Abstract: Vascular stiffness is a mechanical property of the vessel wall that affects blood pressure, permeability, and inflammation. As a result, vascular stiffness is a key driver of (chronic) human disorders, including pulmonary arterial hypertension, kidney disease, and atherosclerosis. Responses of the endothelium to stiffening involve integration of mechanical cues from various sources, including the extracellular matrix, smooth muscle cells, and the forces that derive from shear stress of blood. This response in turn affects endothelial cell contractility, which is an important property that regulates endothelial stiffness, permeability, and leukocyte–vessel wall interactions. Moreover, endothelial stiffening reduces nitric oxide production, which promotes smooth muscle cell contraction and vasoconstriction. In fact, vessel wall stiffening, and microcirculatory endothelial dysfunction, precedes hypertension and thus underlies the development of vascular disease. Here, we review the cross talk among vessel wall stiffening, endothelial contractility, and vascular disease, which is controlled by Rho-driven actomyosin contractility and cellular mechanotransduction. In addition to discussing the various inputs and relevant molecular events in the endothelium, we address which actomyosin-regulated changes at cell adhesion complexes are genetically associated with human cardiovascular disease. Finally, we discuss recent findings that broaden therapeutic options for targeting this important mechanical signaling pathway in vascular pathogenesis. (Circ Res. 2015;116:895-908. DOI: 10.1161/CIRCRESAHA.116.305720.)

Key Words: cardiovascular diseases ■ cell adhesion ■ cellular mechanotransduction ■ inflammation ■ permeability

Blood vessel stiffening is an important aspect of vascular inflammation. Moreover, vascular stiffness is strongly associated with increased risk and progression of cardiovascular diseases. The development of vascular inflammation and disease is often the result of a prolonged and highly multifaceted process. In this overview, we focus on mechanistic events that occur at the level of the endothelium, the monolayer of cells that lines the luminal side of blood vessels and controls vascular permeability and inflammation.

The regulation of vascular stiffness occurs at different levels in vascular tissue, which are functionally connected (Figure 1): (1) systemic (ie, by changes in blood pressure); (2) at the level of the vessel wall (ie, caused by contraction of vascular smooth muscle cells (VSMCs), extracellular matrix (ECM) changes, or changes in blood flow); (3) within individual endothelial cells (ECs; ie, changes in cytoskeletal contractility induced by inflammatory cytokines, or adhesion of leukocytes). ECs respond to changes in stiffness by biochemical and structural adaptation. This mechanotransduction response not only includes signals that promote but also signals that limit, endothelial monolayer permeability. Excessive vascular wall stiffening disturbs this balance and causes increased permeability and inflammation. Conversely, the mechanotransduction response in ECs and VSMCs further drives cellular contractility and vascular wall stiffening, which will aggrevate this phenomenon. Such positive feedback puts the cardiovascular system at risk of self-amplifying molecular events into systemic responses that underlie vascular pathologies, including pulmonary arterial hypertension, cardiac hypertrophy, and kidney failure.

We surmise that targeting EC mechanotransduction responses represents a promising approach to combat stiffness-associated vascular diseases and inflammation. Relevant questions are what are the events that trigger endothelial contractility? How does endothelial contractility regulate migration of inflammatory cells? How do molecular events in individual ECs relate to vascular pathology? These aspects will be discussed in detail. We will start with a brief clinical perspective that links vascular stiffening to human disease. Next, we will discuss the molecular basis of EC contractility (which occurs in response to...
vascular stiffening) and the various external cues that regulate this. In addition, we will discuss the relationship between endothelial contractility and leukocyte extravasation and finally address (future) possibilities to therapeutically target ECs to reduce chronic, stiffness-related, vascular inflammation.

Systemic Regulation of Vascular Stiffness

Arterial stiffness is strongly associated with high blood pressure, as well as with aging, and serves as an independent predictor of human cardiovascular disease. An elevation in arterial stiffness increases systolic blood pressure, thereby promoting cardiac hypertrophy, and decreases the diastolic blood pressure which is a trigger for developing myocardial ischemia. Increased PWV accurately estimates arterial wall stiffness and has recently been included in official hypertension guidelines. PWV values increase with age, and increases in aortic PWV is a good predictor of cardiovascular risk. Aortic PWV is also promoted by other diseases, which include diabetes mellitus, metabolic syndrome, and renal disease. The stiffness of the great pulmonary arteries is elevated in several forms of pulmonary hypertension, which affects hemodynamics in proximal arteries and right heart afterload, thereby increasing blood pressure and flow pulsatility. Another example showing the importance of arterial stiffness on end-organ function is demonstrated in the brain, especially in the pathogenesis of elderly. Here, PWV is associated with cerebral small-vessel disease and cognitive deterioration.

Various other approaches to assess the rigidity of vascular tissue, such as atomic force microscopy (AFM), have shown that aging vessels and atherosclerotic lesions are stiffer than healthy vasculature. Although we do understand the pathophysiology of large arterial stiffness, blood pressure, and microcirculatory damage, we mostly lack insights in the molecular response to arterial stiffness. Current antihypertensive drugs are aimed to reduce arterial stiffness by modulating blood pressure, whereas specific arterial wall properties, or EC responses to stiffness, are not specifically targeted. An obvious approach to counteract stiffness-related vascular disease is targeting the subsequent response of ECs to vascular stiffening. Important pathways that regulate vessel wall stiffness, the mechanoresponse of ECs to that, and the related inflammatory response are discussed below.

Figure 1. Vascular stiffness is regulated at different related levels. **A**, At the level of the vasculature, systemic processes, such as hypertension or aging, can drive vascular stiffness. **B**, Within the vascular wall, contraction or calcification of vascular smooth muscle cells (VSMCs) can increase vessel stiffening. In addition, extracellular matrix (ECM) deposition and composition can contribute to local stiffness, as does the presence of laminar or turbulent flow. **C**, Within vascular endothelial cells (ECs), the Rho–Rho kinase regulated contractile machinery regulates cellular stiffness and endothelial permeability. This is initiated by integrins (1), receptors and ion channels (2), and adherent leukocytes (3). Cellular stiffness can enhance leukocyte influx and potentiate vascular inflammation. Note that stiffness changes at the cellular level also feedback into altered vessel wall dynamics and stiffness, which in turn regulate blood pressure. Thus, there is a causal relationship between molecular events in ECs and stiffness-related pathologies in the vasculature.
Molecular Basis of Endothelial Responses to Vascular Stiffening

Stiffening of the vascular wall directly affects 2 crucial EC functions: endothelial barrier function and the interaction of the endothelium with leukocytes (explained in detail in the paragraphs below). The mechanisms by which mechanical stiffening is transduced into cellular responses is collectively called mechanotransduction. We will now first briefly mention the key molecular events that occur during endothelial mechanotransduction and then we will elaborate on the consequences of such mechanotransduction pathways for endothelial permeability, leukocyte–EC interactions and inflammation.

Rho-Actomyosin–Induced Endothelial Contractility

The physical properties of cells are mostly determined by their cytoskeletal networks and these, in turn, affect important cellular functions. Cytoskeletal networks respond to forces, such as disturbed flow, vessel wall stiffening, or blood pressure, that cause their deformation.15 In vitro reconstituted actomyosin networks show a force-induced reversible stiffening response depending on the magnitude of the applied forces.16 Moreover, in response to extracellular mechanical stiffening, cells reinforce their adhesive connections with their environment by activating intracellular pathways that promote actomyosin contraction.17 Just like in other cell types, in ECs, the actomyosin cytoskeleton is a major determinant of cell stiffness. Treatment of ECs with cytochalasins to perturb actin polymerization significantly reduces cellular stiffness.18,19 Similarly, treatment with myosin inhibitors, such as blebbistatin, strongly reduces cellular stiffness, linking motor protein activity directly to cellular stiffening.20,21

Thus, the integrity of the actin network and the myosin-based contraction of F-actin are important determinants of EC stiffness. Moreover, ECs on rigid ECM substrates increase activity of the small GTPase Rho.12,22 GTP-loading of Rho results in the activation of downstream effectors, including the Rho kinase (also known as Rho-associated coiled coil-containing protein kinase). Rho kinase inhibits the myosin light chain (MLC) phosphatase, promoting phosphorylation of MLC and myosin activity in the actomyosin cytoskeleton.23 Conversely, inhibiting Rho kinase with the broadly used Y-27632 inhibitor relaxes human ECs on stiff substrates.22

In addition to regulating actomyosin contractility directly, Rho kinase phosphorylates and inactivates endothelial nitric oxide (NO) synthase.24 This reduces endothelial production of NO, which is a key regulator of blood pressure and vascular stiffness by inducing smooth muscle relaxation and vasodilation.25 Thus, there is a crucial role of the endothelial Rho–Rho kinase axis in regulating vascular stiffness (Figure 2). A host of EC agonists and stimuli induces contractility via activation of G-protein–coupled receptors, such as integrins, leukocyte ligands, receptors for soluble ligands, and for flow-induced shear force, all feed into common pathways in endothelial cells (ECs). The core of these mechanotransduction pathways is the Rho–Rho kinase–myosin axis, in which inhibition of myosin phosphatase, induced by phosphorylation by Rho kinase, is a key event. Consequent actomyosin-based contractility promotes cellular stiffening. This feeds back into the organization of the membrane-associated receptors and eventually drives endothelial permeability and (chronic) vascular inflammation in cardiovascular disease. In addition, Rho kinase negatively regulates nitric oxide (NO) production, leading to smooth muscle cell (SMC) contraction and vasoconstriction. This will feedback, a.o. through hypertension, onto endothelial mechanosensors. The Rac-nuclear factor kappa B (NFκB) pathway regulates inflammatory genes and expression of leukocyte receptors such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. However, the role of this pathway is complex and may serve to impair or protect endothelial function (see text). ECM indicates extracellular matrix; GPCR, G-protein–coupled receptor; MLCK, myosin light chain kinase; and VSMC, vascular SMC.

Figure 2. Vascular stiffness-induced endothelial responses that regulate permeability and inflammation. Various cell surface receptors, such as integrins, leukocyte ligands, receptors for soluble ligands, and for flow-induced shear force, all feed into common pathways in endothelial cells (ECs). The core of these mechanotransduction pathways is the Rho–Rho kinase–myosin axis, in which inhibition of myosin phosphatase, induced by phosphorylation by Rho kinase, is a key event. Consequent actomyosin-based contractility promotes cellular stiffening. This feeds back into the organization of the membrane-associated receptors and eventually drives endothelial permeability and (chronic) vascular inflammation in cardiovascular disease. In addition, Rho kinase negatively regulates nitric oxide (NO) production, leading to smooth muscle cell (SMC) contraction and vasoconstriction. This will feedback, a.o. through hypertension, onto endothelial mechanosensors. The Rac-nuclear factor kappa B (NFκB) pathway regulates inflammatory genes and expression of leukocyte receptors such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. However, the role of this pathway is complex and may serve to impair or protect endothelial function (see text). ECM indicates extracellular matrix; GPCR, G-protein–coupled receptor; MLCK, myosin light chain kinase; and VSMC, vascular SMC.
this pathway.28 Next, we will discuss key examples of extracellular stimuli that feed into the EC rigidity-regulating machinery.

**Mechanotransduction at the Integrin–Cytoskeleton Interface**

Myosin-based tension on actin-linked proteins regulates both cell–matrix adhesions and cell–cell junctions.17,27,28 Changes in the vascular ECM are transmitted into the cell via integrin-based adhesion complexes. Integrins cluster in focal adhesions (FAs) that are linked via many actin-binding adaptor proteins to the actin cytoskeleton.29 In addition, FAs are signal initiation sites that regulate Rho GTPases and thereby control cytoskeletal dynamics.13,30–33 We recently showed that the endothelial cytoskeleton within human arteries is dominated by F-actin stress fibers that attach to integrin-based FAs. In contrast, F-actin in veins is mainly cortically organized, with few FAs detectable.34 This cytoskeletal organization depends on the mechanical characteristics of the vascular wall because ECs (irrespective of their vascular origin) adopt a venous F-actin distribution when seeded on soft ECM matrices, and an arterial cytoskeletal organization on relative stiffer ECM. Thus, endothelial integrin-based adhesions sense vascular stiffness and translate this parameter into endothelial cytoskeleton organization (Figure 2).

ECM stiffening induces conformational changes, protein recruitment, and signaling events within integrin complexes.32,35 Integrin-mediated mechanotransduction occurs via adaptor proteins, such as talin, FA kinase, α-actinin, and filamin.35–38 Talin, a cytoplasmic protein that interacts with integrin cytoplasmic tails, is crucial for EC adhesion, and its endothelial-specific ablation results in embryonic lethality caused by adhesion defects during developmental angiogenesis.39 ECM stiffening promotes tension-induced conformational changes in talin, which then exposes a binding site for vinculin.40 Recruitment of vinculin to talin subsequently strengthens the integrin–actin connection and supports stiffening of the actin cytoskeleton.30 The importance of vinculin in cardiovascular disease is further evidenced by the association of mutations in the human vinculin gene with cardiomyopathy.41,42

The conformation of another integrin-binding protein, filamin, is also force dependent: mechanical forces or myosin-dependent contractile forces that increase tension on filamin, enhance its affinity for integrins,43 which likely contributes to the reorganization of the actin cytoskeleton in response to ECM stiffness. Finally, proteomic studies show that the abundance of ∼350 of the total of ∼900 proteins found at integrin adhesions change in response to increases or decreases in actomyosin contractility.44,45 Further indicating that a significant portion of integrin complexes are mechanically regulated and interdependent. Interestingly, we find that mutations in 26 proteins of those myosin-dependent, integrin-linked proteins are associated with human (cardio)vascular disease (Table), emphasizing the importance of integrin-mediated signaling in vascular health and disease.

**Stiffness-Induced Regulation of Endothelial Permeability**

The actomyosin cytoskeleton is thus responsive to mechanical signals that derive from integrin–ECM adhesions and vice versa. Moreover, there is cross talk between integrin-based adhesion structures and cell–cell junctions. This is underscored by experiments, which show that stiffening of the ECM raises Rho-dependent pulling forces at cell–cell junctions.28,46 Increased pulling by actomyosin bundles remodels cell–cell junctions.22,28,47 Several (angiogenic or inflammatory) permeability factors trigger signaling pathways in ECs that enhance actomyosin contractility and thereby increase monolayer permeability. The most effective activator of Rho-mediated contraction is the protease thrombin, which acts by cleaving and activating PAR receptors. These G-protein–coupled receptors trigger the dissociation of the Rho activator guanine exchange factor H1 (GEFH1) from microtubules, allowing it to activate Rho.48 Subsequently, Rho, via Rho kinase, increases myosin activity and destabilizes EC–cell adhesions (Figure 2).47,49–53 Thrombin-induced EC contractility, EC-derived traction forces, and monolayer permeability are enhanced on stiffening substrates.52,58 Huynh et al.12 extended these studies to ex vivo measurements using AFM, which showed that aortas from young (∼10 weeks) mice are soft when compared with aortas from older (>21 weeks) mice. This correlated with increased EC permeability and changes in junctional organization in the vessels from older animals, and increased leukocyte transendothelial migration (TEM) across inflamed endothelium. This suggests that in the vessel wall, regions of increased stiffness are primed and more sensitive to permeability factors or local insults, such as leukocyte TEM.

The angiogenic permeability-inducing growth factor vascular endothelial growth factor also activates Rho–Rho kinase signaling toward myosin, which drives EC migration and monolayer permeability.54–56 In this case, Rho is activated via the vascular endothelial growth factor receptor-2 and its associated heterotrimeric G protein Gq/11.57 However, to what extent endothelial rigidity is controlled by vascular endothelial growth factor is unclear. The cytokine tumor necrosis factor (TNF)-α is well known for inducing an inflamed state of the endothelium, which involves gradual cytoskeletal remodeling, activation of nuclear factor kappa B (NFκB), increased expression of inflammatory adhesion receptors, and endothelial permeability. Just like thrombin, TNF-α induces rapid activation of Rho and myosin; however, this does not directly underlie the increased permeability.58 Moreover, neither endothelial rigidity nor integrin-mediated traction forces increase after TNFα treatment,59,60 suggesting that Rho activation per se is not sufficient to induce stiffening of inflamed endothelium.

**Endothelial Stiffness Controls TEM of Leukocytes**

Influx of activated leukocytes (neutrophils and monocytes) into the subendothelial vessel wall represents, next to increased endothelial permeability, a hallmark of inflammation in vascular disease.61,62 AFM measurements show a 5- to 6-fold higher stiffness in the hypocellular fibrosis areas when compared with the cellular fibrosis area of atherosclerotic plaques from ApoE−/− mice.14 Increasing evidence shows that vascular stiffness regulates leukocyte TEM.12,63,64 In addition, genetic deficiency or inhibition of myosin light chain kinase (MLCK) in mice attenuates endothelial permeability, leukocyte TEM, and atherosclerosis.55,66 These data support a causal role for EC stiffness in leukocyte influx. This may occur at the level of (1) transcellular migration, where adherent leukocytes migrate toward the underlying tissue via penetration of EC soma; or (2) paracellular migration where leukocytes induce destabilization...
of cell–cell junctions to allow TEM. 61,62 For both pathways, cytoskeletal rigidity is important. Local differences in rigidity of the EC cortical actin network may act as guidance cues for transcellular migration,67,68 whereas cytoskeleton-derived tension at cell–cell contacts regulates junctional integrity and paracellular migration.69 This may explain why in blood vessels transmigration of inflammatory cells occurs heterogeneously at so-called inflammation hotspots, which may result from local variations in endothelial stiffness.70

Cross Talk Between EC Stiffness and Leukocyte Behavior

Thus, pathological cellular stiffening destabilizes cell–cell junctions in endothelial monolayers and promotes TEM. Like other cells, neutrophils respond to stiffer ECs (which serve as a substrate for adherent leukocytes) with increased cell spreading.64,66,68 Vascular ECM stiffness regulates neutrophil motility, with an apparent rigidity optimum of around 10 kPa.64,66,68 EC surface stiffness is not homogenous. Some studies reported that the periphery of ECs is stiffer when compared with the nuclear area.60,71,72 Others found a different stiffness gradient, with the highest stiffness in the nuclear region.73 Despite these apparent discrepancies, we postulate that during the scanning or crawling phase of transmigration, leukocytes sense and respond to endothelial stiffness gradients. It may well be that durotaxis, ie, migration toward regions of increased stiffness, is a part of the multistep paradigm for TEM (Figure 3A). Durotaxis requires the sensing of substrate

### Table. Integrin-Related Mechanotransduction Proteins and Their Association With Human Cardiovascular Disease

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<td>ACTA2</td>
<td>Actin, α 2, smooth muscle, aorta</td>
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<td>Anillin, actin-binding protein</td>
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<td>Myosin, heavy chain 11, smooth muscle</td>
<td>Thoracic aortic aneurysms and dissections182</td>
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Cardiac phenotypes

<table>
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<tr>
<th>Gene</th>
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<th>Genetic Associations With Human Cardiovascular Disease</th>
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<tr>
<td>Vcl</td>
<td>Vinculin</td>
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<td>Actin, α cardiac muscle 1</td>
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<tr>
<td>GNAI2</td>
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<td>NOTCH2</td>
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<td>RAB23, member RAS oncogene family</td>
<td>Carpenter syndrome215</td>
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<tr>
<td>TRPM4</td>
<td>Transient receptor potential cation channel, subfamily M, member 4</td>
<td>Familial heart block; cardiac conduction defects; cardiac arrhythmia206,207</td>
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Recent mass spectrometry analysis revealed that within integrin-based adhesion complexes, the abundance of ~350 proteins changes in response to increases or decreases in actomyosin contractility.44,45 Using the Online Mendelian Inheritance in Man database, we screened these published actomyosin-regulated proteins for association with human disease.
stiffness by adhesion molecule tugging. The identification of podoprints or adhesive filopodia to probe the EC surface, together with the recent results obtained by pulling on intercellular adhesion molecule-1 (ICAM-1) using magnetic tweezers certainly support a model of leukocyte-mediated tugging to sense and regulate local EC stiffness.

Leukocytes use integrins to adhere firmly to the vascular endothelium. As a result, endothelial integrin ligands, such as ICAM-1 and vascular cell adhesion molecule-1, cluster, which triggers signal transduction within ECs. A major response to clustering of ICAM-1 and vascular cell adhesion molecule-1 is the activation of Rho, which is accompanied by increased EC stiffness. This suggests that leukocyte adhesion initiates positive feedback signaling, which increases actomyosin contractility, remodeling of cell–cell junctions, and leukocyte TEM (Figure 3B). We recently showed that endothelial actin-binding proteins, in particular α-actinin-4, control local EC stiffness. α-Actinin4 connects surface ICAM-1 to the endothelial actin cytoskeleton. This locally increases EC stiffness, which further enhances clustering of ICAM-1. In the absence of endothelial α-Actinin4, neutrophils adhere and spread less on EC surfaces, which strongly reduces TEM. Thus, local EC surface stiffening supports spreading and TEM of neutrophils.

In contrast to the above studies, which show that increased EC stiffness promotes TEM, a recent study proposed the concept of diapedesis by the path of least resistance. This work showed that lymphocytes preferentially transmigrate at sites of lowest F-actin density, suggesting that TEM occurs at sites of low stiffness. Taking these studies together, one could speculate that locally increased EC stiffness is important for the adhesion and crawling phase of leukocyte TEM, whereas the initiation of diapedesis requires local reduction of EC stiffness. A thorough investigation into the mechanism of EC stiffening and its contribution to inflammation, or related processes such as lymphocyte trafficking, stem cell homing, or cancer cell metastasis, remains to be established.

In addition to ECs providing signals to adherent leukocytes, there is a reverse communication from adherent leukocytes to ECs. During leukocyte adhesion and paracellular transmigration, endothelial contractility and traction forces that are exerted on ECM are increased. In particular at sites of leukocyte transmigration, endothelial traction forces are higher when compared with those in neighboring EC of the monolayer. In agreement with this, repetitive pulling on adherent anti-ICAM-1–coated magnetic beads using magnetic tweezers increased local EC stiffness. This stiffening response was accompanied by Rho activation and sensitive to inhibitors of actomyosin-based contractility and Rho kinase. The authors identified the RhoGEF LARG (RhoGEF12) as a key upstream activator of Rho. Because adherent and migrating leukocytes most likely exert pulling forces through their integrins on the endothelial ligands, subsequent activation of Rho signaling might support TEM by increased stiffening of ECs.

Regulation of EC Stiffness and Inflammation by Flow

Shear Forces and Endothelial Rigidity

Shear stress is a mechanical dragging force derived from the velocity and viscosity of the blood which is exerted on the apical side of the vascular endothelium. Atherosclerosis and, in particular, atherosclerotic plaque regions represent an important pathological entity where the relationship between blood flow–induced shear stress is linked to spatially restricted regulation of vascular stiffness and inflammation. Laminar flow, which induces EC alignment and occurs in straight portions of the vascular tree, suppresses inflammation and is atheroprotective. Conversely, atherosclerotic plaques mainly form at sites of disturbed flow (ie, at arterial bifurcations or at sites of vascular damage). Early studies using AFM on healthy aorta showed that stiffness depends on the location relative to the aortic bifurcation supporting the notion that local stiffness is regulated by flow patterns. In line with this, the induction of laminar or turbulent flow rapidly regulates Rho-dependent contractility, and flow-induced alignment of ECs requires activity of the actomyosin cytoskeleton. As a consequence, EC–cell junction organization, cell–ECM adhesions, and their derived-traction forces change in response to shear stress.

Shear stress–induced cytoskeletal remodeling depends on a mechanotransduction complex that includes the adhesion receptors platelet endothelial cell adhesion molecule 1 (PECAM-1) and VE-cadherin. Results from FRET (fluorescence resonance
energy transfer)-based tension sensors indicate that shear stress induces a shift from myosin-based tension on VE-cadherin to vimentin-dependent tension on PECAM-1 receptors. Shear stress–activated PECAM-1 signals to cell–ECM adhesions and changes their conformation. Although PECAM-1–mediated mechanosignaling induces transient activation of Rho, the activation of integrins represses Rho-mediated stiffening of ECs in the long run, which supports its atherosuppressive function. Two regulators of Rho GTPases have been implicated in flow-induced cytoskeletal changes. In bovine aortic EC, induction of flow transiently inactivates Rho, followed by strong activation, together with MLCK activation and formation of F-actin fibers. This latter response required the inactivation of p190RhoGAP. Application of tensional force on PECAM-1, using magnetic tweezers, activates Rho in bovine aortic ECs. Using pull-down assays with a GEF-binding mutant of RhoA, both GEFH1 and the GEF LARG were found to be force activated. Thus, local activation of stiffness-inducing pathways, i.e., through tension on PECAM-1, induces a global response in the EC.

Among the events that have been studied in detail in flow-exposed ECs is the nuclear translocation and activation of the proinflammatory transcription factor NFκB. NFκB drives the expression of ICAM-1 and vascular cell adhesion molecule-1. Short-term, laminar flow–induced nuclear translocation of NFκB and EC alignment depends on the activity of the small GTPase Rac1. Moreover, induction of laminar flow activates Jun kinase, which controls inflammatory genes in bovine aortic ECs cultured on fibronectin, upregulated in inflamed vessels, but not on other ECM proteins. Intriguingly, bovine aortic ECs cultured on basement membrane extract activate an atrophoprotective, cAMP-dependent signaling pathway. Additional studies showed that activation of Rac1, and activation of the effector protein PAK, is required for long-term laminar flow–induced activation of NFκB and increased permeability of EC monolayers on fibronectin. Because turbulent flow stiffens ECs, and stiffer ECs show increased permeability, these data indicate that increased stiffness causes a flow-induced endothelial inflammatory phenotype. Importantly, turbulent atherogenic flow stimulates the deposition of fibronectin into the subendothelial matrix of mouse aorta. The increased fibronectin, in turn, drives vascular remodeling in vivo (i.e., matrix deposition; VSMC proliferation). This mechanism may explain the relationship between turbulent flow induced at atheroprone sites which, in conjunction with flow-induced EC stiffening, stimulates vascular inflammation. It is important to note, however, the complex regulation of NFκB signaling in this process. Preshocking of HUVECs (human umbilical vein endothelial cells) by long-term laminar flow promoted TNFα-mediated activation of NFκB, but this activated cytoprotective rather than proinflammatory transcripts. This explains why laminar shear stress prevents TNFα-induced inflammation, for example, in the descending aorta, but not in the aortic arch.

**Shear Stress–Regulated Ion Channels**

Early experiments have shown that mechanical stretching of endothelial plasma membranes leads to an influx of ions via mechanosensitive channels. Such influxes, of Ca2+ in particular, not only identify cellular membranes under mechanical tension, but downstream signaling by Ca2+ also activates MLCK and thereby regulates the mechanoresponse. In blood vessels, many mechanosensitive ion channels respond to changes in blood flow, including the subfamily of vanilloid transient receptor potential (TRPV) channels. In a rat model for arteriovenous fistula, it was shown that increased shear stress up-regulates endothelial expression of TRPV1, downstream Ca2+-calmodulin-dependent kinase II signaling and NO synthase, which in turn controls endothelial rigidity. Similarly, shear stress–induced mechanosignaling to Ca2+ and to NO synthase can occur through the TRPV4 channel, and TRPV4 itself is required for the rapid vascular permeability response in acute lung injury. Interestingly, force-induced opening of Ca2+ channels is controlled by the organization and pulling of the F-actin cytoskeleton linked to integrin-based adhesions. This suggests that once the vascular wall stiffens, mechanosignaling via ion channels might be enhanced. Indeed, a role for TRPV4 in a positive feedback loop was shown to drive alignment of ECs in response to cyclic stretching on flexible substrates. To date not much is known about a possible relationship between mechanosignaling of endothelial ion channels and trafficking of inflammatory cells, albeit that a rise in intracellular calcium was the first signaling event shown to be required for efficient leukocyte TEM. Whether this requires force-sensitive channels remains to be investigated.

The most direct link between endothelial mechanotransduction responses and vascular stiffness is provided by endotheliamediated production of the vaso-relaxant NO. Interestingly, the mineralocorticoid hormone aldosterone regulates the activity of F-actin–associated epithelial sodium channel (ENaC), which promotes endothelial stiffening by regulating NO production and osmotic pressure. Gain-of-function mutations in ENaC are linked to Liddle syndrome, an inherited form of severe hypertension. ECs derived from a Liddle syndrome mouse model display increased stiffness and membrane expression of ENaC. This strongly suggests that there exists a feedback mechanism between endothelial stiffening and osmotic pressure–induced vascular stiffening. Moreover, the pathophysiological phenomenon of increased cortical rigidity in ECs, associated with reduced NO release and inflammation, was termed stiff EC syndrome, which further emphasizes the connection between EC stiffness and vascular disease. The aldosterone antagonist spironolactone, which is used as antihypertensive drug and suppresses cardiac fibrosis, suppresses such endothelial stiffening. This is because spironolactone blocks aldosterone-induced transport of ENaC to the plasma membrane. Furthermore, the ENaC blocker amiloride reduces hypertension and patients with Liddle syndrome benefit from treatment with amiloride. Recent ex vivo analysis of aortae from aged mice shows increased ENaC expression and increased stiffness when compared with the aortae from younger animals. Moreover, the age-induced stiffening was reduced by inhibition of ENaC with amiloride. Importantly, the activity of ENaC is stimulated by Rho, in an F-actin–dependent fashion, which links endothelial cytoskeletal changes to ENaC-mediated cell stiffening. This indicates that multiple inputs downstream from mechanical changes regulate ENaC and endothelial stiffening, which in turn feed back into control of NO production and vasoconstriction.
Regulation of Endothelial Stiffening by the Vascular Wall

Vascular Smooth Muscle Cells

An important contribution to vascular stiffness is derived from the activity of VSMCs. Using AFM measurements, Qiu et al.\(^{132}\) found that VSMCs from old long-tailed macaques (aged, \(\approx 25\) years) showed a significant increase in cellular stiffness when compared with cells from young animals (\(\approx 6\) years). In these experiments, isolated cells were mixed with collagen and incorporated in a reconstituted arterial tissue model to show that the measured stiffness differences are indeed cell-intrinsic and not derived from aged ECM components. Inhibitor studies show that the elevated stiffness in old VSMCs was dependent on the activity of the actomyosin cytoskeleton.\(^{132}\) In a subsequent study, this group showed that VSMCs isolated from spontaneous hypertensive rats displayed increased actomyosin-dependent stiffness when compared with VSMCs from normotensive animals.\(^{133}\)

Thus, systemic hypertension results in a cell-autonomous stiff VSMC phenotype. Furthermore, the integrin-mediated interaction of VSMCs with the ECM in the vascular wall determines both the stiffness of the cells and of the aortic tissue. In line with this, Saphirstein et al.\(^{134}\) showed that inhibitors of integrin signaling reduced vasoconstrictor-induced VSMC and aortic stiffness.

Extracellular Matrix

Just as in other tissues, the biomechanical properties of the vascular ECM vary depending on the location in the vascular network.\(^{83}\) In addition, calcification, local fragmentation of elastin fibers, and increased deposition of ECM components are associated with aging vessels, increased vessel wall stiffness, and cardiovascular disease.\(^{135-138}\) Mouse models show that during aging, the average rigidity of the ECM of blood vessels is increased \(\leq 2\)- to 3-fold,\(^{12}\) which is sufficient to promote endothelial permeability and transmigration of inflammatory cells.\(^{12,22,59}\)

The stiffness of the subendothelial matrix of de-endothelialized bovine carotid arteries was similar to the stiffness of the endothelium of intact arteries.\(^{139}\) This suggests that the composition and stiffness of the subendothelial ECM, determined by several factors as discussed above, are directly reflected by the stiffness of the endothelium. Consequently, changes in vascular wall stiffness will be equally visible at the luminal side of the endothelium and are sensed by adherent leukocytes\(^{12}\) and by adherent platelets.\(^{140}\)

The expression of collagens and of lysyl oxidase (Lox), an enzyme that cross links and strengthens ECM fibers, is strongly upregulated in VSMCs that are cultured on rigid substrates (25 kPa) when compared with cells on softer matrices.\(^{141}\) Intriguingly, addition of ApoE and ApoE-high-density lipoprotein blocked these stiffness-induced increases in collagen, fibronectin, and Lox mRNA. Conversely, lack of ApoE in mice stiffens the thoracic aorta and promotes atherosclerosis. Reduction of this arterial stiffness in ApoE\(^{-/-}\) mice by a Lox inhibitor, also reduced collagen crosslinking and atherosclerosis, despite high cholesterol levels.\(^{141}\) Thus, the atheroprotective role of ApoE lies both in efficient cholesterol transport and protection from arterial stiffening.

Another recent study showed that treatment of mouse lungs with the inflammatory agent lipopolysaccharide strongly increased the stiffness of the wall of microvessels, as measured by AFM, which correlated with increased expression of fibropectin, collagen, and Lox.\(^{142}\) This was accompanied by an inflammatory response that could be inhibited by a lipoxin analog, eLX4, which reduced stiffening of the lung vessels. In line with this, in vitro studies using human pulmonary EC showed that inhibition of Lox reduces stiffness-enhanced, TNF-, and lipo-polysaccharide-induced inflammatory signaling.\(^{143}\) Thus, in the lung vasculature like in the aorta, ECM remodeling contributes to vascular stiffness and lung inflammation.

Calcification

Another vascular event in inflammation and cardiovascular disease is the calcification of the vessel wall.\(^{144}\) This process, which strongly increases with age,\(^{137,145}\) occurs at various locations in the vasculature and is particularly prevalent in atherosclerotic plaques. Several mechanisms underlie arterial calcification.

One of these involves VSMCs or activated pericytes differentiating toward an osteogenic phenotype, mainly under the influence of bone morphogenic protein 2.\(^{146,147}\) Bone morphogenic protein 2 is secreted by VSMCs and ECs, and its expression increases in advancing atherosclerotic plaques. In addition, calcification is linked to vascular inflammation and the influx of macrophages, which promote formation of microcalcifications and also induce VSMC differentiation.\(^{137,148}\) Concomitant release of proinflammatory cytokines and chemokines further increases local inflammation and leukocyte influx, representing another example of a positive feedback loop that drives vascular inflammation and cardiovascular disease.

Targeting Rho–Rho Kinase Pathway to Suppress Vascular Stiffness and Hypertension

Taken together, Rho–Rho kinase–mediated actomyosin contractility, be it in ECs or in VSMCs, is an important event that controls vascular stiffening via feedback mechanisms (Figure 2).

There is a significant body of the literature linking Rho kinase to hypertension, in line with the known relationship between vascular stiffening and hypertension. Interestingly, a longitudinal analysis in \(>1700\) volunteers (Framingham Heart Study) showed that arterial stiffening precedes incident hypertension.\(^6\)

Measuring the carotid-femoral PWV in volunteers, Noma et al.\(^{140}\) showed that elevated Rho kinase activity was strongly associated with higher arterial stiffness and age. Moreover, inhibition of Rho kinase with Fasudil increased vasorelaxation, which lowers blood pressure. Similarly, in a mouse model for diet-induced obesity, Weisbrod et al.\(^{150}\) observed that arterial stiffness develops before diminished NO function and an increase in hypertension. This process was reversible because switching the mice back to a regular diet reduced arterial stiffness and blood pressure to normalized levels within 2 months. These findings strongly suggest that stiffness-induced vascular cell behavior causes cardiovascular disease and that targeting this response might serve as therapy.

Most studies that address the role of Rho kinase activity in hypertension make use of a few, well-established pharmacological inhibitors. The widely used compound Y-27632 was identified in 1997 in a screen for agents that promote smooth muscle relaxation.\(^{151}\) In that study, it was already shown that administration of Y-27632 significantly reduced blood pressure in rat models for hypertension. Subsequent studies using the
Rho kinase inhibitor Fasudil have shown that inhibition of Rho kinase reduces blood pressure and endothelial dysfunction in spontaneous hypertensive rats, as well as pulmonary hypertension in monocrotaline-treated rats. Similarly, clinical trials in patients with pulmonary arterial hypertension confirmed the beneficial effects of inhibiting Rho kinase with Fasudil. It is important to note that in some experiments the Rho–Rho kinase pathway in both ECs and SMCs will be targeted.

In addition to Y-27632 and Fasudil, HMG-CoA reductase inhibitors (statins) reduce hypertension. This may be because statins inhibit, as part of their effects on cholesterol metabolism, also the post-translational lipidation of Rho-like GTPases. For Rho, this results in impaired membrane targeting, activation and signaling toward Rho kinase. Interestingly, several studies showed that Rho kinase is also regulated at the level of its expression: in blood vessels of spontaneous hypertensive rats and in pulmonary arterial smooth muscle cells from patients with pulmonary arterial hypertension, elevated expression of Rho kinase was observed, which might underlie, in part, the increased activity of the Rho–Rho kinase pathway in hypertension.

There is recent information that link endogenous regulators of Rho signaling to hypertension. Angiotension II–induced hypertension in mice was alleviated by the deletion of the RhoA activator Arhgef1 from SMC. In hypertensive patients, but not in healthy controls or Bartter/Gitelman patients (a kidney disorder accompanied by normal to low blood pressure), increased mRNA and protein levels of p63RhoGEF were detected, albeit that this was measured in mononuclear cells from peripheral blood. In a broader screen for regulation of RhoGEF expression in hypertension, Cario-Toumaniantz et al. showed that the vasoconstrictor angiotensin II, used in vitro on mesenteric artery SMCs or applied in vivo to induce hypertension in rats, induced a significant downregulation of 9 of 28 analyzed RhoGEFs. Intriguingly, this effect was sensitive to Fasudil, which indicates that in VSMCs, activation of the Rho–Rho kinase axis triggers a negative feedback loop to limit the consequences of vasoconstriction.

Conversely, genetic deletion of Arhgap42 (also known as GRAF3), which is a RhoA inactivating protein that is specifically expressed in SMC, increases blood pressure. This increase was sensitive to Y-27632 treatment in vivo and the loss of GRAF3 in cultured SMC was accompanied by increased Sphingosine-1-P–induced RhoA activity. Another means of regulating Rho output is through the related GTPase Rac1. In many cell types, Rac1 and RhoA have opposing effects on the actomyosin cytoskeleton and indeed, deletion of Rac1 in SMC was recently found to mimic a RhoA activation phenotype by inducing hypertension in mice which is sensitive to inhibition by Fasudil. Thus, in VSMCs, a Rac1-dependent signaling pathway limits Rho–Rho kinase activity, maintaining VSMCs pliability and protecting against increased arterial stiffening and blood pressure.

Conclusions

Cellular mechanotransduction responses are key events in vascular homeostasis, and dysregulation of these pathways is an important cause of cardiovascular disease. During the past 10 to 15 years, several mechanotransducers in ECs have been identified. In addition, the regulation of the actin cytoskeleton via the Rho–Rho kinase axis was shown to be not only a central mechanotransductive signaling pathway in ECs but also as a promising therapeutic target to improve vascular function. The recent studies that link regulators (GEFs and GAPs [GTPase activating proteins]) of Rho-mediated contraction to hypertension not only bridge fundamental molecular insights with pathology but also provide new ways to address targeting this pathway to limit vascular stiffening and inflammation. This is important for the development of treatment options for damaged vasculature not only in age-related vascular disease but also to heal vasculature in obesity, diabetes mellitus, kidney disease, and after chemotherapy or radiotherapy. We expect that targeting vascular stiffness, by translating basic knowledge into effective drugs, holds great promise for the treatment of patients with cardiovascular disease.

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Disclosures

None.

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