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REVIEW

Rho GTPases in the regulation of pulmonary vascular barrier function

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Abstract Pulmonary endothelial permeability is an important determinant of vascular adaptation to changes in oxygen tension, blood pressure, levels of growth factors or inflammatory cytokines. The Ras homologous (Rho) family of guanosine triphosphate phosphatases (Rho GTPases), key regulators of the actin cytoskeleton, regulate endothelial barrier function in response to a variety of environmental factors and signalling agents via the reorganization of the actin cytoskeleton, changes in receptor trafficking or the phosphorylation of junctional proteins. This review provides a brief summary of recent knowledge on Rho-GTPase-mediated effects on pulmonary endothelial barrier function and focuses in particular on their role in pulmonary vascular disorders, including pulmonary hypertension, chronic obstructive pulmonary disease, acute lung injury and acute respiratory distress syndrome.

Keywords Rho GTPases · Pulmonary endothelial permeability · Endothelial barrier function · Actin · Lung disease

Abbreviations

ADMA	Asymmetric dimethylarginine
AJ	Adherens junction
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
Cx	Connexin
DDAH	Dimethylarginine dimethylhydrolase
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GJ	Gap junction
GTP	Guanosine triphosphate
GTPase	GTP phosphatase
HGF	Hepatocyte growth factor
HIF1α	Hypoxia inducible factor 1 alpha
HPAECs	Human pulmonary artery endothelial cells
LPS	Lipopolysaccharide
MLC_{20}	Myosin light chain 20
MLCK	Myosin light chain kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
OxPAPC	Oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylcholine
PAECs	Pulmonary artery endothelial cells
PAH	Pulmonary arterial hypertension
PAK	p21-activated protein kinase
PH	Pulmonary hypertension
PKC	Protein kinase C
PKG	Protein kinase G
Rho	Ras homologous
ROCK	Rho kinase
ROS	Reactive oxygen species
S1P	Sphingosine-1 phosphate
TJ	Tight junction
TNF-α	Tumour necrosis factor alpha
VASP	Vasodilator-stimulated phosphoprotein
VE-cadherin	Vascular endothelial cadherin

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VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens protein 1

Lung-function: general overview

Pulmonary circulation is a low-pressure high-volume circulation that responds to hypoxia with vasoconstriction. It is exposed to the highest oxygen tension of all vascular beds (Stan 2009) and its function impacts the whole circulatory system, as the entire cardiac output passes through the lung with every heartbeat. Only a 0.3-μm-thick barrier separates the capillary blood from the alveolar gas (West 2013). The integrity of the pulmonary endothelial barrier is important in maintaining the dry interstitium and alveolar airspaces that are necessary for effective gas exchange. It also affects vascular tone, inflammation, coagulation and angiogenesis. Vascular leakage can be initiated by a number of environmental and chemical factors including sudden or sustained changes in oxygen tension, mechanical injury and agents produced by cells in response to injury, cancer or inflammation, such as vascular endothelial growth factor (VEGF), thrombin and histamine.

Proteins and liquids are transported across the endothelium via transcellular or paracellular routes. Transcellular routes, involving the active transport of molecules larger than 3 nm in radius, are mediated by caveolae, vesiculo–vacuolar organelles and fenestrations (Weis 2008; Wang and Dudek 2009). Paracellular permeability, usually induced in pathological situations, is caused by the disruption of intercellular junctions and allows the passive transport of larger volumes of liquids and molecules of radii smaller than 3 nm across the endothelium (Weis 2008; Wang and Dudek 2009).

Endothelial cells are connected to each other by three different junctional complexes, comprising (1) adherens junctions (AJ), (2) tight junctions (TJ) and (3) gap junctions (GJ; Bazzoni and Dejana 2004). Vascular endothelial cadherin (VE-cadherin) is the major structural protein of AJ in endothelial cells (Dejana et al. 2009). The VE-cadherin cytoplasmic tail binds β-catenin or plakoglobin, which associates with a number of actin-binding proteins such as α-catenin, vinculin, α-actinin and eplin (Dejana et al. 2009). This complex stabilizes AJ anchorage to the actin cytoskeleton.

TJ are composed of claudins, occludins and junctional adhesion molecules. The link between the TJ proteins and the actin cytoskeleton is modulated by intermediate signalling proteins (catenins, zonula occludens 1 protein [ZO-1]) and is subject to regulation by kinases and phosphatases (Bazzoni and Dejana 2004; Dejana et al. 2009).

GJ form channels between adjacent cells and allow the exchange of small signalling molecules such as cyclic nucleotides, calcium, adenosine triphosphate and inositol 1,4,5-trisphosphate between cells. In the vascular system, the core proteins of these channels are connexins (Cx) 37, 40 and 43 (van Kempen and Jongsma 1999; Parthasarathi and Quadri 2009). GJ are located in close proximity to the TJ and AJ that form the endothelial barrier and share common linker proteins that bind to the actin cytoskeleton (Derangeon et al. 2009).

The association of the actin cytoskeleton to junctional proteins, integrins and their extracellular ligands is essential for the maintenance of endothelial barrier function. The barrier is regulated by a balance between competing contractile forces, which generate centripetal tension and adhesive tethering forces created by cell-cell and cell-matrix adhesions (Wojciak-Stothard and Ridley 2002). Contractility of the actin cytoskeleton is controlled directly by myosin light chain kinase (MLCK) and Ras homologous (Rho) guanosine triphosphate (GTP) phosphatases (GTPases).

Rho GTPases

Rho GTPases are key regulators of cytoskeletal dynamics (Hall 1998; Hall and Lalli 2010) and affect several vascular processes such as endothelial permeability (Beckers et al. 2010), cell motility (Ridley 2001), angiogenesis (Bryan and D'Amore 2007), nitric oxide (NO) production (Takemoto et al. 2002), smooth muscle contractility (Somlyo and Somlyo 2003), cell proliferation, differentiation and apoptosis (Vega and Ridley 2008; Pedersen and Brakebusch 2012). Rho proteins share approximately 30 % homology with the Ras family of proteins and 80-90 % homology with each other (Hall 1998).

Rho GTPases are activated by a number of factors known to affect endothelial permeability, such as thrombin, histamine, angiotensin II, endothelin-1 (ET-1), VEGF, tyrosine kinase receptors or integrin clustering. In addition, Rho proteins are also activated by mechanical and physical stimuli such as shear stress, stretch, pressure and hypoxia (Wojciak-Stothard and Ridley 2002; Wojciak-Stothard 2008).

Rho GTPases cycle between an active GTP-bound and an inactive guanosine diphosphate (GDP)-bound conformation. Inactive GDP-bound Rho proteins remain in the cytosol in complex with guanine nucleotide dissociation inhibitors (GDIs). Upon phosphorylation triggered by signalling mediators, GDIs dissociate from Rho GTPases allowing them to interact with guanine nucleotide exchange factors (GEFs). GEFs activate the exchange of GDP for GTP allowing Rho GTPases to interact with their downstream effectors (Jaffe and Hall 2005). GTPase-activating proteins (GAPs) mediate the inactivation of GTPases. Apart from GTP/GDP binding, Rho GTPases can be regulated through isoprenylation, carboxylmethylation, oxidation, direct phosphorylation or ubiquitination (Storck and Wojciak-Stothard 2013). Isoprenylation of the C-terminus of Rho GTPases enhances

their binding to the cell membrane, a characteristic that is important for interaction with signalling effectors (Gao et al. 2009).

In this review, we describe the implications of the dysregulation of Rho GTPase signalling in pulmonary vascular barrier dysfunction and highlight some of the potential therapeutic strategies.

RhoA, RhoB and Rho kinase

The basal activity of RhoA is important in the maintenance of inter-endothelial junctions by promoting membrane localization of the AJ protein, VE-cadherin and by strengthening endothelial cortical actin via its effector diaphanous in a profilin-dependent manner (van Nieuw Amerongen et al. 2007; Spindler et al. 2010). However, excessive activation of RhoA and its effector Rho kinase (ROCK), by agents such as thrombin, tumour necrosis factor alpha (TNF-α), oxidative or mechanical stress, is associated with a decrease in endothelial barrier function (Wojciak-Stothard and Ridley 2002; Wojciak-Stothard 2008; Beckers et al. 2010; Spindler et al. 2010). This is caused by an increase in the contractile forces that pull the endothelial intercellular junctions apart (Wojciak-Stothard and Ridley 2002; Wojciak-Stothard 2008; Beckers et al. 2010; Spindler et al. 2010).

Activation of actomyosin contractility by RhoA results predominantly from a ROCK-mediated increase in the level of myosin light chain (MCL_{20}) phosphorylation (Somlyo and Somlyo 2003; Connolly and Aaronson 2011). ROCK can also facilitate actomyosin contractility by increasing the levels of intracellular calcium, following inhibition of voltage-gated potassium channels (Aaronson et al. 2006). RhoA can also compromise endothelial barrier function by reducing the expression of endothelial NO synthase (eNOS) and NO generation by endothelial cells (Takemoto et al. 2002). Conversely, NO might inhibit RhoA by reducing its stability and membrane localization (Sauzeau et al. 2000, 2003).

RhoB, a protein 85 % homologous to RhoA, has also recently been implicated in the regulation of pulmonary endothelial barrier function. RhoB expression and activity can be increased by numerous agents including oxidative stress, tyrosine kinases, the transforming growth factor beta/bone morphogenetic protein/Smad pathway and growth factors such as fibroblast growth factor, epidermal growth factor and platelet-derived growth factor (Huang et al. 2007; Kajimoto et al. 2007; Vardouli et al. 2008; Wojciak-Stothard et al. 2012). Similar to RhoA, RhoB can interact with ROCK, increase MLC_{20} phosphorylation and promote actin polymerization (Conway et al. 2004; Fernandez-Borja et al. 2005). Under hypoxic conditions, RhoB appears to have a complementary effect to RhoA on actomyosin contractility and pulmonary endothelial permeability (Wojciak-Stothard et al. 2012). Whereas RhoA acts as a major activator of ROCK-mediated serine MLC_{20} phosphorylation, RhoB promotes actin filament formation by interacting with a mammalian homologue of *Drosophila* diaphanous, a protein known to induce actin nucleation (Wojciak-Stothard et al. 2012). RhoB can also activate the pro-inflammatory transcription factor, nuclear factor kappa B (Rodriguez et al. 2007), an event of potential importance in the regulation of endothelial barrier function during inflammatory responses.

Rac1 and Cdc42

Rac1 and Cdc42 generally have endothelial-barrier-protective effects and their activation co-incides with the formation of intercellular adhesions, whereas RhoA activity is reduced (Wojciak-Stothard and Ridley 2002; Beckers et al. 2010; Spindler et al. 2010). GTP-bound Rac1 and Cdc42 bind to and allow the autophosphorylation and activation of p21-activated protein kinases (PAK). Phosphorylation of MLCK by PAK results in the inhibition of the phosphorylation of MLC_{20} in vitro and in vivo (Bokoch 2003), counteracting actomyosin contraction. Whereas basal levels of PAK activity are required for the maintenance of the junction-associated cortical actin rim, excessive activation of PAK can activate extracellular signal-regulated kinase (Erk) and induce barrier breakdown (Stockton et al. 2004, 2007).

Physiological activation of Rac by barrier-protective molecules (i.e., sphingosine-1 phosphate [S1P], NO) and specific mechanical stimuli (physiological level of laminar shear stress, low magnitude cyclic stretch) enhances the peripheral actin cytoskeleton and improves endothelial cell monolayer integrity (Garcia et al. 2001; Vouret-Craviari et al. 2002; Birukov et al. 2002; Dudek et al. 2004; Mehta et al. 2005; Birukova et al. 2006, 2007a, 2007b). Rac1 also enhances endothelial NO production by increasing eNOS mRNA and protein levels or by stimulating the uptake of eNOS substrate, L-arginine (Sawada et al. 2008). Whereas basal levels of Rac1 activity are endothelium-protective, sustained Rac1 activation can lead to endothelial dysfunction associated with the generation of reactive oxygen species (ROS; Hordijk 2006). Rac1 is a part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, the main source of ROS generation in the vasculature (Hordijk 2006).

Cdc42 appears to have a unique role in endothelial barrier restoration, possibly as a result of the regulation of VE-cadherin turnover in endothelial cells (Spindler et al. 2010). For instance, the delayed activation of Cdc42 following the activation of cells with thrombin contributes to the reassembly of inter-endothelial junctions and the re-establishment of barrier integrity (Kouklis et al. 2004).

Rho GTPases in endothelial barrier dysfunction in lung diseases

Dysregulation of Rho GTPase signalling by mechanical stress, hypoxia or inflammation is a shared feature of many lung diseases such as pulmonary hypertension (PH), asthma, acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). Below, we present evidence of abnormal Rho GTPase signalling in endothelial barrier dysfunction in selected examples of lung disorders. A summary of the effects of various regulatory factors on the activity of Rho GTPases and pulmonary endothelial barrier function is presented in Table 1. Signalling pathways leading to changes in the organization of the actin cytoskeleton and endothelial junctional integrity are illustrated in Fig. 1.

Pulmonary hypertension

Pulmonary hypertension (PH) is a condition characterized by abnormal remodelling of small pulmonary arteries, leading to increased pulmonary vascular resistance and right heart hypertrophy (Schermuly et al. 2011). The current classification is based on similar pathogenesis, clinical features and therapeutic options and has five main sub-categories: (1) pulmonary arterial hypertension (PAH); (2) PH resulting from left heart disease; (3) PH attributable to lung diseases and/or hypoxia; (4) chronic thromboembolic pulmonary hypertension (CTEPH); (5) PH with unclear multifactorial mechanisms (Archer et al. 2010; Schermuly et al. 2011).

Endothelial dysfunction is believed to be an early component of the disease and involves a decrease in endothelial barrier function, a decrease in the production of vasorelaxants such as NO and prostacyclin and an increase in the production of vasoconstrictors such as ET-1 or thromboxane (Budhiraja et al. 2004; Archer et al. 2010; Burton et al. 2011; Schermuly et al. 2011). RhoA and Rho kinase are activated in the pulmonary vasculature of PH patients and animals (Wojciak-Stothard 2008).

PH is a disease of multifactorial origin and a number of different stimuli implicated in the pathogenesis of PH converge on Rho GTPase signalling pathways. Hypoxia activates RhoA and inhibits Rac1 and Cdc42 in cultured pulmonary endothelial cells (PAECs; Wojciak-Stothard et al. 2005). This change might be transient and reversible by reoxygenation or might be sustained and irreversible depending on the duration of hypoxic exposure (Wojciak-Stothard et al. 2005, 2006). Endothelial cells isolated from pulmonary arteries of chronically hypoxic hypertensive piglets show increased

Table 1 Ras homologous (Rho) GTPases mediate the effects of endothelial-barrier-disrupting and barrier-protective agents in the lung (*EC* endothelial cell, *ROCK* Rho kinase, *Y-27632* ROCK inhibitor, *FVIIa* Factor VIIa, *TNF* tumour necrosis factor, *PKC* protein kinase C, *LPS* lipopolysaccharide, *OxPAPC* oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylcholine, *S1P* sphingosine-1 phosphate, *HGF* hepatocyte growth factor, *ADMA* asymmetric dimethylarginine, *NO* nitric oxide, *cGMP* cyclic guanosine monophosphate, *DDAH* dimethylarginine dimethylhydrolase, *VEGF* vascular endothelial growth factor, *ET* endothelin)

Factor	EC permeability	Rho GTPases	Preventive agent	Reference
Thrombin	↓	↑ RhoA/ROCK	Angiopoietin, FVIIa, 5 % cyclic stretch, Y-27632	van der Heijden et al. 2011
Bacterial toxin (listeriolysin)	↓	↑ RhoA/ROCK	Lectin-like domain of TNF, PKC inhibitor GÖ6976	Xiong et al. 2010
Adenosine Acute	↑	↑ Rac1	–	Lu et al. 2012
Sustained	↓	↑ RhoA		
Endotoxin (LPS)	↓	↑ RhoA/ROCK	OxPAPC, S1P Y-27632, Cdc42	Ma et al. 2004; McVerry et al. 2004; Zhao et al. 2009; Gorovoy et al. 2007; Ramchandran et al. 2008)
Mechanical ventilation	↓	↑ RhoA/ROCK	Iloprost, HGF, S1P, OxPAPC	McVerry et al. 2004; Nonas et al. 2006; Nonas et al. 2008; Birukova et al. 2008
OxPAPC	↑	↑ Rac1/Rap1	–	Birukova et al. 2007a; Birukova et al. 2011
Fe^{2+}	↓	↑ RhoA/ROCK	Y-27632	Gorbunov et al. 2012; Cinel et al. 2012
S1P	↑	↑ Rac1, Cdc42	–	Dudek et al. 2004
Hypoxia	↓	↑RhoA/ROCK, ↑RhoB ↓ Rac1, Cdc42	C3-transferase (RhoA inhibitor), Y-27632, manumycin a (farnesyl trasnferase inhibitor)	Wojciak-Stothard et al. 2005, 2012
ADMA	↓	↑RhoA ↓ Rac1, Cdc42	NO, cGMP, DDAH, Rotigaptide	Wojciak-Stothard et al. 2009; Tsang et al. 2014
VEGF	↓	↑ Rho, Rac1, Cdc42	Physiological cyclic stretch, HGF	Birukova et al. 2008
Cigarette smoke	↓	↑RhoA/ROCK	Y-27632, ET_A-ET_B receptor antagonists, antioxidant N-acetylcysteine	Milara et al. 2010

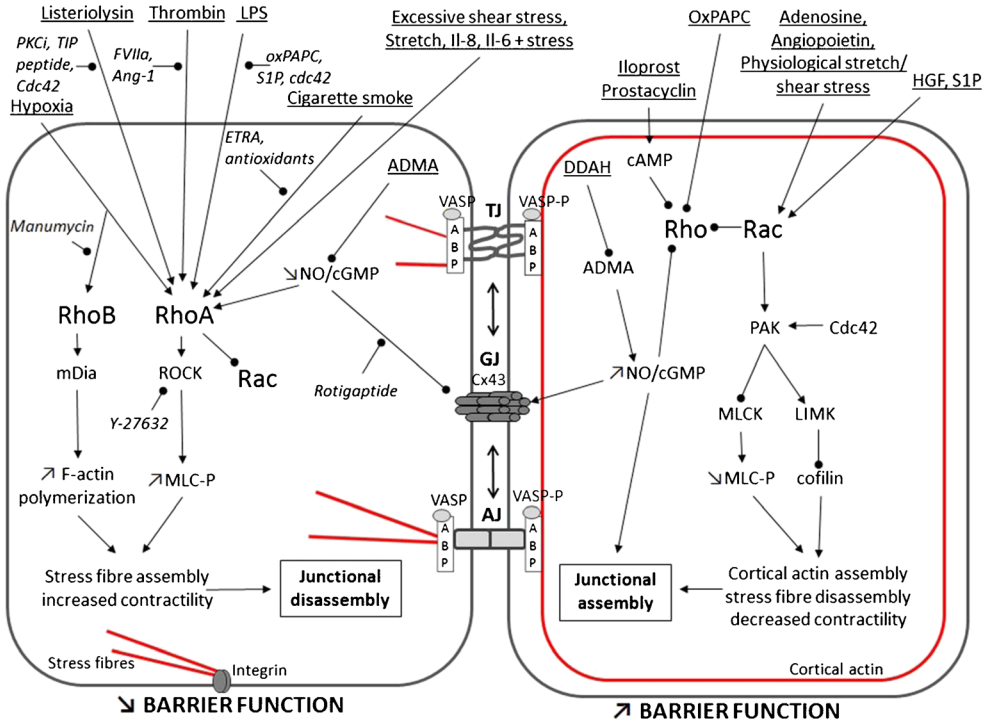

Fig. 1 Proposed mechanisms of Rho-GTPase-mediated changes in the organization of the actin cytoskeleton and endothelial junctional integrity. Details regarding signalling mediators shown in this diagram are provided in the text (*arrows* activation/upregulation, *lines with a black circular ending* downregulation/inhibition, *open arrows* an increase or decrease depending on arrow direction, *ABP* actin-binding proteins, *ADMA* asymmetric dimethylarginine, *AJ* adherens junction, *Ang-1* angiopoietin-1, *cGMP* cyclic guanosine monophosphate, *DDAH* dimethylarginine dimethylhydrolase, *mDia* mammalian diaphanous, *ETRA* endothelin receptor antagonist, *FVIIa* factor VIIa, *GJ* gap junction, *HGF* hepatocyte growth factor, *Il-6* interleukin-6, *Il-8* interleukin-8, *LIMK* LIM kinase, *LPS* lipopolysaccharide, *MLC-P* phosphorylated myosin light chain, *MLCK* myosin light chain kinase, *NO* nitric oxide, *OxPAPC* oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylcholine, *PAK* p21-activated protein kinase, *PKCi* PKC inhibitor, *Rho* Ras homologous, *ROCK* Rho kinase, *S1P* sphingosine-1 phosphate, *TIP* TNF-derived tonoplast intrinsic protein, *TJ* tight junction, *VASP* vasodilator-stimulated phosphoprotein, *VASP-P* phosphorylated VASP)

permeability associated with the activation of RhoA and the inhibition of Rac1 (Wojciak-Stothard et al. 2006). Regulation of RhoA and Rac1 during hypoxia/reoxygenation depends on the activity of NADPH oxidase, phosphoinositide 3 kinase and intracellular ROS production (Wojciak-Stothard et al. 2005). Activation of RhoA by hypoxia in PH rat lungs depends on superoxide generation (Broughton et al. 2010). However, RhoA activation in cultured pulmonary endothelial and smooth muscle cells is ROS-independent (Chi et al. 2010) suggesting that the mechanism is cell-type-/tissue-specific. RhoA/ROCK can also be activated by the Src family of tyrosine kinases in agonist- and hypoxia-induced stimulation of pulmonary arteries in rats (Wang et al. 2001; Knock et al. 2008).

RhoB is rapidly and transiently upregulated in response to stress conditions. Both RhoA and RhoB are upregulated by hypoxia and the inhibition of either RhoA or RhoB prevents hypoxia-induced stress fibre formation and an increase in endothelial permeability in human pulmonary endothelial cells (HPAECs), indicating that both GTPases are important (Wojciak-Stothard et al. 2012). Inhibition of RhoB farnesylation prevents hypoxia-induced pulmonary endothelial permeability in vitro (Wojciak-Stothard et al. 2012). RhoB also stabilizes hypoxia inducible factor 1 alpha (HIF-1α) in HPAECs and therefore might impact pulmonary endothelial permeability induced by factors acting downstream of HIF-1α, such as VEGF. The mechanism of RhoB-induced stabilization of HIF-1α in endothelial cells will require further studies. In glioblastoma cells, RhoB prevents proteolytic degradation of HIF-1α by the Akt/glycogen synthase kinase-3β pathway (Skuli et al. 2006). Rac1, which activates the RhoB promoter (Huelsenbeck et al. 2013), has been shown to stabilize HIF-1α in hypoxic Hep3B cells (Hirota and Semenza 2001).

VEGF is important for the maintenance of pulmonary vascular homeostasis and protects against chronic hypoxia-induced PH in rats (Partovian et al. 2000; Tuder and Yun 2008). However, VEGF expression is increased in remodelled hypertensive arteries and is prominent in plexiform lesions in PH, suggesting that VEGF contributes to vascular pathology (for a review, see Tuder et al. 2000). VEGF induces pulmonary endothelial permeability in vitro and in vivo (Bates 2010) as a result of the rapid endocytosis of vascular endothelial cadherin (Gavard and Gutkind 2006). This process is initiated by VEGF-receptor-2-induced activation of the small GTPase Rac through a Src-dependent pathway. Rac1 activation

promotes PAK-mediated phosphorylation of VE followed by internalization of the protein into clathrin-coated vesicles and a breakdown of inter-endothelial junctional integrity. Interestingly, the VEGF-induced decrease in endothelial barrier function is preceded by a Rac1-dependent transient enhancement of the endothelial barrier (Seebach et al. 2005).

Inhibition of NO signalling in PH is associated with increased plasma and tissue levels of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), mainly because of a decrease of its metabolism by the dimethylarginine dimethylhydrolase (DDAH) enzymes (Arrigoni et al. 2003; Millatt et al. 2003; Pullamsetti et al. 2005). ADMA activates RhoA and inhibits Rac1 in cultured endothelial cells, causing pulmonary endothelial barrier dysfunction, cell motility defects and abnormal angiogenesis in vitro and in vivo (Wojciak-Stothard et al. 2005, 2007, 2009). ADMA-induced RhoA activation in PAECs results from a decrease in NO/cyclic guanine monophosphate/protein kinase G (PKG) signalling and reduced levels of Ser188-phosphorylated RhoA, whereas Rac1 downregulation is associated with reduced phosphorylation of the PKG substrate scaffolding protein, vasodilator-stimulated phosphoprotein (VASP). Phosphorylation of RhoA on Ser188 weakens membrane binding because of electrostatic repulsion with negatively charged phospholipids and also results in an increased affinity for Rho-GDIs. Rho-GDI is sequestered in the cytosol, disabling downstream signalling (Lang et al. 1996; Sauzeau et al. 2000). Phosphorylation additionally plays a role in regulating the levels of cellular RhoA by inhibiting ubiquitin-mediated proteasomal degradation (Rolli-Derkinderen et al. 2005). VASP associates with AJ (Vasioukhin and Fuchs 2001) and TJ (Comerford et al. 2002) and links intercellular junction proteins with the actin cytoskeleton (Krause et al. 2003). Mice lacking proteins of the VASP family consistently die from oedema formation attributable to defective vascular barrier function (Furman et al. 2007). VASP-deficient endothelial cells show increased permeability and reduced Rac1 activity under basal conditions (Schlegel et al. 2008). Although the way that the NO/PKG-induced phosphorylation of VASP activates Rac1 is not fully understood, interactions of VASP with Rac1 regulatory proteins such as p120Ras GAP, GTP exchange factors for Rac1 or the TJ component ZO-1, are likely to play a role (Comerford et al. 2002; Schlegel et al. 2008).

In addition to the Rho/Rac1-mediated reorganization of the actin cytoskeleton and of AJ, ADMA reduces the expression, activation and membrane localization of a GJ protein, Cx43, in PAECs (Tsang et al. 2014). These changes are associated with decreased expression and phosphorylation of c-jun and increased pulmonary endothelial permeability in vitro and in vivo (Tsang et al. 2014). Interestingly, endothelial-like cells derived from the peripheral blood of patients with idiopathic PAH exhibit abnormal DDAH1/Cx43 signalling and increased permeability in vitro, highlighting the potential importance of this pathway in the disease. Rotigaptide, an antiarrhytmic drug that enhances Cx43 function, prevents ADMA-induced pulmonary endothelial leakage in vitro and in vivo (Tsang et al. 2014). GJ proteins might affect pulmonary endothelial permeability by facilitating the assembly of AJ and TJ (Nagasawa et al. 2006) and/or mediating the exchange of secondary signalling mediators such as Ca^{2+} or cyclic nucleotides between cells in the lung capillary bed (Parthasarathi et al. 2006).

Chronic obstructive pulmonary disease

ROCK is activated by agents known to contribute to the pathogenesis of chronic obstructive pulmonary disease such as inflammatory cytokines (e.g., interleukin-6 [IL-6] and monocyte chemoattractant protein-1) or cigarette smoke (Fukumoto and Shimokawa 2011; Sakai et al. 2011). Cigarette smoke has been shown to induce ET-1 release in a ROS-dependent manner and to cause endothelial barrier dysfunction in vitro by activating the RhoA/ROCK pathway in HPAECs (Milara et al. 2010). These effects are attenuated by endothelin receptor antagonists, antioxidant N-acetylcysteine and ROCK inhibitor Y-27632 (Milara et al. 2010). In contrast, in another study, cigarette smoke has been shown to induce ROS and to increase endothelial permeability by the inhibition of RhoA signalling (Lu et al. 2011). Although the activation of RhoA/ROCK is commonly associated with an increase in endothelial permeability, the inhibition of RhoA can also compromise endothelial barrier function as a baseline level of active RhoA is essential for the maintenance of intercellular junctions (Beckers et al. 2010). ROS activate RhoA in cells by the direct oxidation of two cysteine residues located in the redox-sensitive motif of the protein (Aghajanian et al. 2009) but can also inhibit RhoA by the formation of an intramolecular disulfide bridge that prevents GTP binding (Heo et al. 2006). The differential effects of ROS on RhoA activity have been proposed to depend on ROS levels and on the balance of oxidizing and reducing agents within the cell (Lu et al. 2011). Physiological levels of ROS and a high reduction potential tend directly to oxidize and activate RhoA, whereas high levels of ROS and a low reduction potential tend to inhibit RhoA by the formation of disulfide bridges (Aghajanian et al. 2009; Lu et al. 2011).

ALI and ARDS

Vascular leakage is a hallmark of ALI and ARDS induced by direct or indirect mechanical, toxic, infectious or inflammatory challenges to the lung (Kumar et al. 2009). Several activators of RhoA/ROCK including endotoxin, IL-6, thrombin, ROS and mechanical stress have been implicated in pulmonary endothelial barrier dysfunction and oedema formation in

these conditions (Maniatis and Orfanos 2008). Endotoxin-induced lung oedema in mice can be attenuated by the ROCK inhibitor, Y-27632 (Gorovoy et al. 2007), or by the endothelium-specific over-expression of Cdc42 (Ramchandran et al. 2008). Y-27632 has also been shown to attenuate ALI in septic rats (Cinel et al. 2012). Plasma levels of redox-reactive non-transferrin bound iron can increase under various pathophysiological conditions, including those associated with ALI (Gorbunov et al. 2012). The addition of [Fe^{2+}] increases pulmonary endothelial permeability in vitro; this can also be attenuated by Y-27632 (Gorbunov et al. 2012).

Thrombin, one of the mediators of ALI, increases RhoA activity by activating p115-RhoGEF (Birukova et al. 2004). Fluorescent resonance energy transfer analysis has revealed an initial rise in RhoA activity at the cell periphery, followed by a shift of activity towards the cytosolic F-actin filaments, accompanied by the disruption of junctional integrity and intercellular gap formation (Szulcek et al. 2013).

The effects of thrombin can be attenuated by a number of factors. Factor VIIa, a clotting protease that binds to tissue factor, protects the endothelium from thrombin-induced barrier dysfunction in a Rac1-mediated manner (Sen et al. 2011). Angiopoietin-1 has also been shown to attenuate the effects of thrombin by increasing Rac1 activity, which enforces VE-cadherin organization and reduces RhoA activity in human pulmonary microvascular endothelial cells (van der Heijden et al. 2011).

The activation of RhoA/ROCK by a stretch induced by mechanical ventilation might constitute a "second hit" to Rho-independent lung injury induced by factors such as IL-6 (Birukova et al. 2012b). RhoA/ROCK activation in this model can be attenuated by the prostacyclin analogue, iloprost, which probably acts via the protein-kinase-A-mediated phosphorylation of Rho inhibitor, RhoGDI, or the negative regulation of RhoA by the cAMP/Epac/Rap1/Rac pathway (Birukova et al. 2010).

The mechanochemical environment can significantly affect the severity of ALI/ARDS (Birukov 2009). An 18 % cyclic stretch enhances thrombin-induced Rho activation, whereas a 5 % cyclic stretch promotes Rac activation, critical for the recovery of endothelial barrier function (Shikata et al. 2005; Birukova et al. 2006). Another Rac1-activating agent, hepatocyte growth factor (HGF), has been shown to prevent endothelial barrier dysfunction induced by a cyclic stretch and VEGF (Birukova et al. 2008). A combination of physiological cyclic stretch preconditioning and HGF has been demonstrated to attenuate VEGF-induced barrier dysfunction via the downregulation of the Rho pathway. These results highlight the importance of the mechanochemical environment in the control of Rho GTPase activity and lung endothelial permeability in ALI/ARDS.

The severity of permeability oedema during infection with the Gram-positive bacterium, *Listeria monocytogenes*, correlates with the levels of the cholesterol-binding pore-forming toxin, listeriolysin, which it produces (Rose et al. 2001; Repp et al. 2002; Munder et al. 2005). Listeriolysin-induced permeability is accompanied by an increased ROS generation, RhoA activation and MLC phosphorylation and can be completely inhibited by the protein kinase C (PKC) α/β inhibitor GÖ6976, indicating a crucial role for PKC in the induction of barrier dysfunction. The TNF-derived tonoplast intrinsic protein, which mimics the lectin-like domain of the cytokine, blunts listeriolysin-induced hyperpermeability in vitro, upon inhibiting PKC-α activation, ROS generation and MLC phosphorylation and upon restoring the RhoA/Rac1 balance. These results indicate that the lectin-like domain of TNF has a potential therapeutic value in protection from listeriolysin-induced pulmonary endothelial hyperpermeability (Xiong et al. 2010).

The platelet-derived phospholipid S1P can improve pulmonary endothelial barrier dysfunction in ALI by inducing Rac-dependent rearrangement of cortical actin (Abbasi and Garcia 2013; Dudek et al. 2004; McVerry et al. 2004)). S1P-induced cortical rearrangement of actin involves PAK activation, the phosphorylation and activation of LIM kinase and the subsequent inactivation of actin-severing protein, cofilin (Garcia et al. 2001). The importance of S1P signalling in the regulation of pulmonary vascular permeability has also been demonstrated by Zhao et al. (2009). Paracrine release of S1P by bone-marrow-derived endothelial progenitor cells in co-culture with pulmonary microvascular endothelial cells helps to re-anneal endothelial AJ and prevent lipopolysaccharide (LPS)-induced pulmonary endothelial leakage in vitro and in vivo (Zhao et al. 2009). The protective mechanism involves the activation of Rac1 and Cdc42 (Zhao et al. 2009).

The levels of phospholipid oxidation products, specifically oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine (OxPAPC), derived from lipoproteins and membranes of cells undergoing oxidative stress or apoptosis are increased in inflammatory diseases including atherosclerosis, lung inflammation and tissue injury (Birukova et al. 2012a). Low concentrations of OxPAPC (5–20 mg/ml) enhance pulmonary endothelial barrier function in vitro and in vivo and reduce inflammation in animal models of acute lung injury caused by LPS or mechanical stress (Ma et al. 2004; Nonas et al. 2006, 2008). Protective effects of OxPAPC involve the enhancement of the peripheral actin cytoskeleton and of AJ and TJ mediated by Rac and Rap1 GTPases (Birukova et al. 2007a, 2011).

Plasma levels of adenosine are increased in response to lung injury (Lu et al. 2012). Acutely elevated adenosine has been shown to protect against pulmonary oedema in various animal models of ALI. This effect is thought to be mediated through transporter- and receptor-A2 and to involve the activation of Rac1, possibly via G-protein-coupled receptors (Lu et al. 2012). Whereas acute exposure of the lung to adenosine

is protective, sustained adenosine exposure decreases endothelial cell barrier function, elevates cellular ROS levels and activates p38, c-jun N terminal kinases and RhoA (Lu et al. 2012).

Concluding remarks

Basal activity of Rho GTPases is required for the maintenance of normal pulmonary endothelial barrier function. Pathological activation of Rho GTPases by hypoxia, NO deprivation, inflammatory cytokines or mechanical stress leads to imbalance in the activities of RhoA, Rac1 and Cdc42 and causes profound changes in the structure and function of endothelial AJ, TJ and GJ. Improved understanding of the temporal and spacial activity changes of Rho GTPases in the pulmonary vasculature in response to stress conditions is required for the success of future therapeutic efforts in the treatment of endothelial barrier dysfunction in lung diseases.

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