



Endothelial barrier disruption and recovery is controlled by substrate stiffness[☆]

Anna A. Birukova^{a,*}, Xinyong Tian^a, Ivan Cokic^a, Yvonne Beckham^b,
Margaret L. Gardel^b, Konstantin G. Birukov^a

^a Lung Injury Center, Section of Pulmonary and Critical Medicine, Department of Medicine, University of Chicago, Chicago, IL 60637, USA

^b Department of Physics, University of Chicago, Chicago, IL 60637, USA

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ABSTRACT

Circulating barrier disruptive agonists bind specific cell membrane receptors and trigger signal transduction pathways leading to the activation of cell contractility and endothelial cell (EC) permeability. Although all cells in tissues including vascular EC are surrounded by compliant extracellular matrix, the impact of matrix stiffness on agonist-induced signaling, cytoskeletal remodeling and EC barrier regulation is not well understood. This study examined agonist-induced cytoskeletal and signaling changes associated with EC barrier disruption and recovery using pulmonary EC grown on compliant substrates of physiologically relevant (8.6 kPa) stiffness, very low (0.55 kPa) and very high (42 kPa) stiffness. Human pulmonary microvascular and macrovascular EC grown on 0.55 kPa substrate contained a few actin stress fibers, while stress fiber amount increased with increasing matrix stiffness. Thrombin-induced stress fiber formation was maximal in EC grown on 42 kPa substrate, diminished on 8.6 kPa substrate, and was minimal on 0.55 kPa substrate. These effects were linked to a stiffness-dependent increase in thrombin-induced phosphorylation of the Rho kinase target, myosin light chain phosphatase (MYPT1), and regulatory myosin light chains (MLC). Surprisingly, EC barrier recovery and activation of Rac GTPase-dependent barrier protective signaling reached maximal levels in EC grown on 8.6 kPa, but not on 0.55 kPa substrate. In conclusion, these data show a critical role of extracellular matrix stiffness in the regulation of the Rac/Rho signaling balance during onset and resolution of agonist-induced EC permeability. The optimal conditions for the Rho/Rac signaling switch, which provides an effective and reversible EC cytoskeletal and permeability response to agonist, are reached in cells grown on the matrix of physiologically relevant stiffness.

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Introduction

The vascular endothelium functions as a semi-selective barrier for macromolecule transport across the vessel wall. EC respond to external stimuli by cytoskeletal rearrangements and activation or inhibition of contractile machinery, which determine the barrier enhancing or barrier disruptive EC response (Dudek and Garcia, 2001; Mehta and Malik, 2006). The activation of Rho GTPase and Rho-associated kinase (Rho kinase) is a key mechanism of EC permeability induced by barrier-disruptive and inflammatory agonists (Birukova et al., 2004, 2012b; Essler et al., 1998; Kakiashvili et al., 2009). Rho signaling is further potentiated in agonist-stimulated EC monolayers exposed to pathologic cyclic stretch (Birukova et al., 2006b). Activated Rho kinase phosphorylates

and inactivates myosin light chain phosphatase (MLCP) by phosphorylating Thr⁶⁹⁵, Ser⁸⁹⁴, and Thr⁸⁵⁰ (Essler et al., 1998; Fukata et al., 2001) leading to the accumulation of phosphorylated regulatory myosin light chains (MLC), actomyosin contraction and disruption of the endothelial barrier (Birukova et al., 2004; van Nieuw Amerongen et al., 2000).

In turn, the recovery of EC monolayer integrity is controlled by Rac signaling. Physiologic activation of Rac by barrier-protective molecules (Birukova et al., 2007a, 2007b; Garcia et al., 2001; Vouret-Craviari et al., 2002) or physiologic mechanical forces (Birukov et al., 2002; Birukova et al., 2006b) enhances the peripheral actin cytoskeleton, induces peripheral redistribution of focal adhesions, and enhances the EC barrier. These effects are mediated by Rac1-mediated activation and phosphorylation of several Rac effectors including p21-associated kinase (PAK1) and cortactin (Birukova et al., 2010b; Lee et al., 2006; Uruno et al., 2001).

Although all cells in various tissues are surrounded by compliant extracellular matrix, the role of matrix stiffness in cell responses to circulating bioactive molecules has not been previously appreciated. In contrast to experiments in cell cultures grown on rigid substrates with stiffness in the GPa giga-pascals range (plastic, glass), cells in situ are surrounded by compliant extracellular matrix, and matrix stiffness varies in the range of 1 kPa in brain to ~30 kPa in precalcified

Abbreviations: EC, endothelial cells; HPAEC, human pulmonary artery endothelial cells; HLMVEC, human lung microvascular endothelial cells; MLC, myosin light chain; MYPT, myosin-associated phosphatase; PAK1, p21-activated kinase.

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* Corresponding author at: Lung Injury Center, Section of Pulmonary and Critical Medicine, Department of Medicine, University of Chicago, 5841 S. Maryland Ave, Office N-611, Chicago, IL 60637, USA. Fax: +1 773 834 2683.

E-mail address: kbirukov@medicine.bsd.uchicago.edu (A.A. Birukova).

bone, and ~100 kPa in calcified sites of atherosclerotic rabbit thoracic artery (Flanagan et al., 2002; Liu et al., 2010; Matsumoto et al., 2002; Suki et al., 2005). In lung tissue, the estimated stiffness range in the alveolar wall is ~5 kPa (R-45), although local stiffness variations in the lung parenchyma are within 0.5–3 kPa range and may increase 6–8 fold in fibrotic conditions (Liu et al., 2010). Emerging studies demonstrate that matrix stiffness affects cell signaling, cytoskeletal organization, levels of intercellular and intracellular force generation (Aratyn-Schaus et al., 2011; Krishnan et al., 2011; Maruthamuthu et al., 2011; Yeung et al., 2005), and may even define a fate of progenitor cells directing them towards neuronal, muscle or bone lineages (Engler et al., 2006). Alterations in matrix stiffness are associated with pathologic conditions. Increased matrix stiffness has been implicated in various pathologies including cardiovascular disease, diabetes, aging and tumor progression (Cameron and Cruickshank, 2007; Chan and Dart, 2011; Levental et al., 2009), and contributes to lung fibrosis by stimulating the Rho pathway of myofibroblast differentiation (Huang et al., 2012; Liu et al., 2010).

Although the active role of matrix stiffness in the control of cell phenotype and intracellular signaling has been recognized, understanding of substrate stiffness-dependent regulation of endothelial permeability and barrier recovery remain limited. This study investigated the role of matrix stiffness on the agonist-induced cytoskeletal remodeling, activation of Rho and Rac signaling and recovery of macrovascular and microvascular EC grown on substrates with very low (0.55 kPa), physiologically relevant (8.6 kPa); and very high (42 kPa) (corresponding to fibrotic tissue) stiffness.

Materials and methods

Reagents and cell culture

Unless specified, biochemical reagents were obtained from Sigma (St. Louis, MO). Reagents for immunofluorescence were purchased from Molecular Probes (Eugene, OR). Antibodies to phospho-Thr⁸⁵⁰ myosin-associated phosphatase (MYPT) were purchased from Millipore (Billerica, MA); antibody to diphospho-Ser¹⁹/Thr¹⁸ myosin light chain (MLC) was from Cell Signaling Inc. (Beverly, MA); phospho-Ser⁴²³-PAK1 and phospho-Tyr⁴²¹-cortactin antibody were from BD Transduction Laboratories (San Diego, CA). Human pulmonary artery endothelial cells (HPAEC) and human lung microvascular endothelial cells (HLMVEC) were obtained from Lonza (Allendale, NJ), maintained in a complete culture medium according to the manufacturer's recommendations and used for experiments at passages 5–7.

Preparation of polyacrylamide (PAA) substrates for endothelial cell cultures

PAA substrates were prepared on glass coverslips with an acrylamide/bis-acrylamide ratio to obtain gels with shear elastic moduli of 0.55 kPa, 8.6 kPa and 42 kPa and coated with collagen as characterized previously (Aratyn-Schaus et al., 2010; Yeung et al., 2005). Collagen was covalently attached to the top surface of the PAA hydrogel by using the bifunctional crosslinker sulfo-SANPAH (Pierce Thermo Scientific, Rockford, IL).

Immunofluorescence and image analysis

Endothelial monolayers plated on glass cover slips were subjected to immunofluorescence staining with Texas Red phalloidin to visualize F-actin, as described previously (Birukova et al., 2006a, 2010a). The integrated fluorescence density of Texas Red phalloidin was measured using MetaMorph software. The results were normalized in each experiment.

Western blot analysis of MYPT, MLC, cortactin and PAK1 phosphorylation

Analysis of MYPT and MLC phosphorylation was used to monitor activation of Rho signaling, and levels of phosphorylated cortactin and PAK1 were measured as readouts of Rac activation, as previously described (Birukova et al., 2004, 2006b).

Statistical analysis

Results are expressed as mean ± SD of three to six independent experiments. Experimental samples were compared to controls by unpaired Student's *t*-test. For multiple-group comparisons, a one-way variance analysis (ANOVA) and post hoc multiple comparison tests were used. *P* < 0.05 was considered statistically significant.

Results

F-actin arrangement in control and thrombin-stimulated macrovascular and microvascular EC is controlled by substrate stiffness. Confluent and subconfluent EC cultures were grown on PAA hydrogels of different stiffness, as described in **Materials and methods**. Both, confluent and subconfluent HPAEC and HLMVEC grown on low stiffness matrix (0.55 kPa) developed a fine network of actin filaments evenly distributed across the cell area. Increasing matrix stiffness caused gradual increase in actin stress fiber formation. In subconfluent EC cultures grown on 42 kPa substrate, more pronounced actin filament concentration at the cell periphery and formation of a distinct actin stress fiber rim were observed (Figs. 1A, 2A, and 3A – left panels). Thrombin-induced stress fiber formation was observed in confluent and subconfluent HPAEC and HLMVEC and increased with increasing substrate stiffness (Figs. 1–3 – panels B). Of note, stiffness-dependent increase in stress fibers under basal conditions was less expressed in EC monolayers as compared to subconfluent culture. These data may indicate additional influence of cell–cell communications on cell responses to substrate stiffness. Most pronounced recovery of actin cytoskeletal structure at 30 min after thrombin treatment was observed in HLMVEC on 8.6 kPa substrate. Interestingly, partial dissolution of stress fibers after 30 min of thrombin stimulation and appearance of lamellopodia, which reflect the onset of EC monolayer recovery after thrombin challenge, was significantly reduced in EC grown on 42 kPa PAA hydrogels (Figs. 3A,B).

Substrate stiffness dependent activation of Rho and Rac signaling in acute phase of thrombin-induced EC barrier disruption and during barrier recovery. We monitored levels of MYPT1 and MLC phosphorylation as the biochemical parameters reflecting the activation of thrombin-induced Rho pathway of cytoskeletal remodeling and EC barrier disruption (Birukova et al., 2004). Increasing substrate stiffness progressively enhanced thrombin-induced MYPT1 and MLC phosphorylation (Figs. 4A,B,C). EC grown on substrate with the highest stiffness (42 kPa) exhibited the highest levels of MYPT1 and MLC phosphorylation under both, control and thrombin-stimulated conditions, which also remained elevated for a longer time. Quantitative analysis of thrombin-induced MYPT and MLC phosphorylation in HPAEC grown on substrates with different stiffness is shown in Fig. 4D.

Physiologically relevant substrate stiffness supports most efficient activation of Rac signaling during EC barrier recovery after thrombin. Several cytoskeletal Rac effectors, such as the Arp2/3 complex, p21Arc, p21-activated kinase (PAK1), and cortactin control cortical actin structure (Borisy and Svitkina, 2000; Weed and Parsons, 2001). Increased autophosphorylation of PAK1 at Thr⁴²³, and cortactin phosphorylation at Tyr⁴²¹ is mediated by Rac activation and has been previously observed during EC barrier recovery (Birukova et al., 2012a). Thrombin challenge did not affect PAK1 and cortactin phosphorylation state at an early time point (5 min data not shown), but significantly increased PAK1 and cortactin phosphorylation at 30 min after thrombin treatment, the time point corresponding to the monolayer recovery phase

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