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Endothelial barrier disruption and recovery is controlled by substrate stiffness

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Abstract

Circulating barrier disruptive agonists bind specific cell membrane receptors and trigger signal transduction pathways leading to 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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Keywords

endothelium; hydrogels; stiffness; cytoskeleton; thrombin; monolayer recovery; Rho signaling; MLC; PAK1; cortactin

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). 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; Birukova et al., 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 Thr^{695}, Ser^{894}, and Thr^{850} (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, recovery of EC monolayer integrity is controlled by Rac signaling. Physiologic activation of Rac by barrier-protective molecules (Birukova et al., 2007a; Birukova et al., 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 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 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-Thr^{850} myosin-associated phosphatase (MYPT) were purchased from Millipore (Billerica, MA); antibody to diphospho-Ser^{19}/Thr^{18} myosin light chain (MLC) was from Cell Signaling Inc (Beverly, MA); phospho-Ser^{423}–PAK1 and phospho-Tyr^{421}–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; Birukova et al., 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., 2006b; Birukova et al., 2004).

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 comparisons 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 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 (Figures 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 (Figures 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 (Figure 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 (Figure 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 Figure 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 Thr^{423}, and cortactin phosphorylation at Tyr^{421} 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 (Figure 5A,B,C). Interestingly, maximal levels of PAK1 and cortactin phosphorylation reflecting stimulation of Rac signaling were observed in EC grown on the substrate of physiologically relevant stiffness, while cortactin and PAK1 phosphorylation on very soft substrate was diminished compared to cells on 8.6 kPa substrate (Figure 5D). Because we used only one physiologically relevant stiffness index, we cannot exclude that more optimal stiffness conditions may exist in this range and promote even more rapid monolayer recovery than on 8.6 kPa substrate.

Discussion

This study shows substrate stiffness dependent actin cytoskeletal arrangement in microvascular and macrovascular EC. Both cell types grown on very soft substrates exhibited less F-actin stress fibers, while cells grown on physiologically relevant or very stiff substrates exhibited a stiffness-dependent increase in stress fibers. In addition, lung microvascular and macrovascular EC grown on very stiff substrate showed dramatic circumferential accumulation of stress fibers. These data are in agreement with other observations, which revealed increased stress fiber content in fibroblasts grown on substrates with increasing stiffness (Yeung et al., 2005). Thrombin stimulation of pulmonary EC exhibited modest effects on stress fiber formation when cells were grown on very soft substrate. Furthermore, thrombin stimulation induced a gradual increase in stress fiber formation in EC grown on 0.55 kPa, 8.6 kPa and 42 kPa substrates. Surprisingly, we observed occasional lamellopodia formation in EC on very soft matrix at early times of thrombin treatment. These results may reflect spatial dysregulation of Rho-Rac signaling upon agonist stimulation of EC grown on very soft matrices.

A stiffness-dependent increase in stress fiber formation was paralleled by activation of MYPT1 and MLC phosphorylation. MYPT and MLC are downstream effectors of Rho. Rho GTPase signaling can be activated by thrombin via receptor-dependent mechanism (Birukova et al., 2004) or locally at the focal adhesions via recrutitment or activation of focal adhesion-associated mechanosenstitive guanine exchange factors (Guilluy et al., 2011). Thus, the observed stiffness-potentiated Rho activation in control and thrombin-stimulated cells reflected by phosphorylation state of MYPT and MLC may be controlled by mechanosensing at focal adhesions and translated to actin cytoskeleletal changes driven by MLC phosphorylation. Taken together, these data demonstrate that enhancement of thrombin-induced stress fiber formation is driven by Rho-dependent barrier disruptive signaling, which increases unidirectionally with increase in substrate stiffness. In turn, EC monolayer recovery and reestablishment of intercellular contacts and a peripheral actin cytoskeletal rim after thrombin is associated with upregulation of Rac signaling (Birukova et al., 2012a; Tauseef et al., 2008). Growing EC on very stiff substrate (42 kPa) delayed the disappearance of stress fibers and lamellopodia formation during the recovery phase after

thrombin stimulation (30 min), while most efficient recovery was observed in EC grown on the 8.6 kPa substrate. Differences in morphological changes of EC grown on physiologic and very stiff substrates were associated with significantly reduced levels of Rac signaling in EC on very stiff substrate monitored by levels of phosphorylated PAK1 and cortactin. Surprisingly, activation of Rac effectors at later times after thrombin stimulation in EC grown on the 0.55 kPa substrate was lower than in cells on the 8.6 kPa substrate. These results were obtained using macrovascular EC. However, although the impact of macrovascular endothelium in development pulmonary edema is less evident than microvascular EC, the molecular mechanisms of barrier recovery in EC from both vascular beds share common features and critically depend on activation of cortactin and Rac1 GTPase pathway (Birukova et al., 2007c; David et al., 2011; Tauseef et al., 2008). Taken together, these results demonstrate that agonist-induced Rho signaling uniformly increases with increasing substrate stiffness, while activation of Rac signaling by EC during recovery is biphasic: it is reduced on substrates with very low or very high stiffness and stimulated in substrates of physiological stiffness. Delayed activation of Rac signaling after rapid activation of Rho-dependent EC permeability is a key mechanism driving EC barrier recovery (Tauseef et al., 2008). However, the mechanism of such a Rho/Rac switch is poorly understood, and precise mechanisms orchestrating temporal changes in Rho and Rac activities remain to be defined.

Control of intracellular signaling by substrate stiffness is under active investigation. Intracellular and extracellular mechanical forces generated by the actomyosin cytoskeleton and extracellular matrix induce activation of protein kinases and small GTPases located at focal adhesions and cell-cell junctions via a process of mechanotransduction (Orr et al., 2006). Mechanical activation of focal adhesions may stimulate Rho signaling (Bershadsky et al., 2006). Therefore, decreased mechanical loading of focal adhesions in EC grown on very soft matrix may dampen full activation of Rho by this mechanism. The mechanism of maximal Rac activation in EC grown on 8.6 kPa substrate post-thrombin treatment is not clear. Similar to Rho, Rac can also be regulated by focal adhesion complexes. Rac signaling, critical for cell adhesion, protrusion dynamics (Nayal et al., 2006), endothelial barrier restoration and barrier enhancement (Birukova et al., 2008), was stimulated by activated PAK1 localized in focal adhesions (Birukova et al., 2008; Nayal et al., 2006). In turn, pathologic cyclic stretch decreased Rac activation (Katsumi et al., 2002) suggesting mechanical control of Rac signaling by mechanical forces. Current published studies testing Rac signaling activated by mechanical stimulation were performed on plastic or silicone substrates with very high, non-physiologic stiffness. Thus, the role of substrate stiffness in the physiologic range on Rac awaits further investigation.

In conclusion, the results of this study demonstrate a monophasic, stiffness-dependent increase of Rho signaling in EC upon thrombin treatment and biphasic stiffness dependent effects on delayed activation of Rac signaling in thrombin-stimulated EC grown on compliant substrates. Our data show that EC grown on substrates with physiologically relevant stiffness display a range of Rho and Rac activation, which ensures most efficient permeability response and rapid barrier recovery in physiological conditions *in vivo*. We speculate that changes in the lung vascular endothelial mechanical microenvironment in

pathological settings of acute lung injury, and inflammation of chronic conditions (lung fibrosis, emphysema, diabetes) may contribute to severity of lung barrier dysfunction and promote chronic changes in vascular permeability initiated by circulating pathologic mediators. Thus, better characterization of the vascular mechanical microenvironment in health and disease and efforts aimed at normalization of lung vascular mechanical properties may improve resolution of ALI and restoration of lung barrier properties.

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Non-standard 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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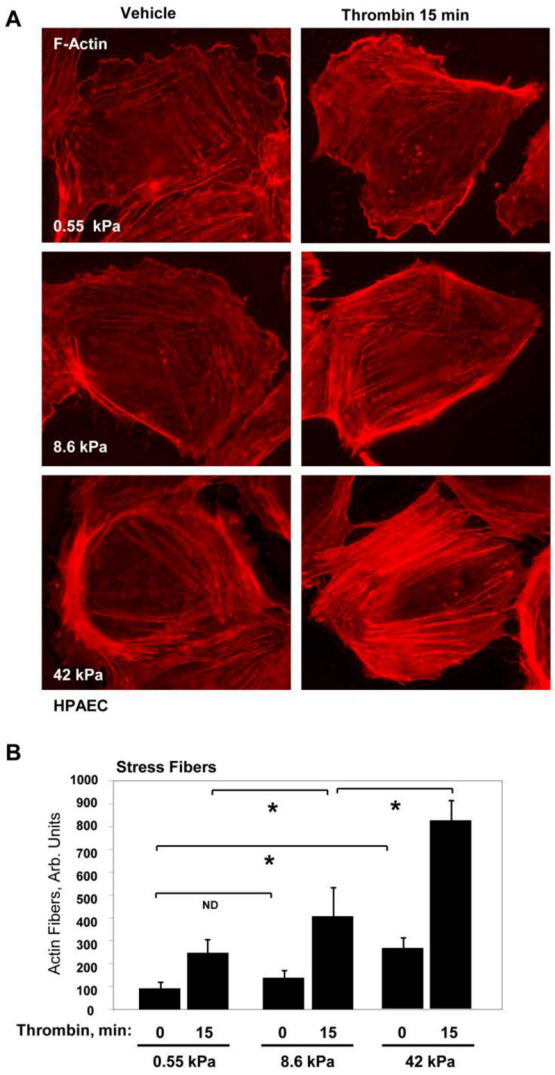

Figure 1. Stiffness dependent stress fiber formation in subconfluent human pulmonary artery endothelial cells (HPAEC)

A - Cells were grown on collagen-I coated polyacrylamide gels of different stiffness (0.55 kPa, 8.6 kPa, and 42 kPa) and treated with thrombin (0.3 U/ml, 15 min). F-actin was visualized by immunofluorescence staining with Texas Red-conjugated phalloidin. **B -** Quantitative image analysis of thrombin-induced stress fiber formation in HPAEC under conditions depicted in panel A. Quantification of F-actin fluorescence intensity was performed as described in the Materials and Methods. *P<0.05, n=4 independent experiments.

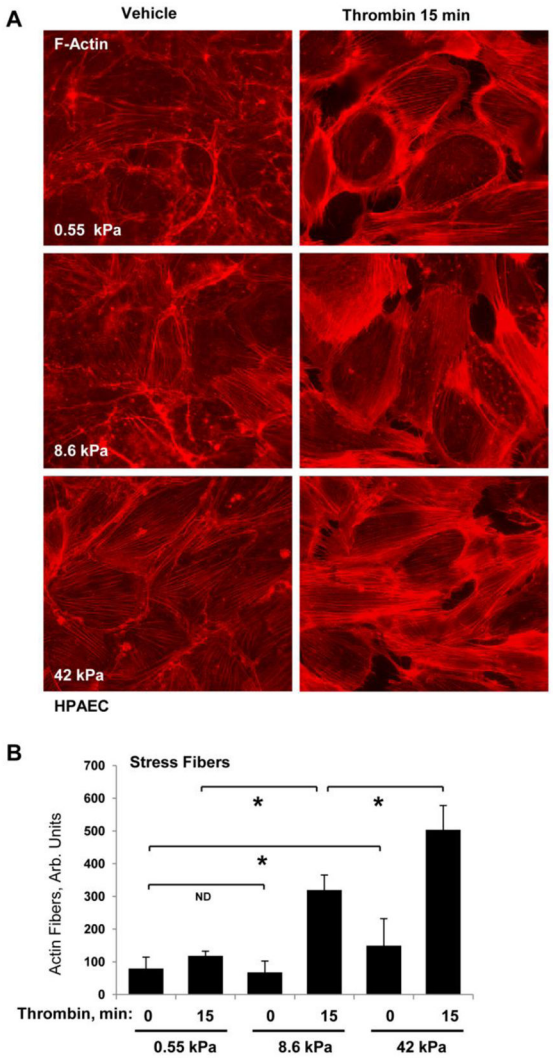

Figure 2. Stiffness dependent stress fiber formation in confluent thrombin-stimulated HPAEC monolayers

A - Cell monolayers were grown on collagen-I coated polyacrylamide gels of different stiffness (0.55 kPa, 8.6 kPa, and 42 kPa) and treated with thrombin (0.3 U/ml, 15 min). F-actin was visualized by immunofluorescence staining with Texas Red-conjugated phalloidin. **B -** Quantitative image analysis of thrombin-induced stress fiber formation in HPAEC under conditions depicted in panel A. Quantification of F-actin fluorescence intensity was performed as described in the Materials and Methods. *P<0.05, n=3 independent experiments.

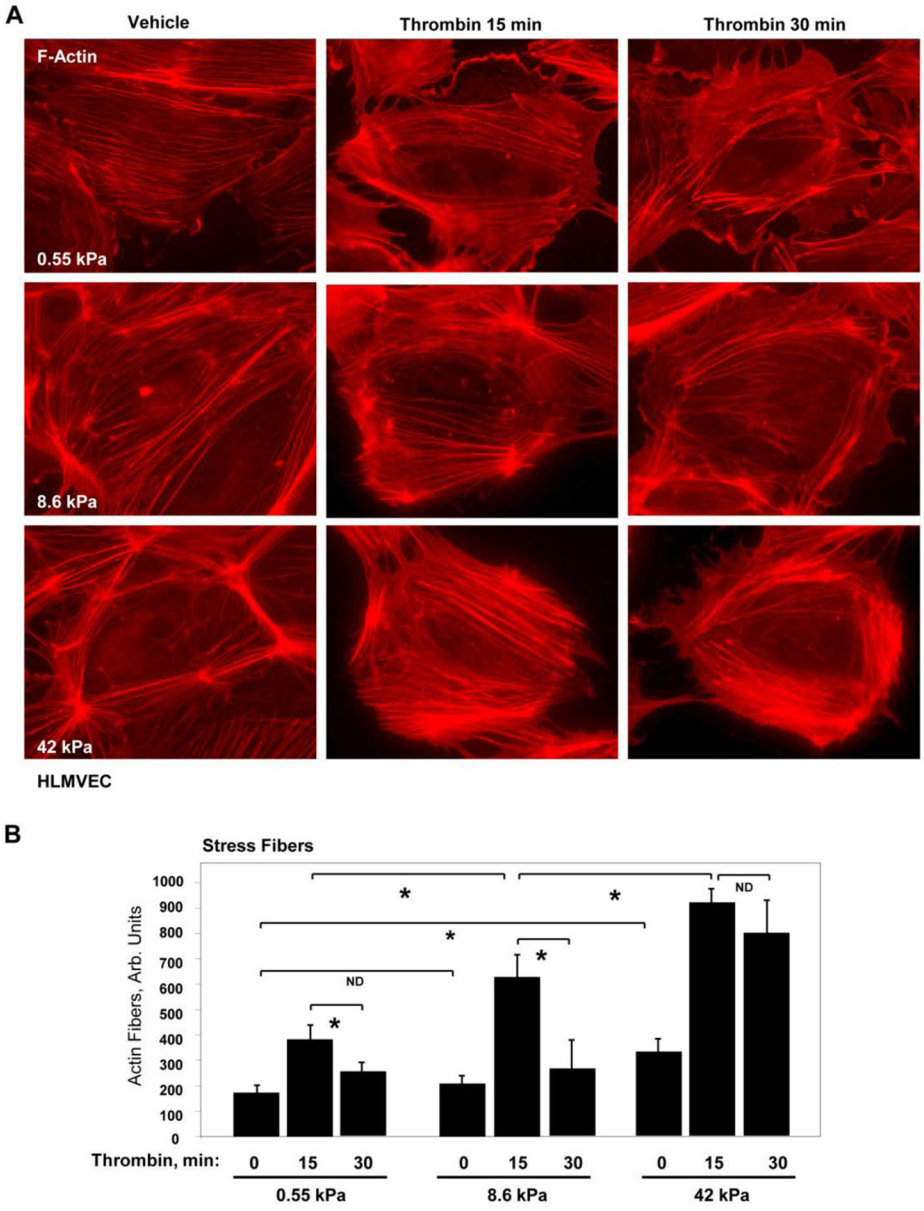

Figure 3. Substrate stiffness dependent F-actin cytoskeletal remodeling in control and thrombin-stimulated human lung microvascular EC (HLMVEC)

A - Cells were grown on collagen-I coated polyacrylamide gels of different stiffness (0.55 kPa, 8.6 kPa, and 42 kPa) and treated with thrombin (0.3 U/ml, 15 min and 30 min). F-actin was visualized by immunofluorescence staining with Texas Red-conjugated phalloidin. **B -** Quantitative image analysis of thrombin-induced stress fiber formation in under conditions depicted in panel A. Quantification of F-actin fluorescence intensity was performed as described in the Materials and Methods. *P<0.05, n=4 independent experiments.

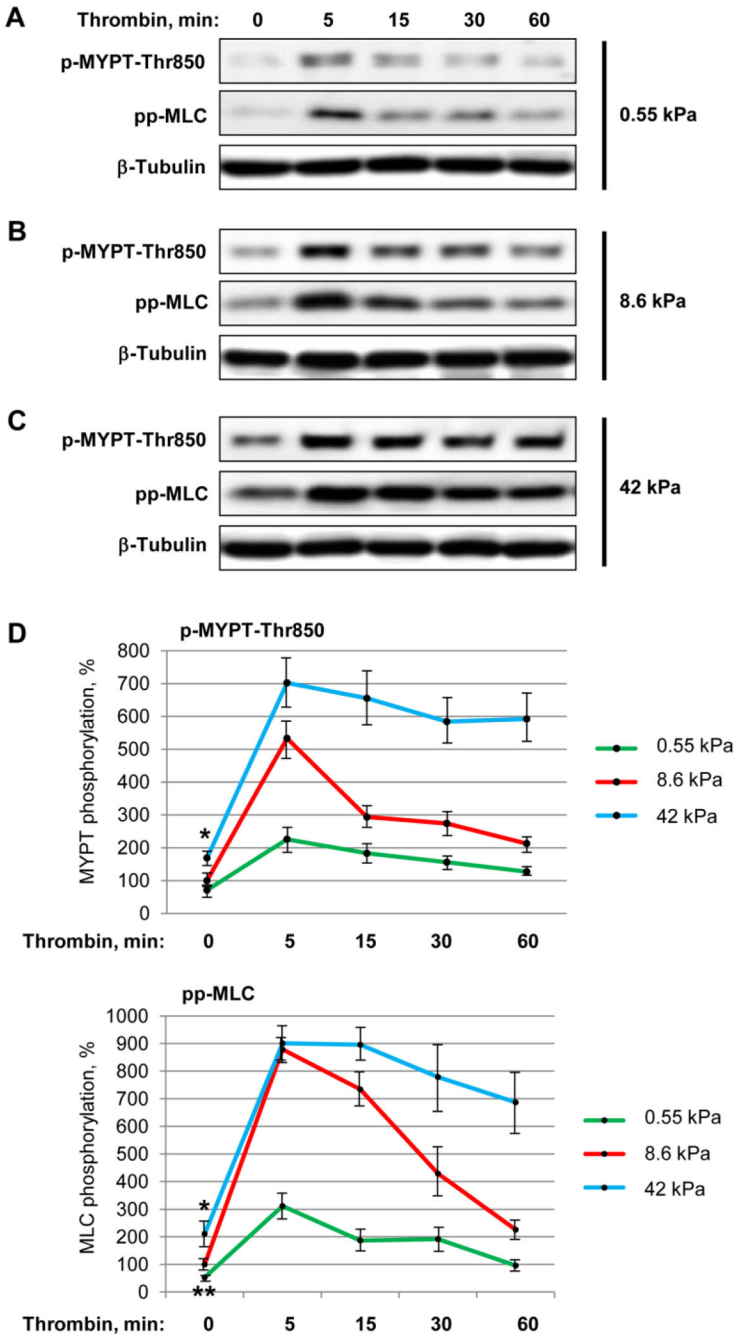

Figure 4. Stiffness dependent activation of Rho signaling in thrombin-stimulated endothelium

HPAEC were grown on collagen-I coated polyacrylamide gels of different stiffness: **A** - 0.55 kPa; **B** - 8.6 kPa; and **C** - 42 kPa; and treated with thrombin (0.3 U/ml) for indicated periods of time. Rho-kinase mediated phosphorylation of myosin light chain phosphatase (MYPT1) at Thr-850 and myosin light chain phosphorylation at Ser-19/Thr-18 was detected by immunoblotting with phospho-site specific antibodies. Tubulin staining was used as normalization control. **D** - Quantitaive densitometry analysis of thrombin-induced MYPT1 and MLC phosphorylation dynamics in HPAEC grown on matrices with different stiffness.

*P<0.05, 0.55 kPa vs. 42 kPa; **P<0.05, 0.55 kPa vs. 8.6 kPa; n=3 independent experiments.

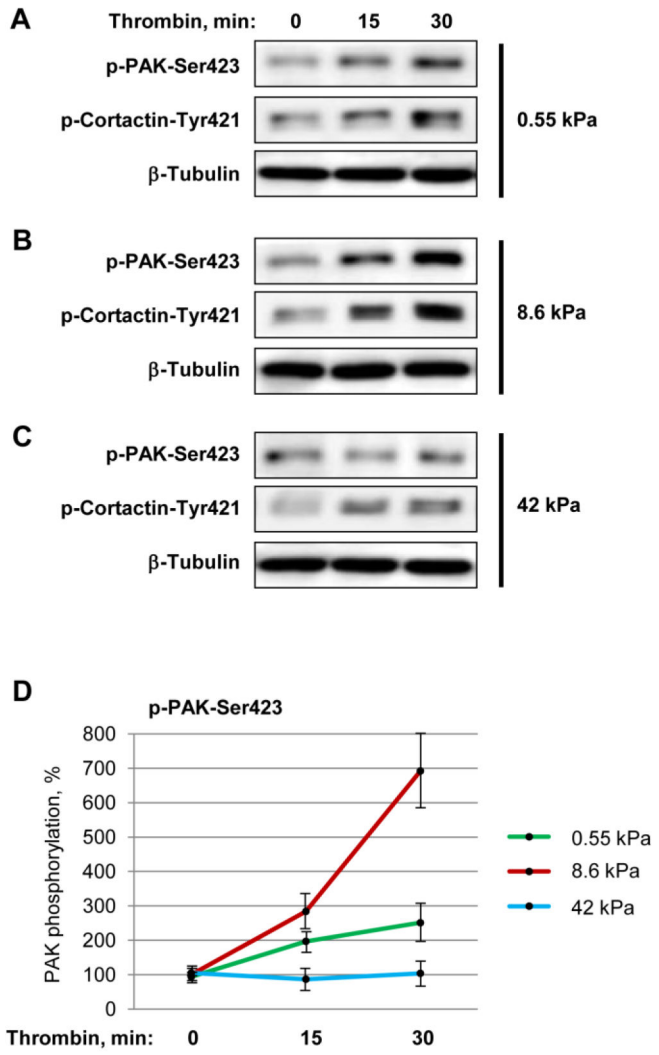

Figure 5. Stiffness dependent activation of Rac signaling in HPAEC during recovery after thrombin

Cells were grown on collagen-I coated polyacrylamide gels of different stiffness: **A -** 0.55 kPa; **B -** 8.6 kPa; and **C-** 42 kPa, and treated with thrombin (0.3 U/ml) for 15 min or 30 min. Autophosphorylation of Rac target PAK1 at Thr^{423} and cortactin phosphorylation at Tyr^{421} reflecting activation of Rac signaling was detected by immunoblotting with phospho-site specific antibodies. Tubulin staining was used as normalization control. **D -** Quantitaive densitometry analysis of delayed PAK1 phosphorylation in thrombin-stimulated HPAEC grown on matrices with different stiffness. *P<0.05, n=3 independent experiments.