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Rho GTPases, Statins, and Nitric Oxide

Role of Small GTPases in Endothelial Cytoskeletal Dynamics and the Shear Stress Response

Rho Kinases in Cardiovascular Physiology and Pathophysiology

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Rho GTPases and Signaling by Endothelial Receptors

The Rac and Rho Hall of Fame: A Decade of Hypertrophic Signaling Hits

Anne Ridley, Guest Editor

Role of Small GTPases in Endothelial Cytoskeletal Dynamics and the Shear Stress Response

Eleni Tzima

Abstract—Fluid shear stress caused by blood flow is a major determinant of vascular remodeling and arterial tone and can lead to development of atherosclerosis. The endothelial monolayer in vivo acts as a signal transduction interface for hemodynamic forces; these forces determine the shape, cytoskeletal organization, and function of endothelial cells, allowing the vessels to cope with physiological or pathological conditions. The Ras superfamily of GTPases have been revealed to be master regulators of many cellular activities. In particular, the GTPases RhoA, Rac1, and Cdc42 are known to regulate cell shape changes through effects on the cytoskeleton, but their ability to influence polarity, microtubule dynamics, and transcription factor activity is just as significant. Shear stress modulates the activity of small GTPases, which are critical for both cytoskeletal reorganization and changes in gene expression in response to shear stress. The goal of this article is to review what is known about Ras and more so about Rho GTPases in mechanotransduction and the responses of cells to fluid flow. Several distinct signaling pathways can be coordinately activated by flow, and small GTPases are strongly implicated in some of them; thus possible connections will be explored and a unifying hypothesis offered. (*Circ Res.* 2006;98:176-185.)

Key Words: GTPases ■ shear stress ■ endothelium ■ cytoskeleton

Fluid shear stress, the frictional force from blood flow, is crucial in regulation of vascular remodeling and restructuring of vessels, blood pressure, arteriogenesis, cardiac embryogenesis, and atherogenesis.¹⁻⁵ Responses of endothelial cells (ECs) to flow play an important role in regulating vascular performance in health and disease. Atherosclerotic lesions preferentially develop in regions of low and variable shear stress at vessel branch points, bifurcations, and regions of high curvature, whereas high levels of laminar flow are atheroprotective.^{6,7}

Shear stress induces several signaling cascades in ECs including: opening of K⁺ and Ca²⁺ channels,⁸⁻¹⁰ activation of heterotrimeric G proteins,¹¹ production of NO,¹² tyrosine phosphorylation of proteins such as Shc, c-src, and focal adhesion kinase (FAK),^{13,14} activation of mitogen-activated protein kinase (MAPK),¹⁵ protein kinase C (PKC),¹⁶ and C-Jun-N-terminal kinase (JNK),¹⁴ release of reactive oxygen species (ROS),¹⁷ and activation of transcriptional regulators such as c-fos, c-jun, c-myc, and nuclear factor (NF)-κB.¹⁸ Slower responses include increased expression of genes for

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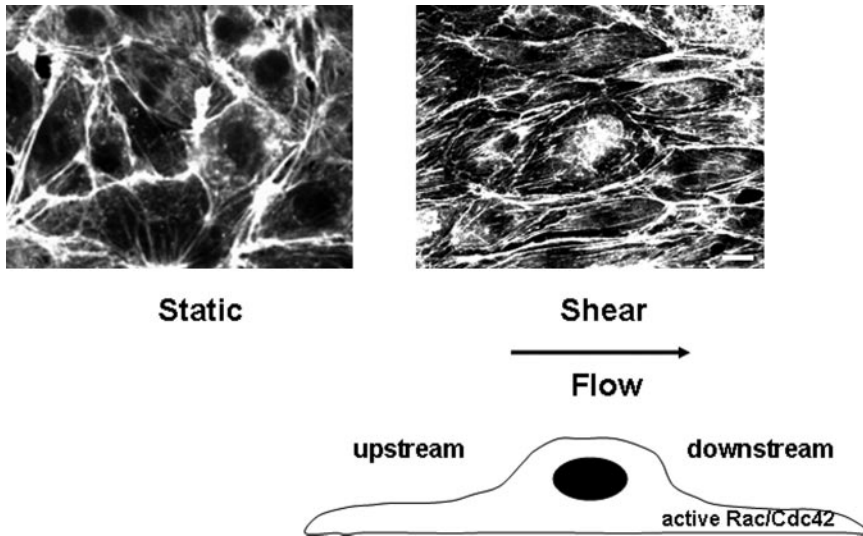


Figure 1. Shear stress–induced rearrangement of the cytoskeleton. Bovine aortic endothelial cells were fixed and stained with rhodamine-phalloidin to visualize actin filaments before (static) or after 16 hours of shear stress. Schematic diagram of an endothelial cell showing the downstream and upstream regions relative to the direction of flow. Scale bar, 15 μm .

intercellular cell adhesion molecule (ICAM)-1, nitric oxide synthetase (NOS), platelet-derived growth factor (PDGF), tissue factor, transforming growth factor (TGF)- β , and monocyte chemoattractant protein (MCP)-1^{18–22} and decreased expression of the vasoconstrictor endothelin 1 (Et-1).^{23,24} The hallmark of the EC response to fluid shear stress is the rearrangement of the microfilaments and microtubules^{25,26} and their elongation along the direction of flow²⁷ (Figure 1).

An intriguing aspect of the endothelial hemodynamic interface that is directly relevant to vascular pathophysiology is the recruitment of leukocytes from the blood. In the larger arteries, monocyte adhesion, spreading, and migration across the endothelium occur early in atherogenesis where intimal monocyte-derived macrophages are 1 of the most prominent characteristics of lesion development.²⁸ Monocyte adhesion and transmigration occur at sites of complex hemodynamics where there are steep cyclical gradients of macroscopic and microscopic shear stress.²⁹

The cellular mechanisms for sensing flow and transducing its signal are still unclear, but it has been proposed that shear stress is transmitted from the apical surface through the cytoskeleton to points of attachment that allow cells to resist the drag forces; points of attachment therefore experience changes in mechanical tension and could serve as mechanotransducers.⁶ This concept has been proposed for both focal adhesions that anchor cells to the subendothelial basement membrane and intercellular adhesions.^{30,31} Because of their interaction with specific signaling molecules already implicated in signal transduction, 4 candidates have been proposed as likely mechanotransducers: integrin-matrix interactions, specialized membrane microdomains, ion channels, and G proteins (reviewed by Traub and Berk¹⁶). Recently, we identified a mechanosensory complex comprised of PECAM-1, which directly transmits mechanical force, VE-cadherin, which functions as an adapter, and VEGFR2, which activates phosphatidylinositol 3-kinase (PI3K).³² This complex regulates conformational activation of integrins, which initiates both alignment (an adaptive response to laminar shear) and activation of NF- κB (which promotes atherogenesis in disturbed shear).^{33,34}

There are at least 150 small GTPases encoded by the human genome, and the various subclasses of this protein superfamily (including the Ras, Rho, Arf, Rab, and Ran GTPases) have been implicated in almost all aspects of cell biology, including proliferation, differentiation, cytoskeletal organization, vesicle trafficking, nucleocytoplasmic transport, and gene expression.^{35,36} These small GTPases can be considered as “molecular switches,” whose cycling between active and inactive forms is regulated stringently by cellular factors. Over the past few years, different laboratories have addressed the role of small GTPases in shear stress responses. Although the regulatory processes are not fully understood, several signaling pathways that are activated by shear stress have been identified. Among these signaling pathways, the ones regulated by Rho small GTPases stand out as the most studied and most interesting and will thus be the focus of this review.

Rho family small GTPases, including Rho, Cdc42, and Rac, belong to the Ras superfamily of proteins that cycle between an active GTP-bound form and an inactive GDP-bound form, thereby functioning as molecular switches to turn on/off the downstream signal transduction processes.³⁷ Rho proteins have been found to regulate many cellular activities besides the cytoskeleton and cell adhesion, such as cell polarity, endocytosis, vesicle trafficking, progression through the cell cycle, differentiation, oncogenesis, and gene transcription.³⁸ This review has been organized into 3 broad, often overlapping topics relevant to the responses of the endothelium to blood flow: (1) morphological rearrangements and directionality, (2) gene expression, and (3) permeability changes and transendothelial migration.

Role of Ras GTPases

The small G protein Ras has been identified as an early link between rapid mechanotransduction events and the effects of shear stress on downstream signal-transduction cascades.⁶ Ras mediates the effects of receptor and nonreceptor tyrosine kinases in mitogenic signaling pathways and regulates G protein-dependent activation of extracellular signal-regulated kinase (ERK) and JNK.^{39,40}

Effects on Gene Expression

There are several lines of evidence that support the hypothesis that p21ras plays critical roles in the responses of vascular EC to fluid shear stress, especially in regulating endothelial gene expression. First, the guanine nucleotide exchange on Ras, ie, the conversion of Ras-GDP to Ras-GTP, was promoted by fluid shear stress.¹⁴ Second, the dominant negative mutant of p21ras, RasN17, inhibited the shear-induced signal transduction pathway, including ERK, JNK, and its downstream c-Jun transcriptional activity.^{14,15,39} Third, RasN17 also abrogated the expression of genes such as MCP-1, which is upregulated through the transcription factor activating protein 1 (AP-1) acting on the 12-*O*-tetradecanoyl-13-phorbolacetate-responsive element (TRE).¹⁴ In addition, shear stress-induced early growth response (Erg)-1, an immediate early gene whose expression is a common theme in vascular injury,⁴¹ is mediated via the Ras/Raf/ERK pathway.⁴² Notably, it was shown that p60src is a common upstream mediator for both the Ras-JNK and the Ras-ERK pathways in ECs in response to shear stress⁴³ and that shear-induced Ras activation of the ERK and JNK pathways can be inhibited by the tyrosine kinase inhibitors genistein and herbimycin A.^{39,44} Furthermore, Davis et al described a Src-Ras-ERK pathway upstream of eNOS transcription.⁴⁵

Upstream Signaling Pathways

The mechanism through which shear stress activates Ras is not clear, although it has been suggested by Chen et al that the assembly of Shc-Grb2-Sos provides a route for shear-induced activation of Ras.⁴⁶ In contrast, a recent study by Gudi et al has shown that rapid activation of Ras in human ECs by temporal gradients in shear stress is mediated by Gβγ subunits dissociated from flow-activated Gα_q.⁴⁷ These data are consistent with previous reports that shear stress activation of Ras is mediated by Gβγ subunits.³⁹ Taken together, these results suggest that the shear-induced MAPK activation pathway begins with shear-induced activation of Gα_q- and Gβγ-mediated stimulation of Ras.

As most signaling pathways are not linear, the expectation is most likely that shear-induced Ras activation is the result of signaling pathway crosstalk or parallel mechanotransduction pathways.

Rac1 Signaling

Rac is required at the front of the cell to regulate actin polymerization and membrane protrusion and assembly of membrane ruffles depends on Rac activity.⁴⁸ One of the fastest structurally identifiable EC responses to shear stress is the formation of ruffles/lamellipodia in the direction of flow^{49–51} (Figure 1). This observation coupled to the realization that Rac regulates many other signal transduction pathways in addition to those linked to the cytoskeleton, fuelled interest in the role of Rac in shear stress signaling.

Effects on Polarity and Alignment

Biochemical and immunofluorescence studies in ECs indicate that Rac1 GTPase is activated by shear stress. Using pull-down assays we showed that shear stress can transiently activate Rac within 30 minutes and then Rac activity returns

to basal levels.³⁴ The key observation is that visualization of activated Rac by using the FLAIR (FLuorescence Activation Indicator for Rho proteins) technique⁵² reveals that fluorescence resonance energy transfer (FRET) signals are localized primarily at the downstream edges of cells that have been sheared³⁴ (Figure 1). In contrast, expression of the Rac nucleotide exchange factor Vav activates Rac1, but few cells show polarization of the FRET signal. Interestingly, blockade of new integrin binding to the extracellular matrix (ECM) strongly inhibits the shear stress-induced increase in Rac1 activity, whereas the residual FRET signal shows no preferred direction, suggesting that new integrin binding to ECM determines the localized activation of Rac1 in response to shear stress.

The role of Rac in EC responses to shear stress has been further explored by assaying the effect of Rac1 inhibition on stress fiber alignment in the direction of flow. ECs expressing dominant negative Rac (N17Rac) show the typical decrease in actin staining followed by recovery of stress fibers³³; however, F-actin orientation is largely random. Even after longer times of shear stress, the orientation of actin stress fibers in cells expressing N17Rac is significantly inhibited. Importantly, cells expressing dominant active Rac (V12Rac) show high levels of Rac activity; however, they lack polarization toward the downstream edge and are unable to align in the direction of flow. Consistent with this, cytoskeletal reorientation in response to sustained shear stress was abolished in cells overexpressing either dominant negative Rac 1 or a dominant negative construct of its downstream target, p21-activated kinase (PAK)-1.⁵³

In a more detailed study, investigators showed that Rac became activated within 5 to 30 minutes after shear stress stimulation and was required for respreading and alignment of ECs in the direction of flow.⁵⁴ Rac was also required for shear stress-induced orientation of cell migration and N17Rac reduced cell migration speed under flow. Interestingly, although PI3Ks can act upstream of Rac in cell migratory responses⁵⁵ and are important for chemotaxis in some cell types,^{56,57} they are not required for shear-induced changes in cell polarity but contribute to cell migration speed.⁵⁴

In a model of EC migration under flow, it was shown that transfection of BAECs with N17Rac inhibited lamellipodial protrusion and cell migration under static and shear conditions, whereas V12Rac induced lamellipodia in all directions and attenuated the shear-induced migration. These studies suggest that an appropriate level of Rac activity and polarized lamellipodial protrusion are important for cell migration under static and shear conditions.⁵¹

Taken together, these data show that shear stress activates Rac1 in a polarized manner and that Rac1 activity has to be spatially restricted in order for cells to align and/or migrate in the direction of flow.

Effects on Gene Expression

Several *trans*-acting factors are activated by shear stress and subsequently induce the expression of target genes. NF-κB was the first such factor to be described.^{18,58–60} NF-κB is composed of protein dimers of the Rel/NF-κB family, with

the p50/p65 dimer being the predominant form in vivo. In unstimulated cells, Rel/NF- κ B dimers are sequestered in the cytoplasm by binding to members of the I κ B family of inhibitor proteins. On activation, I κ B is degraded and NF- κ B translocates to the nucleus, where it regulates the transcription of multiple target genes by binding to promoter elements in many genes.⁶¹ An NF- κ B consensus promoter was identified as the shear stress response element (SSRE) within the PDGF gene that mediated induction of PDGF by flow.^{61,62} Activated NF- κ B was identified in smooth muscle cells, macrophages, and ECs of human atherosclerotic tissue specimens⁶³ as well as in humans with unstable angina pectoris,⁶⁴ suggesting a pathophysiological role for NF- κ B in atherosclerosis.⁶⁵

Rac1 is widely known to act upstream of reactive oxygen species (ROS) production in a variety of cell types and Rac1 contributes to ROS production in response to shear stress.⁶⁶ ROS production in response to shear stress leads to increased expression of ICAM-1 gene.⁴² It has been shown previously that Rac mediates a cytokine-stimulated, redox-dependent pathway necessary for NF- κ B activation.⁶⁷ Additionally, Rac, Rho, and Cdc42 induce transcriptional activity of NF- κ B by phosphorylation of I κ B,⁶⁸ and activation of Rac induces NF- κ B binding and activity and enhances expression of cyclin D1.⁶⁹ Toll-like receptor 2-mediated NF- κ B activation also depends on Rac⁷⁰ and integrin signaling to NF- κ B is mediated by Rac.⁷¹

We showed that Rac1 mediates flow-induced stimulation of NF- κ B; as in cells transfected with N17Rac, flow induced minimal translocation of NF- κ B from the cytosol to the nucleus and minimal transcriptional activity of NF- κ B.³⁴ Rac1-dependent NF- κ B activation is required for the subsequent flow-induced surface expression of ICAM-1,³⁴ which is involved in the recruitment of leukocytes to atherosclerotic plaque.^{72,73} Recently, it has been shown that the subendothelial ECM modulates NF- κ B activation by flow and that ECs plated on fibronectin or fibrinogen activate NF- κ B in response to flow, whereas cells on collagen or laminin do not.⁷⁴ As flow-induced NF- κ B activation is downstream of conformational activation of integrins and mediated by Rac 1,³⁴ it would be of interest to determine whether the ECM-dependent activation of NF- κ B is under the control of Rac and whether flow-induced activation of Rac is itself ECM dependent.

Effects on EC Permeability

Shear stress enhances barrier protective effects on the endothelium as revealed by increased transendothelial resistance.^{75,76} EC permeability changes induced by barrier-disruptive and barrier-protective agonists are associated with specific patterns of cytoskeletal remodeling.^{77,78} Furthermore, because endothelial permeability depends on the integrity of intercellular junctions as well as actomyosin-based cell contractility, Rho and Rac, have emerged as key regulators acting antagonistically to regulate endothelial barrier function: Rho increases actomyosin contractility, which facilitates breakdown of intercellular junctions, whereas Rac stabilizes endothelial junctions and counteracts the effects of Rho.⁵⁴ In this regard, it was recently discovered that shear stress induced

activation of Rac and mediated barrier enhancement in human pulmonary ECs.⁷⁸ That said, activated Rac also promotes cell scattering, which involves the breakdown of cell-cell junctions. Although at first glance this dual role of Rac seems incompatible, modulation of the effector pathways downstream of Rac is likely to be critical for the decision to scatter versus remaining as a well-organized tissue. IQGAP is implicated in stabilizing adherens junctions downstream of Rac and Cdc42,⁷⁹ whereas PAK activation is linked to increased EC permeability.⁸⁰ Thus, conditions that favor interaction of Rac with PAK would lead to junctional disruption, whereas those that favor IQGAP would lead to junctional stabilization. Investigation of the mechanisms by which specific downstream effectors are selectively activated by shear stress will be an important direction for future work.

Upstream Signaling Pathways

Regulation of Rac activity by shear stress is downstream of new integrin-ligand binding.³⁴ Although the signaling mediators that connect integrins to Rac activation under flow have not been elucidated, it is likely that the same players that mediate adhesion-induced Rac activation are important. In this regard, integrin-mediated adhesion activates Rac⁸¹ and this requires an intact β integrin subunit.^{82,83} In addition, p130cas and paxillin associate with FAK and both have been linked to Rac activation. Tyrosine phosphorylation of p120cas promotes a complex of Crk, ELMO and DOCK180,^{84,85} which is a Rac guanine nucleotide exchange factor (GEF).⁸⁶ Another complex also associates with paxillin: this complex includes PKL (GIT) and Pak-interacting exchange factor (PIX), which is another Rac GEF (reviewed by Turner⁸⁷). Furthermore, integrin-mediated activation of Rac also involves targeting of the GTP-bound protein to sites of adhesion, allowing it to interact with effectors.⁸⁸

Whether all or part these pathways control shear stress-induced activation of Rac remains to be seen.

Rho Signaling

Rho GTPase regulates stress fibers and focal adhesions,⁸⁹ 2 cytoskeletal structures whose assembly is tightly regulated by shear stress. It is therefore not surprising that fluid flow, just like growth factors and adhesion, regulates Rho activity. It has been shown that Rho translocates to the membrane in response to shear stress, presumably indicating its activation.⁹⁰ Expression of dominant negative mutants of Rho and its downstream target Rho-kinase/ROCK inhibited shear stress-induced cell alignment and stress fiber formation in confluent cultures of bovine aortic endothelial cells (BAECs), further suggesting a role for Rho in shear stress signaling.⁹⁰

Effects on Shear Stress-Induced Morphological Rearrangements

Our own work has shown that fluid shear stress induces a transient inactivation of Rho that is followed by an increase that peaks at 60 minutes,³³ similar to that seen when suspended cells are plated on ECM proteins.⁹¹ Visualization of actin filaments revealed that, in sheared cells, the initial phase of low Rho activity is associated with a decrease in stress fibers, whereas the later restoration of Rho activity corre-

sponds to an increase in stress fibers. Reappearance of stress fibers at 60 minutes is accompanied by significant cell alignment in the direction of flow, although maximal alignment requires longer times of exposure to flow.²⁷ Rho activity decreases again at 120 minutes, although actin stress fibers remain. We do not fully understand this result, although very similar effects were seen during adhesion to FN.⁹¹ It may be that Rho is highest during assembly of stress fibers and that lower levels are sufficient for their maintenance.

The observed regulatory effects on Rho activity closely match the requirements for shear stress–induced cell alignment: constitutively activated mutants of RhoA inhibit shear stress–induced alignment of ECs, indicating that this decrease in Rho activity is required for the initial alignment of cells with the direction of flow.³³ In a more detailed analysis of the role of Rho in shear stress directionality, it was shown that Rho is required for the initial shear stress–induced polarization and retraction and finally elongation, but not migration speed.⁵⁴ Importantly, Rho is also required for directional migration of ECs as well as orientation of cell movement induced by shear stress, correlating with its effect on shear stress–induced alignment.⁵⁴

Using the traction force microscopy technique, Shiu et al provide a biophysical basis for the role of Rho in shear stress–induced migration.⁹² They report that shear stress increases the migration speed and Rho activity of the ECs over a range of FN densities and that shear stress enhances the migration speed of ECs by modulating traction force generation through the Rho-p160ROCK pathway. This discrepancy between other reports could be attributable to differences in cell type, flow rate and degree of confluence. A direct assay of subcellular activity of Rho would allow us to correlate sites of Rho activation and traction force generation at the subcellular level and clarify the role of Rho in shear stress induced cytoskeletal changes and migration.

The role of Rho/Rho kinase system in blood flow was also studied in a model of rat mesenteric small arteries in vivo. In small arteries, a chronic blood flow reduction leads to a decrease in the internal diameter and media mass (ie, inward hypertrophic remodeling), and reduced contractile capacity. In response to chronically elevated blood flow, these parameters increase (ie, outward hypertrophic remodeling). The Rho/Rho kinase system was shown to be modulated by shear stress, as flow reduction downregulated many RhoA-related cytoskeletal markers of smooth muscle cell phenotype and inhibition of Rho kinase potentiated the inward hypertrophic remodeling in response to chronically reduced flow.⁹³

Effects on Shear Stress–Induced Gene Expression

The Rho GTPase regulates many other signal transduction pathways in addition to those linked to the actin cytoskeleton. To this end, Rho mediates the shear stress–induced activation of the transcription factor AP-1 through JNK.⁹⁰ Rho also mediates shear-induction of *c-fos* activation,⁹⁴ which controls expression of several shear-inducible genes.^{95,96} This Rho-mediated shear-induction of *c-fos* is dependent on intracellular calcium but not on the Rho effector p160ROCK or actin filaments. Although the inhibition of p160ROCK and its ensuing disruption of actin filaments decreased the basal

c-fos activity in static ECs (no flow), it did not affect the shear-inductive effect. The calcium chelator BAPTA-AM inhibits the shear-induction, as well as the static level, of *c-fos* activity.⁹⁴

The shear stress–induced Rho pathway can also modulate the activity of sterol regulatory element binding proteins (SREBPs) in ECs,⁹⁷ which are key regulators of cellular sterol and lipid homeostasis (reviewed by Brown and Goldstein⁹⁸). These researchers have shown that shear stress activates the Rho-ROCK-LIMK-cofilin pathway, which, in turn, enhances the cytoskeleton and facilitates the transport of SREBP to the nucleus to activate transcription.

Effects on Intercellular Adhesion

The complex series of events involved in cell–cell interactions is a cardinal feature of vascular permeability, paracellular pore formation and leukocyte transendothelial migration. The critical importance of the small GTPase Rho in regulation of the contractile apparatus has been demonstrated in several models of agonist-induced EC barrier dysfunction (reviewed by Dudek and Garcia⁹⁹), as well as in during leukocyte transendothelial migration (reviewed by Wittchen¹⁰⁰). Many agents that promote inflammation and leukocyte transendothelial migration have been shown to activate RhoA in ECs, thereby stimulating EC contraction and junctional opening.⁹⁹ When RhoA is inhibited, monocyte adhesion and spreading on ECs are decreased.¹⁰¹ How Rho becomes activated during adhesion of leukocytes to the endothelium is unclear but may involve Thy-1 (CD90). Thy-1, expressed on ECs, binds Mac-1 on leukocytes, and blocking this interaction prevents leukocyte adhesion and transmigration.¹⁰² Intriguingly, Thy-1 can activate RhoA by decreasing activity of p190RhoGAP¹⁰³ or through $\beta 3$ clustering.¹⁰⁴

Upstream Signaling Pathways

The pathway upstream of Rho inactivation involves rapid shear stress–induced conformational activation of integrins and their increased binding to ECM.³³ These findings are in agreement with those described in the study of Jalali et al, which showed that new connections between integrins and matrix proteins were needed for integrins to associate with Shc and activate JNK in response to shear stress.⁴³

The molecular mechanism(s) responsible for the cross-talk between integrins and the initial downregulation of Rho activity in response to shear stress are still not known. It has been shown that fibroblasts from FAK null mice failed to transiently inhibit Rho activity when plated on FN, whereas reexpression of FAK restored normal Rho regulation.¹⁰⁵ Another report has focused on the role of *c-Src*–mediated integrin signaling in modulating RhoA activity during cell adhesion through tyrosine phosphorylation of p190RhoGAP GTPase activating protein.¹⁰⁶ The same investigators also demonstrated the existence of a protein tyrosine phosphatase Shp-2, sensitive to calpeptin, acting upstream of RhoA.¹⁰⁷ A role for paxillin phosphorylation has also been indicated in this decrease in RhoA activity. When 2 of the paxillin phosphorylation sites (Y31 and Y118) were mutated, the depression in RhoA activity was abolished and the cells

showed premature formation of stress fibers.¹⁰⁸ These authors demonstrated that the phosphorylation of these 2 tyrosines, which is induced by integrin-mediated adhesion, generates a binding site for p120RasGAP, displacing it from its binding partner p190RhoGAP. Evidence was presented that p190Rho freed from p120RasGAP was activated and hence contributed to the decrease in RhoA activity. More recently, a component of cell–cell junctions, vascular endothelial (VE)-cadherin, has been shown to signal through RhoA and the actin cytoskeleton to cross-talk with cell–matrix adhesion.¹⁰⁹ Future studies aiming to determine the signaling events that activate Rho following its transient inhibition by shear stress are of particular interest in elucidating fundamental mechanisms in mechanotransduction.

Cdc42 Signaling

Cdc42 is known to promote formation of actin-rich, finger-like membrane extensions (filopodia).^{110,111} There is now considerable evidence that Cdc42 controls polarity, during establishment of intracellular asymmetry, morphogenesis, and migration (reviewed by Etienne-Manneville and Hall³⁸).

Effects on the Microtubule Cytoskeleton

An early suggestion that Cdc42 might play a role in shear stress–induced signaling came from the observation that Cdc42 translocates to the membrane of ECs subjected to shear stress and mediates activation of JNK and AP-1.⁹⁰ Recently, we have extended this observation, showing that Cdc42 is activated by fluid shear stress and that activation is a consequence of integrins binding to ECM. A novel fluorescence energy transfer assay to visualize Cdc42 activation in single cells shows that Cdc42 activity is polarized in the direction of flow¹¹² (Figure 1).

It was previously shown that dominant negative Cdc42 (N17Cdc42) does not inhibit shear stress–induced alignment and stress fiber formation.⁹⁰ Instead, Cdc42 regulates the microtubule cytoskeleton and, in particular, controls the polarization of the microtubule organizing center (MTOC).^{113,114} Indeed, we found that localized activation of Cdc42 directs the reorientation of the MTOC to a position on the downstream side of the nucleus relative to the direction of flow.¹¹² Importantly, correct spatial activation of Cdc42 rather than activity per se is essential for localization of the MTOC after shear stress. Thus, polarized activity of Cdc42 is critical for correct orientation of MTOC.

This result is particularly interesting in light of the role of Rac in shear stress–induced alignment. The correct spatial activation of Rac is required for the orientation of stress fibers with the direction of flow.³⁴ In addition, although Rho, Rac, and Cdc42 all control the organization of the actin cytoskeleton,¹¹⁵ only Cdc42 was responsible for shear stress–induced MTOC reorientation. In agreement with these findings, Cdc42 was also found to mediate nucleus movement and MTOC polarization in 3T3 fibroblasts under shear stress.¹¹⁶ These results seemingly conflict with those obtained in human umbilical vein ECs (HUVECs), in which N17Cdc42 did not affect directionality of EC movement toward the center of the wound under flow.⁵⁴ These differences could be cell-type specific or attributable to lower levels of shear stress used.

Previous investigators reported that the Par6-PKC ζ complex controls cell polarity.^{113,114} PAR proteins were identified as key regulators of cell polarity in early *Caenorhabditis elegans* development.¹¹⁷ Under flow, the activity of Par6 and PKC also direct the reorientation of the MTOC.¹¹² Altogether, these data define a mechanism through which Cdc42 regulates the microtubule-dependent establishment of cell polarity under shear stress: shear-stimulated integrin dynamics induce polarized Cdc42 activity, which induces MTOC localization through the Par6-PKC ζ complex.

Effects on Gene Expression

Like Rho, Cdc42 regulates the transcriptional activation of the serum response element (SRE) in the c-fos promoter through the serum response factor (SRF).¹¹⁸ Cdc42 is also required and sufficient in the shear stress activation of JNK that leads to the increase in AP-1/TRE activity. In this way, Cdc42 controls expression of several shear stress–inducible genes.

Intermediate Filaments and the Shear Stress Response

Activation of Rho family GTPases also induces collapse of the vimentin intermediate filament (IF) network in fibroblasts.^{119,120} Cdc42-induced vimentin reorganization involves PAK¹²¹ and, in a novel cytoskeletal role, p70 S6K.¹²² The importance of the IF cytoskeleton and, in particular, vimentin in responses of the endothelium to flow are now becoming clearer: vimentin-deficient mice (V^{-/-})¹²³ exhibit a blunted, acute, flow-induced arterial vasodilatation^{124,125} and an altered balance between endothelin-1 and NO. Vimentin also plays an important role in diameter and wall mass changes during flow-induced arterial remodeling¹²⁶ and regulates not only focal contact structure but also function.¹²⁷ The evolutionary advantage of the highly conserved vimentin sequence has been proposed to lie not only within the role of the intermediate filament in cellular motility and contractility but also in its possible role in pathological conditions that require vascular adaptations.¹²⁵

Recently, elegant qualitative and quantitative spatial analyses in living ECs revealed rapid regional intermediate filament (IF) displacement in response to shear stress and that cytoskeletal mechanics are rapidly altered by the onset of fluid shear stress.^{128–130} These data suggest an integrated mechanism of mechanotransduction in which spatial organization of multiple structural and signaling networks regulates cellular responses to an altered hemodynamic environment. Shear stress–induced redistribution of IFs near the basal side of the cell affects the dynamics of focal adhesion sites.¹³ Because the perinuclear ring of vimentin IFs may be directly or indirectly linked to the nuclear lamina,¹³¹ force redistribution under flow may also affect the karyoskeleton, consistent with other mechanical perturbations. Through interactions among nuclear IF proteins, the nuclear lamins, DNA, and histones,^{132,133} changes in gene expression may be directly mediated by flow.

The interplay between Rho GTPases and the IF cytoskeleton during flow remains to be elucidated.

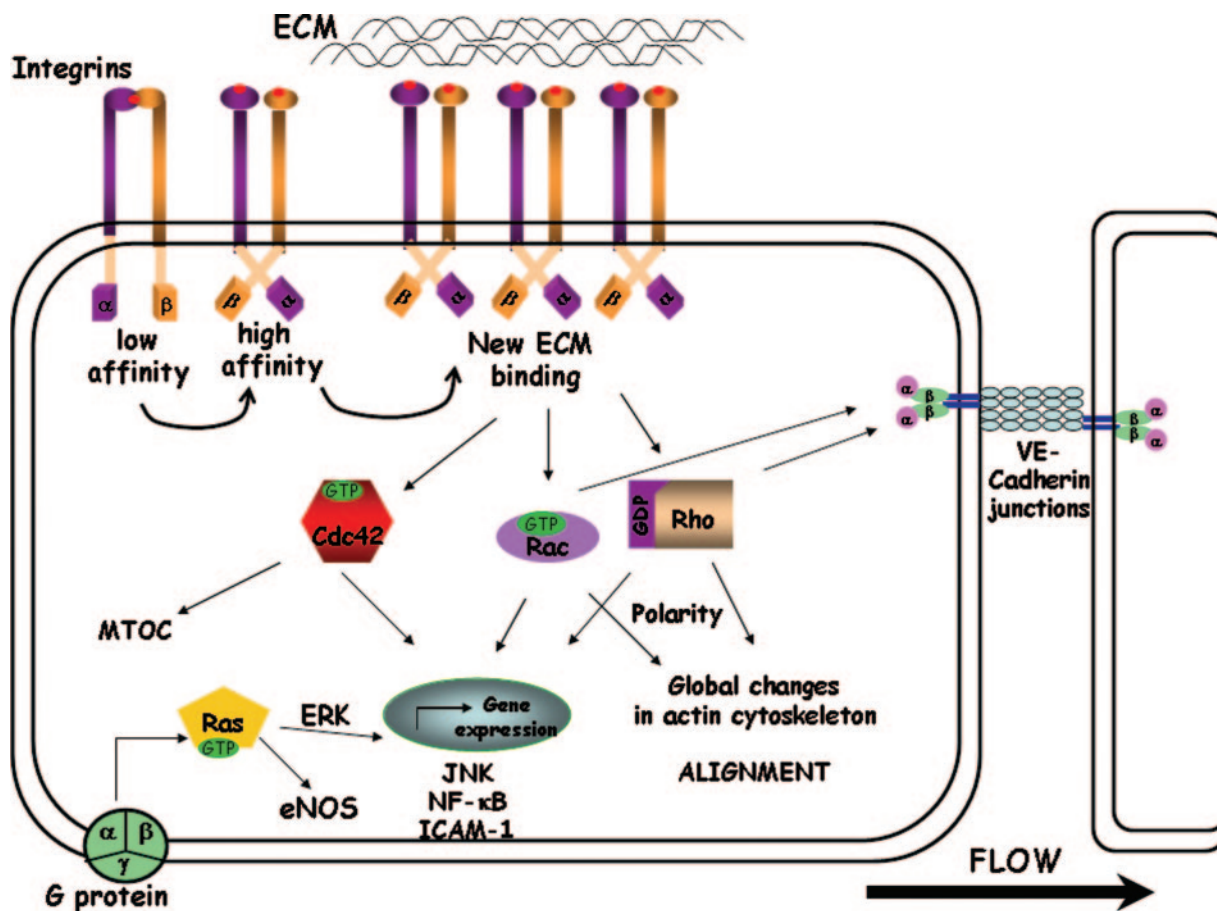


Figure 2. Model for mechanotransduction. Shear stress activates integrins, which bind to ECM. Ligated integrins transiently deactivate Rho, which causes disassembly of stress fibers. Ligated integrins also activate Rac at the downstream edge of the cell, which facilitates alignment of the newly formed stress fibers in the direction of flow. Polarized activation Cdc42 mediates reorganization of the MTOC. GTPases also control gene expression and regulate endothelial junctions. Ras GTPase is activated through G proteins and regulates gene expression under flow through ERK and JNK as well as eNOS activation.

Model of Mechanotransduction

It has become increasingly clear that exogenous force transmission caused by blood flow via filamentous elements linked to membrane surfaces and organelles provide exquisite sensitivity to allow appropriate cellular responses. Although each Rho GTPase mediates distinct signaling networks that are spatially organized, the overall signaling response of the endothelium to flow is integrated. Thus, a unifying model of endothelial mechanotransduction can be proposed based on the relationship between cell adhesions, Rac, Rho, and Cdc42 and actin stress fibers and microtubules during shear stress-induced directional reorganization of the cytoskeleton (Figure 2). To this end, Civelekoglu-Scholey et al formulated a mathematical model¹³⁴ based on the assumption that the cytoskeleton transfers the shear force to the adhesion sites, which allow integrins to be activated. Activated and ligated integrins signal and transiently deactivate Rho, causing disassembly of stress fibers. Ligated integrins also signal and activate Rac, which enhances focal complex assembly. When Rho activity recovers, stress fibers reappear and promote maturation of focal complexes into focal contacts. The elevated level of Rac activity at the downstream edge of the cell is translated into an alignment of the newly formed stress

fibers in the direction of flow. Whereas Rac and Rho control the actin cytoskeleton, polarized activation Cdc42 mediates reorganization of the MTOC. In addition, all 3 GTPases control gene expression and regulate a complex molecular signaling network that modulates endothelial barrier permeability and intercellular communication.

This model aims to represent the role of Ras and Rho GTPases in mediating their effects on the endothelial cytoskeleton and gene expression. Other factors, such as channels and G proteins are also involved in mediating endothelial cell responses to flow and it can be envisaged that differential coupling of signaling mechanisms to the various shear stress receptor “subtypes” may provide flexibility and adaptation to the endothelium.

Conclusions

To sense, transduce and adapt to blood flow, there is a constant need for the EC to coordinate a variety of intracellular activities both spatially and temporally. The Ras and Rho GTPases, each with their numerous targets, are ideally positioned to coordinate and orchestrate such diverse responses as cytoskeleton rearrangements, gene expression, and leukocyte transmigration under flow. The activation of small

GTPases by flow and the identification of specific targets of the signaling cascade will open up important avenues for future investigation. Most importantly, further insights into the mechanistic details of how small GTPases and other signaling molecules cooperate to regulate the endothelial response to fluid shear stress will not only identify new components of signaling pathways but will also provide unpredicted insights into how pathways cooperate with each other during distinct biological responses.

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