their effects via actomyosin-driven microfilament reorganization and cell contact remodeling.^{9–14} EC barrier regulation is controlled by several signaling molecules including Ca²⁺/calmodulin-dependent myosin light chain kinase, Ca²⁺/calmodulin-dependent kinase II, protein kinase C, protein kinase A, and protein tyrosine kinases.⁸ However,

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both barrier-protective and barrier-disruptive processes in ECs are heavily dependent on the activation of the small GTPases Rac and Rho.^{10,14–18}

Recent studies showed that Rac and Rho activities in vascular endothelial and smooth muscle cells can be regulated by mechanical forces.^{19–23} Importantly, shear stress and high-magnitude cyclic stretch (CS) exhibited differential effects on Rho and Rac activation, which were linked to specific actin cytoskeletal remodeling, focal adhesion redistribution, and Rac-mediated endothelial barrier enhancement by shear stress.²⁰ Pathologically relevant levels of CS exhibited synergistic effects on thrombin-induced EC barrier disruption and involved activation of Rho effector, Rho-associated kinase, and myosin light chain phosphorylation.¹⁹ In this study, we tested the hypothesis that CS related to physiological and pathological lung mechanical ventilation is critically involved in the regulation of agonist-induced disruption and recovery of pulmonary EC monolayer integrity. Using a cell culture model of human lung ECs exposed to CS at physiologically and pathologically relevant magnitudes *in vitro*, we investigated magnitude-dependent effects of CS on the recovery of EC monolayer integrity after thrombin challenge and linked these effects with activation of the small GTPases Rho and Rac.

Materials and Methods

Reagents

RhoA and Rac1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), a paxillin antibody was obtained from BD Transduction Laboratories (San Diego, CA), a cortactin antibody was purchased from Upstate Biotechnology (Lake Placid, NY), horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies were obtained from Cell Signaling Inc. (Beverly, MA). Rho and Rac activation kits were purchased from Upstate Biotechnology. Rac inhibitor NSC-23766 was purchased from Calbiochem (La Jolla, CA). All reagents used for immunofluorescent staining were purchased from Molecular Probes (Eugene, OR). Unless specified, all other reagents, including sphingosine 1-phosphate and human thrombin, were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Human pulmonary artery endothelial cells (HPAECs) were obtained from Clonetics, BioWhittaker Inc. (Frederick, MD). Cells were maintained in complete culture medium consisting of Clonetics EBM basic medium containing 10% fetal bovine serum and supplemented with a set of nonessential amino acids, endothelial cell (EC) growth factors, and 100 U/ml penicillin/streptomycin (Clonetics, BioWhittaker Inc.) and incubated at 37°C in humidified 5% CO₂ incubator. Cells were used for CS experiments at passages 6 to 8.

Cell Culture under CS

All CS experiments were performed using FX-4000T Flexercell Tension Plus system (Flexcell International,

McKeesport, PA) equipped with a 25-mm BioFlex loading station, as previously described.^{19,20} Experiments were performed in the presence of culture medium containing 2% fetal bovine serum. Briefly, HPAECs were seeded at standard densities (8×10^5 cells/well) onto collagen I-coated flexible bottom BioFlex plates. Both static HPAEC cultures and cells exposed to CS were seeded onto identical plates to ensure standard culture conditions. After 48 hours of culture, the medium was changed in each plate, and experimental plates with EC monolayers were mounted onto the Flexercell system and exposed to CS of desired magnitude (5% or 18% elongation) and duration (0 to 48 hours). Control BioFlex plates with static EC culture were placed in the same cell culture incubator. When necessary, static controls and CS-exposed HPAECs were treated with thrombin and incubated for 5, 15, 30, or 50 minutes of continuous exposure to both stimuli. At the end of the experiment, cell lysates were collected for Rac and Rho activation assays or for gel electrophoresis and Western blot analysis; alternatively, CS-exposed endothelial monolayers were fixed with 3.7% formaldehyde and used for immunohistochemistry.

Depletion of Endogenous Rac

Pre-designed Rac-specific small interfering RNA (siRNA) of standard purity was purchased from Ambion, Inc. (Austin, TX) in purified, desalted, deprotected, and annealed double-strand form. The following 21-bp duplexes of siRNA were used: sense 5'-GGAGAUUGGUGCUGUAAAAAtt-3' and anti-sense 5'-UUUUACAGCACCAAU-CUCt-3'. Nonspecific, nonsilencing FI-luciferase GL2 duplex fluorescently labeled on the sense strand with 5'-fluorescein (Dharmacon Research, Lafayette, CO) was used as a control treatment. HPAECs were grown to 70% confluence, and the transfection of siRNA (final concentration, 100 nmol/L) was performed using GeneSilencer transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocol. Forty-eight hours later cells were used for the experiments.

Immunofluorescence Staining and Image Analysis

After exposure to CS, ECs were fixed in 3.7% formaldehyde solution in phosphate-buffered saline (PBS) for 10 minutes at 4°C, washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 30 minutes at room temperature, and blocked with 2% bovine serum albumin in PBS for 30 minutes. Incubation with antibody of interest was performed in blocking solution (2% bovine serum albumin in PBS) for 1 hour at room temperature followed by staining with Alexa 488-conjugated secondary antibodies. Actin filaments were stained with Texas Red-conjugated phalloidin diluted in the blocking solution. After immunostaining, the slides were analyzed using a Nikon video imaging system (Nikon Instech Co., Japan) consisting of a inverted microscope Nikon Eclipse TE300 connected to SPOT RT monochrome digital camera and

image processor (Diagnostic Instruments, Sterling Heights, MI). The images were acquired using SPOT 3.5 acquisition software (Diagnostic Instruments) and processed with Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Quantitative analysis of paracellular gap formation was performed as previously described.^{19,24,25} The 16-bit images were analyzed using MetaVue 4.6 software (Universal Imaging, Downingtown, PA). Paracellular gaps were manually marked out, and images were differentially segmented between gaps and cells based on image grayscale levels. The gap formation was expressed as a ratio of the gap area to the area of the whole image. The values were statistically processed using Sigma Plot 7.1 (SPSS Science, Chicago, IL) software. For each experimental condition at least 10 microscopic fields in each independent experiment were analyzed from the different areas of the plate (both central and peripheral). The representative images of EC monolayers closer to the plate periphery were taken to illustrate amplitude-dependent effects of CS on thrombin-induced gap formation, cortactin translocation, and focal adhesion remodeling.

Western Immunoblotting

Immunoblot detection of proteins of interest was performed as described previously.^{19,26} Briefly, protein extracts were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (100 V for 1 hour), and probed with Rho or Rac antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Amersham, Little Chalfont, UK). The relative intensities of the protein in the bands were quantified by scanning densitometry.

Rho and Rac activation assays were performed using commercially available assay kits purchased from Upstate Biotechnology, as we have previously described.^{14,18} Briefly, ECs pre-exposed to CS at selected magnitude and duration were treated with vehicle or stimulated with thrombin for desired periods of time. Then, cell lysates were collected, and GTP-bound Rac or Rho were captured using pulldown assays with immobilized PBD domain and rhotekin, respectively, according to the manufacturer's protocols. The levels of activated small GTPases as well as total Rac and Rho content were evaluated by Western blot analysis and quantified by scanning densitometry of the autoradiography films. The levels of activated proteins Rac or Rho were normalized to total Rac or Rho level for densitometry evaluations.

Statistical Analysis

Results are expressed as means $\pm$ SD of three to five independent experiments. Stimulated samples were compared with controls by unpaired Student's *t*-test. For multiple-group comparisons, one-way analysis of variance followed by the post hoc Fisher's test were used. *P* < 0.05 was considered statistically significant.

Results

Magnitude-Dependent Effects of CS on Thrombin-Induced Gap Formation and EC Monolayer Recovery

We have previously shown magnitude-dependent effects of CS on the acute phase of thrombin-induced EC barrier disruption.¹⁹ In this study, we examined the effects of 5% CS and 18% CS on restoration of EC monolayer integrity after thrombin challenge. Human pulmonary ECs were exposed to 5% or 18% CS for 2 hours followed by continuous thrombin (50 nmol/L) stimulation for 5 minutes (peak of barrier disruption) or 50 minutes (recovery phase). Cells exposed to 2 hours of 5% CS or 18% CS without agonist stimulation revealed similar patterns of cytoskeletal arrangement characterized by circumferential F-actin rim and few central stress fibers oriented in a perpendicular direction to the main distension vector (Figure 1A, top). Consistent with our previous studies,¹⁹ ECs exposed to pathologically relevant CS levels (18% elongation) showed a dramatic increase in the level of paracellular gap formation after 5 minutes of thrombin treatment (shown by arrows), as compared to ECs exposed to physiological CS levels (5% elongation) or ECs in static culture (Figure 1A, middle). Analysis of EC monolayer recovery (after 50 minutes of thrombin challenge) showed that static controls and ECs exposed to 18% CS exhibited more stress fiber bundles and larger paracellular gaps, whereas ECs exposed to 5% CS exhibited less central stress fibers with more pronounced peripheral F-actin staining and demonstrated nearly complete disappearance of paracellular gaps (Figure 1A, bottom). Quantitative analysis of thrombin-induced (50 nmol/L, 5 minutes) gap formation (Figure 1B) confirmed these observations and showed that total gap areas in static controls and or EC monolayers exposed to 5% CS represented 60 to 65% of total gap area observed in EC monolayers exposed to 18% CS. More importantly, physiological CS level accelerated paracellular gap resolution after thrombin stimulation (50 nmol/L, 50 minutes). As demonstrated in Figure 1B, EC monolayers preconditioned at 5% CS showed a 4.8-fold reduction in the gap area after 50 minutes of thrombin stimulation, as compared to a 2.5-fold reduction in static EC cultures and a 2.6-fold reduction in ECs exposed to 18% CS.

Focal Adhesion Remodeling in Pulmonary ECs Exposed to CS and Thrombin Stimulation

Remodeling of EC focal adhesions plays an important role in the regulation of the endothelial barrier by chemical agonists²⁷ and has been described in ECs exposed to CS.^{20,28,29} In this study, we examined focal adhesion rearrangement in ECs exposed to physiological and pathological CS levels and stimulated with thrombin. Focal adhesion-associated protein paxillin was used to monitor CS- and/or agonist-induced focal adhesion re-

Figure 1. Effects of CS preconditioning at 5% and 18% linear distension on thrombin-induced disruption and recovery of pulmonary EC monolayer integrity. **A:** Human pulmonary ECs were exposed to CS for 2 hours followed by 5-minute and 50-minute thrombin stimulation (50 nmol/L) with continuous CS. Actin cytoskeletal remodeling was examined by immunofluorescent staining with Texas Red-conjugated phalloidin. Intercellular gaps induced by thrombin stimulation are marked by arrows. **B:** Quantitative analysis of thrombin-induced gap formation in pulmonary ECs exposed to low- and high-magnitude CS was performed as described in Materials and Methods. Maximal gap formation observed in EC monolayers treated with 50 nmol/L of thrombin for 5 minutes was taken as 100%. Shown are representative results of five independent experiments. **P* < 0.05.

modeling. Thrombin stimulation (50 nmol/L, 5 minutes) caused an increase in the number and size of adhesion plaques in static and CS-preconditioned ECs (Figure 2A, middle). However, during the recovery phase after thrombin stimulation (50 minutes), cells exposed to 5% CS revealed accumulations of paxillin-containing focal adhesions at the cell periphery, whereas ECs preconditioned at 18% CS displayed random distribution of focal adhesions of larger size and numbers, as compared to static cultures and ECs exposed to 5% CS (Figure 2, A and B).

Effects of CS on Cortactin Translocation in Lung ECs during Recovery Phase after Thrombin Challenge

Results of this study show that 5% CS promoted EC monolayer recovery after thrombin stimulation, which was characterized by disappearance of paracellular gaps and restoration of peripheral actin cytoskeleton, whereas high-magnitude CS delayed these processes (Figure 1). We and

Figure 2. Focal adhesion remodeling in CS-preconditioned ECs during endothelial monolayer recovery after thrombin stimulation. **A:** ECs were exposed to 5% or 18% CS for 2 hours followed by stimulation with 50 nmol/L of thrombin for 5 minutes or 50 minutes. Immunofluorescence analysis of focal adhesion remodeling after thrombin stimulation in static cultures (**left**) and ECs exposed to 5% (**middle**) or 18% CS (**right**) was performed using paxillin staining as described in Materials and Methods. **B:** Higher magnification images of CS-exposed HPAECs after 50 minutes of thrombin stimulation. **White arrows** show peripheral accumulation of paxillin in ECs preconditioned at 5% CS. Random distribution of paxillin-containing focal adhesions in ECs preconditioned at 18% CS after 50 minutes of thrombin stimulation is marked by **filled arrows**. Shown are representative results of five independent experiments.

others have previously shown that enhancement of EC barrier induced by laminar shear stress or chemical agonists is associated with peripheral accumulation of a regulator of actin polymerization, cortactin.^{18,20,30–33} ECs exposed to 5% CS or 18% CS exhibited diffuse cortactin distribution before and after 5 minutes of thrombin (50 nmol/L) stimulation (data not shown). During the recovery phase (after 50 minutes of thrombin challenge), nonstretched cells showed weak accumulation of cortactin at the cell periphery (Figure 3, left). Remarkably, ECs preconditioned at 5% CS exhibited robust cortactin accumulation at the cell periphery (Figure 3, middle), whereas no noticeable peripheral cortactin accumulation was observed after 50 minutes of thrombin treatment in ECs preconditioned at 18% CS (Figure 3, right).

Accumulation of cortactin at the cell periphery had been previously linked to activation of the small GTPase Rac by chemical agonists (sphingosine 1-phosphate and oxidized phospholipids)^{10,18} and laminar shear stress.^{20,23} In the next series of experiments, we directly examined effects of 5% and 18% CS on the activation of small GTPases Rac and Rho.

Effects of 5% and 18% CS on Rac and Rho Activation

ECs were subjected to 5% CS during 15 minutes or 30 minutes followed by measurements of Rac and Rho

Figure 3. Cortactin distribution in CS-preconditioned ECs during monolayer recovery after thrombin stimulation. Human pulmonary ECs were exposed to 5% CS or 18% CS for 2 hours followed by stimulation with 50 nmol/L of thrombin for 50 minutes. Immunofluorescent detection of cortactin translocation after thrombin stimulation in static cultures and ECs exposed to CS was performed using anti-cortactin antibody. Areas of cortactin accumulation are marked by **arrows**. Shown are representative results of three independent experiments.

activation using *in vitro* pull-down assays as described in the Materials and Methods section. EC exposure to 5% CS increased Rac activity by 30 minutes of CS stimulation (Figure 4A), but did not affect Rho activity (Figure 4B). ECs stimulated with sphingosine 1-phosphate (1 μ mol/L, 5 minutes) or thrombin (50 nmol/L, 5 minutes) were used as positive controls for Rac and Rho activation, respectively. In contrast, we have previously demonstrated that 18% CS induced Rho activation at 15 minutes and 30 minutes of CS exposure without affecting Rac activation.²⁰ Comparison of Rac and Rho activation by 5% and 18% CS is summarized in Figure 4C and shows differential activation of Rac and Rho by physiological and pathological CS levels. Figure 4C also indicates that in the absence of additional co-stimulation with agonists, CS alone induced significantly lower levels of Rac/Rho activation, as compared to agonist stimulation controls.

Figure 4. Differential activation of Rac and Rho by 5% and 18% CS. Human pulmonary ECs were exposed to CS at 5% elongation for the indicated periods of time, and Rac (**A**) and Rho (**B**) activation was measured as described in Materials and Methods. The graphs below depict results of quantitative analysis of Rac and Rho activation by scanning densitometry of the autoradiography films, normalized to the total Rho or Rac content in the cell lysates, and expressed in relative density units (RDU). Sphingosine 1-phosphate (0.5 μ mol/L) and thrombin (50 nmol/L) served as positive controls for Rac and Rho activation, respectively. **C:** Bar graphs represent comparative analysis of Rac and Rho activation in response to 5% CS and 18% CS stimulation for 30 minutes. Shown are representative results of three independent experiments. * $P < 0.05$.

Effects of Short-Term CS Preconditioning on Thrombin-Induced Rho and Rac Activation

To further explore crosstalk between mechanical and chemical stimuli in the regulation of Rho and Rac activity, we exposed human pulmonary ECs to CS at 5% or 18% elongation for 2 hours followed by thrombin stimulation (50 nmol/L, 5 minutes). At this time point, CS-induced cytoskeletal remodeling in EC monolayers is generally complete.¹⁹ Rho activation after 5 minutes of thrombin treatment was not significantly different in ECs preconditioned for 2 hours at 5% CS and static controls (Figure 5A). Importantly, thrombin-induced Rho activation was significantly increased in ECs preconditioned for 2 hours at 18% CS, as compared to static controls and ECs exposed to 5% CS (Figure 5A). Next, we examined the effects of CS and thrombin on Rac activity. Thrombin stimulation of static HPAECs caused a decrease in basal Rac activity by 5 minutes (Figure 5B), correlating with reciprocal Rho activation and paracellular gap formation (Figures 1 and 5A). The increase in Rac activation was first seen after 30 minutes of thrombin stimulation. This early activation of Rac may contribute to further processes of barrier recovery (Figure 1; thrombin, 50 minutes). Importantly, after 50 minutes of thrombin stimulation, Rac activity was significantly increased above basal levels, which correlated with recovery of endothelial monolayer integrity observed after 50 minutes of thrombin challenge (Figure 1). Based on these data, in further experiments we analyzed Rac activation after 50 minutes of thrombin stimulation, the time point that best represented Rac-dependent cytoskeletal remodeling and monolayer recovery process. To further explore magnitude-dependent effects of CS on Rac activation during monolayer recovery after thrombin challenge, cells exposed to 5% CS or 18% for 2 hours were treated with thrombin (50 nmol/L) for 50 minutes. EC preconditioning at 5% CS increased Rac activation observed at 50 minutes of thrombin stimulation, as compared to static controls (Figure 5C). In contrast, preconditioning at 18% CS significantly decreased Rac activation as compared to the levels observed at this time point for both static and 5% CS-preconditioned ECs (Figure 5C).

Figure 5. Effects of 5% and 18% CS preconditioning on thrombin-induced Rho and Rac activation. **A:** Human pulmonary ECs were exposed to 5% CS or 18% CS for 2 hours followed by thrombin stimulation (50 nmol/L, 5 minutes) and measurements of Rho activation. **B:** Static human pulmonary ECs were exposed to thrombin (50 nmol/L) for 5 minutes, 30 minutes, 50 minutes, or left untreated (0 minutes) followed by measurements of Rac activity. **C:** Human pulmonary ECs were exposed to 5% CS or 18% CS for 2 hours, stimulated with thrombin (50 nmol/L, 50 minutes) and Rac activities were measured during EC recovery phase after thrombin challenge (50 minutes). Rho and Rac activation was assessed by *in vitro* pulldown assays, quantified by scanning densitometry of the autoradiography films, normalized to the total Rho or Rac content in the cell lysates, and expressed as the RDU. Shown are representative results of three independent experiments. * $P < 0.05$.

Effects of Rac Down-Regulation on EC Monolayer Integrity under CS and EC Monolayer Recovery after Thrombin Stimulation

Previous results show that HPAEC monolayers exposed to both 5% CS and 18% CS maintained their integrity,¹⁹

and few gaps per 10 microscopic fields were typically found in static and stretched HPAECs without thrombin stimulation. In the next experiments we performed Rac protein depletion using the siRNA-based approach described in our previous studies.¹⁸ Rac depletion (confirmed by Western blot shown in Figure 6A) caused noticeable gap formation in HPAEC cultures exposed to both 5% CS and 18% CS (Figure 6B, bottom), which was not observed in the cells transfected with nonspecific RNA (Figure 6B, top). To directly examine if Rac activity is critical for EC monolayer recovery after thrombin challenge, HPAECs exposed to 5% CS or 18% CS were pretreated with vehicle (PBS) or Rac pharmacological inhibitor NSC-23766 (200 μ mol/L, 1 hour), which specifically down-regulates Rac activity with no effect toward Cdc42 and Rho GTPases.³⁴ NSC-23766 reduced the basal Rac activity in the HPAECs exposed to 5% CS without thrombin treatment and significantly attenuated Rac activation after 50 minutes of thrombin stimulation (Figure 7A). Thrombin challenge induced pronounced paracellular gap formation after 5 minutes of stimulation in both, vehicle- and NSC-23766-pretreated cells exposed to either 5% CS or 18% CS (Figure 7B, left). Importantly, preincubation with NSC-23766 significantly attenuated recovery of HPAEC monolayer integrity after 50 minutes of thrombin stimulation in HPAECs exposed to both, 5% CS and 18% CS (Figure 7B, right). Similar to CS-preconditioned cells, down-regulation of Rac activity in static ECs resulted in delayed monolayer recovery after thrombin challenge as well as inhibition of cortactin translocation during recovery phase (data not shown).

Effects of Physiological CS Preconditioning on EC Barrier Dysfunction Induced by Thrombin and Step-Wise Increase in CS Amplitude

Lung ECs exposed to physiologically relevant stretch (5% elongation) exhibited better barrier protective properties as compared to thrombin-stimulated static EC cultures and cells exposed to high-magnitude CS (Figures 1, 2, 3, and 5). We next examined effects of physiological CS preconditioning on EC responses to acute elevation of CS amplitude and agonist stimulation. ECs preconditioned at 5% CS for 24 hours were subjected to step-wise increase in CS amplitude to 18% elongation during 30 minutes followed by thrombin (50 nmol/L) stimulation. After 5 minutes or 50 minutes of thrombin challenge cytoskeletal remodeling and paracellular gap formation in this experimental group were compared to thrombin-stimulated nonstretched cells, cells exposed to 24 hours of 5% CS, and cells exposed to 30 minutes of 18% CS without prior CS-preconditioning. F-Actin remodeling and paracellular gap formation was assessed by F-actin staining of EC monolayers. Similar to short-term CS experiments (Figure 1), a step-wise increase in CS amplitude in endothelial monolayers exposed to 24-hour CS at 5% elongation did not affect monolayer integrity in the vehicle-exposed monolayers (Figure 8A, top). Thrombin treatment (50 nmol/L, 5 minutes) caused massive stress fibers and paracellular gap formation (shown by arrows) in static culture, less pronounced changes in cells preconditioned at 5% CS,

Figure 6. Effects of Rac protein depletion on the monolayer integrity in HPAECs exposed to CS. **A:** Cells grown in D35 plastic dishes were incubated with siRNA to Rac1 or treated with nonspecific RNA oligonucleotide duplexes for 48 hours, and Rac protein depletion was examined by immunoblotting with corresponding antibody. Control blots represent β -actin expression in ECs treated with siRNA. **B:** HPAECs grown on BioFlex plates were incubated with siRNA to Rac1 (**bottom row**) or treated with nonspecific RNA duplexes (**top row**) for 48 hours as described in Materials and Methods. Cells were next left static (**left**) or were exposed to 2-hour 5% CS (**middle**) or 18% CS (**right**). Actin cytoskeletal remodeling was evaluated by immunofluorescent staining with Texas Red-conjugated phalloidin. Paracellular gaps are shown by **arrows**. Enlarged images of cell interface areas (**insets**) illustrate significant paracellular gaps formed in CS-stimulated EC monolayers with depleted Rac1 (shown by **arrow**), as compared to cells treated with nonspecific RNA duplexes. Shown are representative results of three independent experiments.

and more dramatic cytoskeletal remodeling in cells subjected to 30-minute 18% CS (Figure 8A, middle). Interestingly, chronic CS-preconditioning at physiologically relevant magnitude (5% elongation) did not affect massive gap formation induced by 5 minutes of thrombin exposure in ECs subjected to step-wise CS increases to 18% elongation when compared to 18% CS only. Results of quantitative analysis (Figure 8B) indicate similar levels of thrombin-induced (5 minutes) gap formation in both 5% CS-preconditioned and nonpreconditioned HPAECs subjected to acute 18% CS (30 minutes). Next, EC monolayers were examined during the recovery phase after 50 minutes of thrombin challenge (Figure 8A, bottom). The recovery after thrombin treatment (50 nmol/L, 50 minutes) was characterized by the disappearance of stress fibers and significant reduction in the number of paracellular gaps in static EC culture, and even more dramatic stress fiber dissolution and disappearance of gaps in ECs preconditioned for 24 hours at 5% CS (Figure 8A, second left column). In contrast, less pronounced decrease in stress fibers and gap sealing were observed in ECs exposed to acute 18% CS (Figure 8A, right column) and chronic 18% CS (data not shown), as compared to nonstretched controls. These morphological changes are consistent with the results of experiments with

2 hours of CS (Figure 1A). Remarkably, ECs preconditioned for 24 hours at 5% CS followed by a step-wise increase to 18% CS (30 minutes) revealed more complete monolayer recovery after 50 minutes of thrombin challenge, as compared to thrombin-treated ECs exposed to 18% CS (30 minutes) (Figure 8A, second right column). Taken together with the results of the quantitative analysis of paracellular gap formation (Figure 8B), these data suggest that chronic CS-preconditioning at physiologically relevant magnitude enhances barrier recovery after EC monolayer disruption induced by a combination of acute high-magnitude CS and thrombin.

Effects of Chronic Physiological CS Preconditioning on Thrombin-Induced Rho and Rac Activation

To link effects of physiological CS preconditioning on thrombin-induced disruption of EC barrier with Rho- and Rac-mediated pathways, we exposed HPAECs to chronic (24 hours) CS at 5% or 18% elongation followed by thrombin stimulation for the indicated time periods and analyzed Rho and Rac activities in the cell lysates, as

Figure 7. Effects of Rac inhibition on monolayer recovery in HPAECs exposed to CS and thrombin. **A:** HPAECs were grown on BioFlex plates. Cells exposed to 5% CS were preincubated with Rac inhibitor NSC-23766 (200 μ mol/L, 1 hour) or vehicle. After a total of 2 hours of CS exposure (with or without inhibitor), ECs were stimulated with thrombin (50 nmol/L) for 5 minutes, and Rac activation was assessed by *in vitro* pulldown assays, as described in Materials and Methods. Shown are representative results of three independent experiments. **B:** Cells exposed to 5% CS or 18% CS were preincubated with Rac inhibitor NSC-23766 (200 μ mol/L, 1 hour) or vehicle. After a total of 2 hours of CS exposure (with or without inhibitor), HPAECs were stimulated with thrombin (50 nmol/L) for 5 minutes (**left row**) and 50 minutes (**right row**). Actin rearrangement was analyzed by immunofluorescent staining with Texas Red-conjugated phalloidin. Paracellular gaps are shown by **arrows**. Shown are representative results of three independent experiments.

described above. Chronic CS preconditioning (5% CS, 24 hours) did not change the basal level of Rho activity as compared to static control but significantly attenuated Rho activation after 5 minutes of thrombin stimulation (Figure 9A). In contrast, preconditioning at 18% CS modestly increased a peak of thrombin-induced Rho activation by 5 minutes, as compared to static ECs. Rho activity in ECs preconditioned at 18% CS remained elevated even after 50 minutes of thrombin stimulation, as compared to nonstretched EC cultures (Figure 9B). Measurements of Rac activation at the time point corresponding to the EC monolayer recovery phase (50 minutes of thrombin stimulation) show that preconditioning at 18% CS significantly reduced, whereas 5% CS significantly increased Rac activation as compared to HPAECs cultured under static conditions (Figure 9C). Basal Rac activity

was not significantly different in CS-preconditioned and static EC cultures.

Discussion

The main finding of this study is the beneficial effects of CS preconditioning at physiological amplitudes on the pulmonary EC barrier recovery after challenge with the inflammatory agonist thrombin. In contrast, high-magnitude CS compromised EC barrier recovery after thrombin stimulation, and our data strongly suggest specific roles for the small GTPases Rac and Rho in the magnitude-dependent control of EC barrier disruption and recovery. Assessment of interstitial/vascular distension in the mechanically ventilated lung is extremely complicated because of complexity of local distension patterns in the lung parenchyma. This is especially true during mechanical ventilation of an injured lung when inflammation-induced occlusion of a part of the alveolar tree results in overdistension of the functional alveoli. Elegant studies from Tschumperlin and Margulies^{35,36} have shown that 5 to 12% linear distension corresponds to 60 to 80% of total lung capacity and is associated with physiological levels of mechanical strain experienced by alveolar epithelium and microvasculature during mechanical ventilation at low-tidal volumes, the regimen used in protective lung strategies. In contrast, CS at 17 to 22% linear distension corresponds to 100% of total lung capacity and is relevant to pathophysiological conditions induced by mechanical ventilation at high-tidal volumes and associated with the inflammatory response and acute lung injury *in vivo*, or progressive death of alveolar epithelial cells and increased agonist-induced barrier disruption in the lung endothelial monolayers *in vitro*.^{19,37} Clinical data and experimental observations indicate that besides direct effects on epithelial and endothelial integrity and permeability,^{38,39} mechanical ventilation at high-tidal volumes causes release of inflammatory cytokines, which further exacerbate ventilator-induced lung injury.^{40,41} In addition, mechanical ventilation at high-tidal volumes has been shown to enhance LPS-induced lung injury and vascular leak in animal models.⁴² Our results are highly consistent with clinical observations and results of animal studies and show that in contrast to physiological CS or static conditions, high-magnitude CS (18% elongation) further increased agonist-induced EC monolayer disruption and delayed the restoration of endothelial monolayer integrity. Our results strongly suggest involvement of the small GTPases Rac and Rho in the magnitude-dependent EC barrier regulation.

Small GTPases Rac, Rho, and Cdc42 are critically involved in cell motility, cytoskeletal remodeling^{43,44} and EC barrier regulation^{10,14,16,18,45,46} via activation of downstream effector kinases PAK, mDia, Rho-kinase,^{44,47–49} and nonenzymatic cytoskeletal and cell adhesion effectors such as Arp-2/3 complex, cortactin, N-WASP, paxillin, and PKL/GIT2.^{44,47,50,51} In our previous work we have described a role for Rho-associated kinase (Rho-kinase) in stimulation of myosin light chain phosphorylation and exacerbation of thrombin-induced EC

Figure 8. Effects of long-term physiological CS preconditioning on EC monolayer disruption and recovery induced by 18% CS and thrombin. **A:** Static EC cultures, or CS-preconditioned cells (24 hours, 5% distension) were either immediately treated with thrombin (50 nmol/L) or exposed to 30 minutes of 18% CS before thrombin stimulation. F-actin was visualized after 5 minutes (**middle row**) and 50 minutes (**bottom row**) of thrombin treatment by immunofluorescent staining with Texas Red phalloidin. Intercellular gaps are marked by **arrows**. **B:** Quantitative analysis of thrombin-induced gap formation in static and CS-preconditioned pulmonary ECs on thrombin stimulation was performed as described in Materials and Methods. Shown are representative results of three independent experiments. * $P < 0.05$.

barrier dysfunction by high-magnitude CS¹⁹ and showed that Rho/Rho-kinase is a convergence point (or hub) for agonist- and stretch-mediated signaling, which mediates increased EC permeability. This study demonstrates for the first time differential regulation of Rho and Rac GTPases by physiological and pathologically relevant levels of CS. Our results show that the beneficial effects of physiological CS on the recovery of EC monolayer integrity after thrombin challenge are associated with increased activation of Rac. Furthermore, experiments with Rac down-regulation using a siRNA approach or pharmacological Rac inhibitor demonstrate a direct role for Rac activity in the maintenance of basal monolayer integrity and EC monolayer recovery after agonist challenge in CS-preconditioned lung endothelium. Thus, taken together these results show that high-magnitude CS promotes Rho activation related to the early phase of thrombin-induced Rho activation and EC monolayer disruption, and suppresses Rac activation essential for recovery phase. In contrast to 18% CS, physiological CS causes lower levels of thrombin-induced Rho activation, reduces

EC barrier disruption, and significantly promotes the EC monolayer recovery phase associated with increased Rac activities.

Enhanced EC barrier recovery in pulmonary vascular endothelium preconditioned at 5% CS was associated with increased peripheral F-actin staining and significant accumulation of cortactin at the cell periphery. Cortactin is a key element involved in the regulation of the cortical actin cytoskeleton, and whose translocation to the cell periphery in response to sphingosine 1-phosphate, oxidized phospholipids, or laminar shear stress^{10,18,20,33} correlates with activation of Arp-2/3-mediated actin polymerization,⁵¹ cell motility,⁵² and enhancement of the endothelial barrier.^{18,31} Furthermore, we have shown a Rac-dependent mechanism of cortactin translocation induced by barrier-protective agonists^{18,31} and laminar shear stress.^{20,33} This study shows increased peripheral cortactin accumulation in ECs exposed to 5% CS during the recovery phase after thrombin stimulation and strongly suggests an involvement of the Rac/cortactin mechanism in the lung EC barrier restoration under physiological CS.

Figure 9. Effects of chronic CS preconditioning and thrombin on Rho and Rac activation. After 24-hour preconditioning at 5% CS (**A**) or 18% CS (**B**), human pulmonary ECs were challenged with 50 nmol/L of thrombin for 5 minutes or 50 minutes without interruption of CS stimulation, and Rho activation assays were performed as described in Materials and Methods. HPAEC cultures grown on BioFlex plates without CS and stimulated with thrombin for 5 minutes or 50 minutes served as static controls. **C:** Human pulmonary ECs preconditioned at 5% CS or 18% CS for 24 hours were challenged with vehicle or thrombin (50 nmol/L) for 50 minutes without interruption of CS stimulation, and Rac activation assays were performed. The graphs depict results of quantitative analysis of Rho and Rac activation by scanning densitometry of the autoradiography films, normalized to the total Rho or Rac content in the cell lysates, and expressed in RDU. Shown are representative results of three independent experiments. * $P < 0.05$.

Focal adhesion protein complexes contain several Rac and Rho effectors including paxillin, GIT1, and PKL/GIT2.^{43,50,53–55} We have previously shown a unique peripheral distribution of paxillin, GIT1, and PKL/GIT2 associated with Rac-mediated EC barrier protective response to mechanical factors (laminar shear stress)²⁰ and chem-

ical agonists (sphingosine 1-phosphate).¹³ On the other hand, the Rho-dependent permeability increase in thrombin-stimulated cells was accompanied by an increased number and size of focal adhesions that exhibited random intracellular distribution.^{14,27} Our results show peripheral paxillin distribution in ECs exposed to 5% CS during recovery after thrombin challenge associated with barrier-protective EC potential (Figure 2), whereas preconditioning at 18% CS revealed a pattern of focal adhesion distribution after 50 minutes of thrombin stimulation characteristic for EC monolayers with increased permeability. Thus, different patterns of focal adhesion remodeling in ECs preconditioned at 5% or 18% CS after 50 minutes of thrombin stimulation further support our findings about differential effects of physiological and pathological CS levels on lung EC barrier recovery after agonist stimulation.

Chronic EC preconditioning at physiological CS levels significantly improved EC barrier recovery after acute elevation of CS amplitude to 18% linear elongation and thrombin stimulation, as compared to thrombin-treated ECs under acute 18% CS. These results are consistent with our previous studies, which demonstrated magnitude-dependent CS effects on the expression of specific proteins involved in EC cytoskeletal remodeling and barrier regulation, such as Rho and ZIP-kinase.¹⁹ Thus, increased expression of these signaling molecules may enhance EC responses to agonist stimulation by increased myosin light chain phosphorylation, EC contraction, and barrier dysfunction. Besides a role of CS in agonist-induced EC barrier regulation via induction of key signal molecules, CS preconditioning may affect basal activation of intracellular signaling systems. Studies using 24-hour flow-preconditioning showed that abrupt cessation of flow caused rapid membrane depolarization and increased generation of reactive oxygen species in lung microvascular cells.⁵⁶ Membrane depolarization observed in these experiments was caused by deactivation of ATP-sensitive K^+ channel as result of flow cessation, not anoxia.⁵⁷ These data suggest that mechanical preconditioning may also set new levels of basal activation to cell signaling systems involved in EC remodeling or barrier regulation.

Based on the results of these studies, we speculate that physiological CS stimulates barrier-protective EC potential via Rac-mediated mechanisms, which trigger focal adhesion remodeling, induce cortactin peripheral translocation and activation of cortical actin polymerization, and thus accelerate re-establishment of EC monolayer integrity. In turn, high-magnitude CS suppresses Rac activities and enhances Rho-mediated signaling, which leads to increased EC barrier disruption induced by thrombin via Rho-dependent mechanisms and compromises the Rac-dependent barrier recovery phase in thrombin-challenged lung EC monolayers. Precise mechanisms of CS-induced small GTPase activation are not yet explored, but may involve activation of Rac- and Rho-specific guanine nucleotide exchange factors, which facilitate GDP/GTP exchange in the nucleotide-binding site of Rac and Rho and cause Rac/Rho activation.⁵⁸ A hypothetical mechanism of magnitude-depen-

Figure 10. Hypothetical mechanism of magnitude-dependent regulation of agonist-induced EC permeability by CS. Physiological CS stimulates Rac by activating putative Rac-specific guanine nucleotide exchange factor (GEF). Rac activation triggers focal adhesion peripheral redistribution, induces cortactin peripheral translocation, activation of cortical actin polymerization, and thus accelerates re-establishment of EC monolayer integrity after thrombin challenge. In turn, high-magnitude CS suppresses Rac activities and enhances Rho-mediated signaling, which leads to increased EC barrier disruption and delays barrier recovery induced by thrombin via Rho-dependent activation of stress fiber formation, actomyosin contraction, and random focal adhesion redistribution in thrombin-challenged lung EC monolayers.

dent regulation of EC barrier properties by CS is summarized in Figure 10. Ongoing studies in our laboratory are underway to explore upstream mechanisms of magnitude-dependent regulation of Rho and Rac and further delineate potential impact of other edemagenic agents and inflammatory cytokines associated with acute lung injury on the permeability responses in the pulmonary ECs preconditioned at physiological and pathological CS.

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