Role of GTPases in control of microvascular permeability

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Received 26 November 2009; revised 9 March 2010; accepted 12 March 2010; online publish-ahead-of-print 17 March 2010

Inflammatory mediators increase vascular permeability primarily by formation of intercellular gaps between endothelial cells of post-capillary venules. Under these conditions, endothelial cell–cell contacts such as adherens and tight junctions open to allow paracellular fluid passage. Small guanosine triphosphatases (GTPases) from the ras superfamily, primarily Rho GTPases (RhoA, Rac1, Cdc42) or Rap1 are known to regulate cell adhesion, in part by reorganization of the junction-associated cortical actin cytoskeleton. In this review, we will discuss the role of small GTPases for the maintenance of microvascular barrier functions under resting conditions as well as under conditions of increased permeability and their involvement in signalling pathways downstream of both barrier-stabilizing and inflammatory mediators. Rac1 and Cdc42 are the main GTPases required for barrier maintenance and stabilization, whereas RhoA negatively regulates barrier properties under both resting and inflammatory conditions. For Rac1 and RhoA, contrary functions under certain conditions have also been described. However, Rac1-mediated barrier destabilization in microvascular endothelium appears to be largely restricted to conditions of enhanced endothelial cell migration and thus to be more closely related to angiogenesis rather than to inflammation. Recent studies revealed that cAMP signalling, which is well known to be barrier protective, enhances barrier functions in part via Rap1-mediated activation of Rac1 and Cdc42 as well as by inhibition of RhoA. Moreover, barrier-stabilizing mediators directly activate Rac1 and Cdc42 or increase cAMP levels. On the other hand, several barrier-disruptive components appear to increase permeability by reduced formation of cAMP, leading to both inactivation of Rac1 and activation of RhoA.

Keywords

Endothelial barrier • Rho GTPases • Adherens junctions • Tight junctions • cAMP

This article is part of the Spotlight Issue on: Microvascular Permeability

1. Introduction

Impaired endothelial barrier functions are a hallmark of inflammatory and allergic reactions. Especially in sepsis, dysfunction of the microcirculation contributes to multi-organ failure and death. Under inflammatory conditions, microvascular permeability is selectively increased in post-capillary venules due to different receptor expression in various parts of the vascular tree. This aspect is important since several studies on barrier regulation were performed using cultured endothelial cells derived from the macrovasculature which usually do not contribute to the formation of inflammatory oedema and therefore may differ in the mechanisms involved. During the last 50 years, ample evidence has been provided that endothelial intercellular junctions provide the barrier properties of the endothelium and thus endothelial barrier breakdown is caused primarily by disruption of cell junctions, leading to the formation of intercellular gaps. In this scenario, the major contacts involved are endothelial tight junctions and adherens junctions. Endothelial tight junctions consisting of the tetraspan transmembrane proteins claudin and occludin, the latter of which is most prominent in the endothelium of the blood brain barrier, are thought to limit paracellular permeability by sealing the intercellular cleft (Figure 1).

Adherens junctions apparently provide the mechanical strength of intercellular adhesion by homophilic Ca²⁺-dependent trans-interaction of VE-cadherin. The relevance of cell junctions as bona fide targets of inflammatory mediators is supported by observations that increased permeability in response to the bacterial endotoxin lipopolysaccharide (LPS) was associated with the disruption of endothelial tight junctions or that TNF-α-induced endothelial barrier breakdown in vivo can be blocked by a tandem peptide targeting the VE-cadherin adhesive interface. Notably, both tight junctions and adherens junctions are associated with the cortical actin cytoskeleton via several adaptor molecules such as the tight junction components ZO-1, 2, 3 or the catenin family members α-, β-, and γ-catenin, respectively. However, recent studies revealed that, at least for E-cadherin-containing adherens junctions, a stable mechanical linkage between the cadherin–catenin complex and the actin cytoskeleton is absent because β-catenin inhibits binding of α-catenin to actin. Rather, α-catenin appears to be bound by either β-catenin molecules of the cadherin–catenin complex or

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actin filaments, the latter of which may serve to regulate actin dynamics. Nevertheless, association of cell junctions with the actin cytoskeleton may explain why endothelial barrier-stabilizing mediators strengthen the cortical actin cytoskeleton to enhance the stiffness of the cell periphery,\(^1\) whereas perturbation of actin dynamics strongly alters endothelial barrier functions \(\textit{in vivo}\).\(^2\) Because guanosine triphosphatases (GTPases) of the Rho family are involved in the regulation of cell adhesion and cytoskeletal dynamics,\(^3,4\) they have been recognized early as important regulators of endothelial barrier functions.\(^5\) More recently, Rap1 was also found to stabilize barrier functions \(\textit{in vivo}\).\(^6\)

Recent reviews on small GTPases and endothelial permeability elaborated the molecular mechanisms underlying GTPase function and regulation based on reports from different cell types under various conditions.\(^7\) However, at this stage, involvement of comparable mechanisms in endothelial barrier regulation is largely unknown. Therefore, the aim of this review is to focus on the role of small GTPases in control of microvascular paracellular permeability and to evaluate the relevance of these mechanisms \(\textit{in vivo}\).

2. Small Ras-related GTPases involved in barrier regulation

The Ras superfamily of small (21–25 kDa) GTPases is subdivided into five branches, namely Rho, Ras, Rab, Ran, and Arf.\(^8\) To date, 23 members of Rho family GTPases and 36 Ras family members are known.\(^9,10\) This review primarily focuses on the so far best characterized Rho GTPases RhoA, Rac1, and Cdc42 as well as the Ras family GTPase Rap1 and their role in the regulation of endothelial barrier properties. However, it has to be noted that other less characterized GTPases may also influence endothelial permeability as it was shown recently that the Rho family GTPase Wrch-1 regulates tight junctions in epithelial cells.\(^11\)

By cycling between an active GTP-bound and an inactive GDP-loaded state, small GTPases act as molecular switches in a plethora of signalling pathways regulating a wide array of cellular processes. GTP loading renders high affinity for binding to effector molecules via conformational changes in the switch I and switch II GTPase regions. Thus, only in the activated state, small GTPases bind effectors and allow forwarding of upstream signals. Small GTPases possess low intrinsic GTP-hydrolizing activity, and switching between the active and inactive forms is tightly controlled by three protein classes. Guanine nucleotide exchange factors (GEFs) promote the exchange of GDP to GTP, leading to an active GTPase state, whereas GTPase-activating proteins (GAPs) enhance the intrinsic GTPase activity, thus inactivating the protein.\(^12\) Finally, guanine-nucleotide-dissociation inhibitors (GDIs) sequester the GTPase within the cytosol and stabilize the GDP-bound state.\(^13\) Regulation of small GTPase activity in different cell lines is discussed in detail in a recent comprehensive review.\(^14\) In what follows, we briefly mention some of these mechanisms when found to participate in endothelial barrier regulation.

3. Role of the different GTPases in permeability control

3.1 Rac1 and Rap1 are critical for maintenance and stabilization of the endothelial barrier

There is evidence that Rac1, Cdc42, and Rap1 are involved in the maintenance and stabilization of microvascular endothelial barrier functions, whereas RhoA primarily acts antagonistically to impair barrier properties (Figure 1). For the first studies on the role of Rho GTPases in endothelial barrier regulation, toxin B was used to inhibit RhoA, Rac1, and Cdc42 in parallel. Under these conditions, vascular permeability of intact post-capillary venules \(\textit{in vivo}\) as well as monolayer permeability of different cultured micro- and macrovascular endothelial cells \(\textit{in vitro}\) was drastically increased accompanied by typical hallmarks of endothelial barrier breakdown such as intercellular gap formation and fragmentation of VE-cadherin staining.\(^15,16\) Similar results were obtained using lethal toxin (LT) which inhibits Rac1 together with some other members of Ras-related GTPases.
such as Rap1, Ral, and Ras, strongly indicating that Rac1, which was the only GTPase inhibited by both toxin B and LT, is primarily important for the maintenance of endothelial barrier functions under resting conditions.25,27 Because the bacterial toxins were not specific for Rac1, weak concomitant inhibition of Cdc42 as well as possible inactivation of Rap1 may explain the more severe effects of LT compared with toxin B. However, in more refined studies, treatment with NSC-23766, which interferes with the Rac1-specific GEFs Tiam 1 and Trio, also reduced transendothelial resistance (TER) and caused intercellular gap formation.29 Moreover, since these studies on microvascular endothelium are in general agreement with previous studies in which macrovascular endothelial cells were challenged with constitutively inactive Rac1 mutants,29,30 it can be concluded that, under resting conditions, Rac1 serves to promote endothelial barrier properties.

These observations led us to evaluate whether activation of Rho GTPases (RhoA, Rac1, Cdc42) by cytotoxic necrotizing factor 1 (CNF-1) would be effective to stabilize barrier functions in the presence of pathophysiologically relevant inflammatory mediators in vivo. Indeed, CNF-1 blunted both platelet-activating factor (PAF)- and TNF-α-induced permeability increase in post-capillary venules in vivo.31,32 Similarly, as outlined in the following in detail, various barrier-stabilizing mediators as well as the barrier-protective cAMP signalling pathway were found to reduce vascular permeability at least in part via the activation of Rac1. Especially, barrier enhancement induced by the GTPase Rap1 which is activated via the cAMP-regulated GEF Epac1 and effectively stabilizes barrier functions in vivo.33,34 was shown to be dependent on Rac1 activation and to cause Rac1-mediated cytoskeletal reorganization.28,35–37 Thus, besides its role for the maintenance of barrier properties under resting conditions, activation of Rac1 appears to be a suitable approach to protect barrier functions under inflammatory conditions.

### 3.2 Cdc42 is involved in barrier restoration

It has to be noted that Cdc42 may be equally relevant for barrier maintenance and enhancement like Rac1 (Figure 1). As outlined earlier, modulation of Rac1 activity via toxin B or CNF-1 additionally affects Cdc42, making it impossible to ascribe the effects to one of the two GTPases. Similarly, physiological barrier-stabilizing mediators and signalling pathways such as cAMP apparently activate Rac1 together with Cdc42, presumably via the GEF Yav2.28,36,37 Moreover, because the barrier-stabilizing effect of oxidized phospholipids (OxPAPCs) was blocked by siRNA targeting Cdc42,28 and expression of constitutively active Cdc42 blunted LPS-induced lung vascular permeability in vivo,37 it can be assumed that Cdc42 comparably with Rac1 stabilizes endothelial barrier functions. Nevertheless, since most data on Cdc42 in endothelial barrier regulation in vivo were obtained from whole-lung models rather than from single post-capillary venules, conclusions for microvascular permeability regulation may not be easy to interpret.

In addition, Cdc42 appears to be the primarily relevant GTPase for barrier restoration, indicating that Cdc42 has functions different from Rac1. For example, thrombin was shown to cause delayed activation of Cdc42 which closely paralleled the time course of barrier recovery.40 Moreover, transfection with a constitutively inactive Cdc42 mutant substantially interfered with barrier restoration after thrombin challenge in vivo. Since a VE-cadherin mutant incapable to mediate trans-interaction led to the activation of Cdc42 in parallel to increased permeability in vivo,41 it can be assumed that disruption of adherens junctions may be a trigger for Cdc42 activation. However, under the latter conditions, the constitutively inactive Cdc42 mutant blocked the increase in permeability. Thus, the role of Cdc42 in barrier regulation is obviously more complex, and activation of the GTPase may be protective or disruptive dependent on the trigger of barrier breakdown. Comparably, in Drosophila adherens junctions, Cdc42 as part of the polarity complex was shown to be important for E-cadherin endocytosis,42 a mechanism which on first sight would be assumed to reduce adhesion. In contrast, because inhibition of Cdc42 resulted in junctional fragmentation, Cdc42-regulated E-cadherin turnover appears to be a prerequisite for adherens junction stability.

### 3.3 RhoA activation reduces endothelial barrier functions

It has been known for several years that RhoA activation downstream of several permeability-increasing mediators such as thrombin or TNF-α contributes to increased permeability.15 In addition, RhoA also has a negative function in the regulation of barrier maintenance under resting conditions (Figure 1). Inhibition of Rho kinase reduced baseline permeability of both post-capillary venules and different cultured microvascular endothelial cells in vitro,24,25 which is in line with a previous study in which inhibition of RhoA/ROCK by C3 toxin lowered permeability of bovine macrovascular endothelium.43 In contrast, it was reported recently that baseline activity of Rho kinase was required for barrier maintenance.44 However, since this effect in macrovascular endothelium was only detectable after prolonged incubation (24 h) with a pharmacological Rho kinase inhibitor or when both isoforms of Rho kinase were depleted via siRNA, the relevance of these results for the in vivo situation is yet to be determined. Therefore, RhoA appears to primarily have destabilizing effects on endothelial barrier properties. As outlined in what follows, the underlying mechanism is generally thought to be Rho kinase-mediated inactivation of myosin light chain (MLC) phosphatase leading to increased MLC phosphorylation and actin–myosin contractility.29,45,46

### 3.4 Differential roles of Rho GTPases in microvascular and macrovascular endothelium

Inflammatory barrier dysfunction is confined to post-capillary venules, i.e. the endothelium of the microvasculature. Nevertheless, for in vitro studies, a variety of macrovascular endothelial cells was also used which may explain some of the conflicting data on the role of Rac1 in endothelial barrier regulation. In early studies using macrovascular human umbilical vein endothelial cells (HUVECs), a constitutively active mutant of Rac1 increased monolayer permeability,29 which is in contrast to most studies demonstrating a protective effect of Rac1 activation in both microvascular and macrovascular endothelium.28,30,47 Moreover, some studies on macrovascular endothelium found that Rac1 activation may cause barrier destabilization possibly via the generation of reactive oxygen species (ROS),48,49 a signalling pathway which in microvascular endothelium appears to be confined to VEGF-induced cell migration (as discussed in the following). We have shown in a comparative approach that parallel activation of RhoA, Rac1, and Cdc42 by CNF-1 increased permeability in macrovascular pulmonary artery endothelium (PAEC) but stabilized barrier properties of various microvascular cell types.25 Because
simultaneous inhibition of Rho kinase blunted CNF-1-induced barrier breakdown in PAECs, these data indicate that either the protective effects of Rac1 are limited or that RhoA-induced barrier destabilization is even more prominent in microvascular compared with microvascular endothelium. At present, for RhoA, a barrier-destabilizing effect in both microvascular and macrovascular endothelial cells is well established. Thus, in vitro data on RhoGTPases and endothelial barrier regulation should always be interpreted in conjunction with the cellular background and compared with results obtained in vivo.

4. Mechanisms underlying barrier regulation by small GTPases

4.1 Role of contraction and adhesion for endothelial barrier functions

It is conceivable that paracellular permeability may be regulated via a finely tuned balance between intercellular adhesion, on the one hand, which is primarily mediated by adherens junctions and, on the other hand, centripetal contraction exerted by specific components of the actin cytoskeleton. Small GTPases have been demonstrated to act as key players in regulating both events. Actin–myosin contractility is assumed to be the main mechanism of RhoA-mediated barrier destabilization. Indeed, RhoA activation results in the formation of stress fibres composed of filamentous (F) actin and myosin II.50,51 Direct driving force for stress fibre formation is phosphorylation of MLC which promotes interaction of myosin with actin (Figure 1). MLC phosphorylation state is regulated by MLC phosphatase and MLC kinase. Rho kinase inhibits MLC phosphatase and additionally directly targets MLC, both mechanisms leading to increased MLC phosphorylation and thus increased actin–myosin contractility.46,52 Similarly, Rac1 via PAK was shown to enhance MLC phosphorylation in macrovascular endothelial cells.53,54 It is well established that increased permeability in response to various mediators in vitro is paralleled by formation of stress fibres and increased centripetal tension. This is suggestive for a role of actin–myosin contractility in actively pulling membranes of adjacent cells apart, although stress fibres are spanned between basally located focal adhesions. Nevertheless, this model is supported by very recent observations that, in confluent HUVEC monolayers, actin stress fibres may also connect to adherens junctions.55 Moreover, inhibition of the RhoA/Rho kinase pathway via C3 transferase or Rho kinase inhibitor Y-27632 was effective to block thrombin- and VEGF-induced MLC phosphorylation and to protect endothelial barrier functions in both cultured micro- and macrovascular endothelial cells, indicating a crucial role of actin–myosin-based contractility in vitro.28,45–47,56 Likewise, MLC inhibition or inhibition of myosin-mediated contraction largely reduced barrier destabilizing effects induced by Rac1 inactivation in cultured microvascular cells.57 However, the same inhibitors failed to reduce permeability in response to Rac1 inhibition in single perfused post-capillary venules in vivo. Similarly, Y-27632 or MLC inhibition did not prevent increased hydraulic conductivity in response to PAF, bradykinin, or TNF-α in the same setup. In contrast, another study showed that breakdown of endothelial barrier functions in isolated coronary venules following VEGF treatment was blocked by inhibition of Rho kinase.56 Additionally, transfection with a constitutively active Rho kinase was effective to augment baseline permeability. This indicates that, under conditions in which RhoA is activated, Rho kinase may be involved in the mechanisms leading to increased permeability.59 However, since inhibition of Rho kinase was also found to enhance VE-cadherin-mediated adhesion,57 it is possible that loss of intercellular adhesion rather than actin–myosin-based contractility may be responsible under these conditions.

To explain why actin–myosin contractility was found to be of different relevance for endothelial barrier regulation in vivo and in vitro, the interesting hypothesis has been proposed that cultured endothelial cells display a more contractile phenotype compared with intact microvessels and therefore may rather reflect the situation of inflammatory conditions.60 Indeed, given the fact that cultured cells need to migrate and to divide to form a confluent monolayer similar to intact microvessels, it is likely that the cytoskeleton is more susceptible to contractile stimuli and that junctions are more immature. The latter is supported by the notion that cultured endothelial cells have less stable barrier properties than intact microvessels.3 If this holds true, other mechanisms such as endocytosis of junction components (see what follows) may also be different in endothelial cells in vivo and in vitro.

Therefore, since actin–myosin contractility likely contributes to mediator-induced barrier destabilization in vitro but the role of this mechanism for permeability regulation in vivo is unclear at present, it is possible that Rho GTPases regulate microvascular permeability in vivo primarily on the level of cell junctions. This hypothesis is supported by the findings that increased permeability in response to the inhibition of Rac1 was closely paralleled by loss of VE-cadherin binding27,61 and that VE-cadherin-mediated adhesion was enhanced in situations of improved endothelial barrier properties after the stimulation of the cAMP/Epac/Rap1 pathway.62,63 Available data suggest that stability of cell junctions can be regulated either on the level of the cortical junction-associated actin cytoskeleton which also may involve the microtubule network or directly on the level of the cadherin–catenin and tight junction complex.

4.2 Small GTPases regulate endothelial barrier properties via modulation of cortical actin

The cortical actin band spans the entire circumference of endothelial cells and is composed of F-actin bundles which are thought to be associated with adherens and tight junctions (Figure 1).9 Therefore, it is conceivable that strengthening of the cortical actin cytoskeleton could be effective to enhance endothelial barrier properties by promoting stabilization of junctional proteins. Evidence was provided that several GTPases, first of all Rac1 and Cdc42, utilize this mechanism to regulate permeability.

It is well established that under conditions of improved barrier properties, the actin-binding protein cortactin accumulates at cell borders,32,35,64–66 a process which is dependent on Rac1 activity.67,68 Cortactin is a ubiquitously expressed tyrosine kinase target and has been implicated in cortical actin assembly and reorganization.69 Indeed, functional relevance of cortactin for endothelial permeability was revealed by attenuated responses to barrier-protective stimuli following cortactin knockdown.67,70,71 Alternatively, modulation of coflin phosphorylation is another mechanism to regulate cortical actin fibres. Unphosphorylated coflin binds F-actin and promotes filament severing, which destabilizes the cortical actin cytoskeleton.72 In this context, LIM kinase serves as a central regulator of coflin function by phosphorylating and inactivating this protein. In epithelial cells,
Rac1 and Cdc42, via their specific effectors PAK1 and PAK4, activate LIM kinase, which in turn phosphorylates cofillin to stabilize cortical actin.73 At present, direct evidence for the role of LIM kinase for Rac1/Cdc42-mediated endothelial barrier regulation is lacking. Nevertheless, since, in endothelial cells, overexpression of cofillin resulted in attenuated Rac1-mediated barrier enhancement,74 a contribution of the LIM kinase/cofilin pathway in Rac1- and Cdc42-induced barrier stabilization is possible. Similarly, RhoA effector Rho kinase inhibits cofillin-mediated F-actin disassembly by LIM kinase phosphorylation.75 However, this pathway was proposed to lead to barrier destabilization rather than stabilization since, in macrovascular endothelium (HUVECs), downregulation of LIMK under conditions of elevated RhoA activity blocked the permeability increase induced by thrombin-receptor ligation.75 This would be in line with the general idea that RhoA and Rho kinase primarily negatively regulate barrier properties. In contrast to this, RhoA was shown to strengthen endothelial cortical actin via its effector Dia in a profilin-dependent manner.76–78

In summary, although the exact mechanisms involved are not entirely clear at present, it is conceivable that Rho GTPases can effectively stabilize endothelial barrier properties via the strengthening of cortical actin.

4.3 Microtubules regulate GTPase-dependent actin cytoskeleton remodelling

Interestingly, interference with microtubule polymerization by nocodazole was found to activate RhoA and to increase permeability of endothelial cell monolayers, presumably via Rho kinase-induced stress fibre formation.79 In addition, vasoactive agents such as thrombin and TGF-β triggered microtubule disassembly and barrier breakdown which was attenuated by pharmacological microtubule stabilization.80,81 This links microtubule dynamics to RhoA-mediated actin–myosin contraction. The microtubule-associated protein GEF-H1 likely plays a central role in this context since it acts as a GEF for RhoA when uncoupled from microtubules and is required for thrombin-induced permeability increase.82,83 Moreover, cAMP elevation has been demonstrated to block TGF-β- and nocodazole-induced microtubule disorganization and permeability increase.84,85 This process may involve the cAMP-activated Rap1-GEF Epac, which was shown to strengthen the cortical actin cytoskeleton in a microtubule-dependent manner.85 Unfortunately, the role of Rac1, which is known to be activated via cAMP signalling, has not been investigated in this context. Nevertheless, a scenario is conceivable in which the microtubule system governs the regulation of RhoGTPases downstream of cAMP, which in turn control actin dynamics and stability of cell junctions. Although this integrative model is attractive, in vivo studies are required to evaluate these in vitro findings.

4.4 Small GTPases may regulate barrier functions on the level of junction complexes

Besides their involvement in cytoskeleton dynamics, Rho GTPases may modify endothelial barrier functions directly on the level of cell junctions.

In epithelial cells IQGAP1 was shown to inhibit α-catenin binding to β-catenin resulting in impaired F-actin-linkage to adherens junctions.86 Therefore, it was proposed that Rac1 and Cdc42 regulate cadherin-mediated cell adhesion by sequestering IQGAP1.87 Although data in endothelial cells are still lacking, it is attractive to assume that barrier-protective effects of Rac1 and Cdc42 are at least in part mediated by this mechanism. Similarly, increased VE-cadherin-mediated adhesion following activation of Rap1 may in part be explained by IQGAP1 binding.

Alternatively, endothelial permeability appears to be regulated by phosphorylation and endocytosis of cadherin–catenin complex components. Phosphorylation of VE-cadherin mainly by Src family kinases has been implicated in loss of cell adhesion and endothelial barrier properties.89 In support of this, downregulation of VE-cadherin-associated vascular endothelial tyrosine phosphatase was shown to increase endothelial permeability.90 Furthermore, several barrier-destabilizing mediators which signal via small GTPases such as VEGF, thrombin, or TNF-α induce tyrosine-phosphorylation of VE-cadherin, β-catenin, or p120-catenin.91 The amount of phosphorylation under resting conditions, however, seems to be dependent on junction maturity and declines in confluent monolayers of cultured endothelial cells.92 This aspect may be important to interpret data, which led to the hypothesis that VEGF increases vascular permeability by Rac1-induced phosphorylation and endocytosis of VE-cadherin. It has been shown in cultured macrovascular endothelial cells that, in response to VEGF, Rac1 via PAK induces phosphorylation of VE-cadherin, which in turn leads to β-arrestin recruitment and finally junctional complex endocytosis and increased permeability.93 Under these conditions, Rac1 was activated via the GEF Vav2 in an Src-dependent manner. Additionally, other studies suggested Vav2/Rac1-dependent VE-cadherin phosphorylation and permeability increase via the formation of ROS.94,95 These results are in sharp contrast to other studies showing Rac1-mediated barrier enhancement as discussed earlier. Two potential explanations may reconcile these conflicting findings. First, under conditions in which Rac1 is activated by CNF-1 or downstream of increased cAMP and OxPAPCs, it is possible that Cdc42, which was found to be activated in parallel, may counteract a potentially destabilizing effect of Rac1. Alternatively, as discussed earlier, mechanisms regulating permeability in cultured endothelial cells may differ from the situation in vivo. Therefore, since data from in vivo studies are lacking, it is possible that VEGF, which is an important regulator of endothelial cell migration during angiogenesis, may increase permeability in cultured cells via mechanisms that are not relevant for inflammatory barrier regulation in intact microvessels. More detailed in vitro/in vivo studies are needed to clarify the role of Rac1-dependent VE-cadherin phosphorylation in the regulation of endothelial barrier properties.

Because tight junctions are supposed to be the major cell contact responsible for sealing the intercellular space, it is conceivable that barrier-modulating effects of small GTPases may also target tight junctions. Indeed, a role of GTPases in the regulation of tight junctions is well established for epithelial cells.13 Similarly, in cultured endothelial cells, overexpression of either constitutively active or inactive Rac1 mutants altered localization of the tight junction adapter protein ZO-1.24 Additionally, ZO-1 or claudin5 staining was reduced following treatment with thrombin, histamine, or LPS, i.e. mediators considered to target Rho GTPases.72 Binding of Rho GEF GEF-H1 to the tight junction complex via the adapter cingulin may indicate a direct regulatory role of GTPases, especially since it has been shown that GEF-H1 overexpression increases permeability77 and downregulation attenuates barrier destabilization in response to thrombin treatment.83 However, since it has been recently reported that VE-cadherin regulates claudin5 expression and formation of
5. Regulation of GTPase function

5.1 Nascent junctions and mechanical forces modulate GTPase activity

As discussed earlier, small GTPases function as regulators of cadherin-mediated adhesion and thereby may influence endothelial barrier properties. Vice versa, it is also well known that establishment of cadherin binding induces alterations in the activity of small GTPases in an ‘outside-in’ fashion. Compared with VE-cadherin-deficient endothelial cells, VE-cadherin-reconstituted endothelium showed increased Rac1 activity, whereas activity of RhoA was overall reduced. Similarly, levels of active Cdc42 were also elevated in VE-cadherin-overexpressed endothelial cells. For Rap1, the situation in endothelial cells is unclear at present. At least in epithelial cells, Rap1 is activated by E-cadherin binding and likely plays a role in Cdc42-dependent recruitment of E-cadherin to newly forming junctions. However, although Rap1-mediated effects on endothelial junctions and barrier properties are well established, regulation of Rap1 downstream of VE-cadherin remains to be elucidated.

Interestingly, wildtype VE-cadherin was necessary to induce Cdc42 activation, whereas Rac1 activity was also increased after transfection of mutants lacking the extracellular or the β-catenin-binding domain. Thus, it is not entirely clear which mechanism triggers activation of these GTPases. It is possible that these changes are primarily mediated by p120-catenin, which binds to the juxtamembranous domain of classical cadherins, and was demonstrated to inhibit RhoA and activate Rac1 and Cdc42, respectively. Furthermore, it is likely that GEFs such as Tiam1 or Vav2 participate in the activation of Rac1 and Cdc42 following cadherin ligation. Since VE-cadherin binding appears to activate Rac1 and Cdc42, which in turn are required for adherens junction stability and maintenance of barrier properties, it is conceivable that a positive signalling loop exists which is governed by endothelial adherens junctions. Therefore, inflammatory interruption of this stabilizing loop may be sufficient to cause endothelial barrier breakdown. This aspect may be important since the relevance of destabilizing mechanisms such as actin–myosin-based contractility and endocytosis of junction components for barrier regulation in vivo remains to be elucidated.

As reviewed recently, mechanical forces to which endothelial cells are exposed, such as shear stress or cyclic stretch, were also found to regulate the activity of Rho GTPases and endothelial barrier properties. Shear stress and low amounts of cyclic stretch have been demonstrated to activate Rac1 and to promote recovery of endothelial cells challenged with thrombin, whereas pathological cyclic stretch activates RhoA and further aggravates the thrombin response. By the means of FRET, shear stress-induced Rac1 activation was shown to occur in the direction of flow in an integrin-dependent manner. In contrast, the contribution of intercellular junction molecules to force-dependent changes in GTPase activity remains to be investigated.

5.2 Regulation of small GTPases by barrier-stabilizing mediators

During the last few years, studies from different groups have shown that several barrier-stabilizing mediators activate Rac1 either directly or via increase of intracellular cAMP (Figure 2). This ties the Rac1 pathway to the mechanisms downstream of one of the most potent barrier-protective signalling molecules.

In fact, there is ample evidence that increased endothelial cAMP levels reduce both baseline permeability and the permeability increase in response to inflammatory mediators. This effect of cAMP may be dependent on its generation in specific cellular compartments because predominant increase of membrane-localized cAMP...
strenthened endothelial barrier, whereas cAMP increase by a cytosolically localized adenyl cyclase variant actually destabilizes barrier properties. Nevertheless, permeability increase induced by LT-mediated Rac1 inhibition in post-capillary venules and intercellular gap formation in cultured microvascular endothelial cells were completely blocked when cAMP was increased by combined treatment with forskolin and rolipram. Because this approach also interfered with LT-mediated Rac1 glucosylation, we concluded that CAMP signalling participates in the regulation of Rac1. In line with this, in microvascular endothelium, cAMP was found to activate Rac1, to induce translocation of cortactin to cell borders, and to strengthen the cortical actin cytoskeleton comparable with direct Rac1 activation by CNF-1. O-Me-cAMP, which preferentially triggers Epac/Rap1-mediated CAMP signalling in the presence of NSC-23766 to inhibit Rac1 activation, failed to increase endothelial TER, suggesting that CAMP via Rap1 stabilizes endothelial barrier functions in an Rac1-dependent manner. Moreover, since O-Me-cAMP-mediated Rac1 activation was reduced in microvascular endothelium deficient for vasodilator-stimulated phosphoprotein (VASP), a CAMP/Epac/Rap1/VASP/Rac1 signalling axis appears to exist in microvascular endothelium, which stabilizes endothelial barrier properties. These studies are in support of previous work on macrovascular cells showing that Epac/Rap1 signalling enhanced the endothelial barrier via strengthening of cortical actin and VE-cadherin binding, although activation of Rac1 was not detected to be the key event in these studies.

Similarly, physiological mediators such as prostaglandins (PGE₂, PGI₂) as well as atrial natriuretic peptide (ANP) were shown to support endothelial barrier enhancement by increasing cAMP followed by Epac/Rap1-mediated activation of Rac1 and Cdc42 via the GEFs Tiam1 and Vav2 in vitro and to blunt ventilator-induced lung injury in vivo. Other substances, for example hepatocyte growth factor (HGF) or OxPAPC, which appear under pathological conditions in circulation, are similarly effective in vivo but activate Rac1 and Cdc42 directly via Tiam1 without increasing cAMP. Most recently, Akt-mediated transactivation of the sphingosine 1-phosphate receptor (S1P₁) was found to be involved in this process, supporting previous studies which demonstrated that S1P enhances barrier properties via Rac1 activation. Taken together, all these studies demonstrate that Rac1, in part downstream of increased cAMP, is barrier-protective and involved in barrier enhancement under physiological and pathophysiological conditions.

It has to be noticed that barrier protection may not exclusively be attributed to CAMP but also to cGMP signalling. Recently, in macrovascular cells, barrier destabilization by asymmetric dimethylarginine was demonstrated to be caused by cGMP-dependent Rac1 activation. On the other hand, cGMP signalling has been shown to be important to increase permeability in response to various agonists in vivo. Thus, the role of cGMP in barrier regulation appears to be complex, which is further underscored by the notion that cGMP may activate or inhibit specific isoforms of phosphodiesterases, leading to either permeability increase or decrease.

5.3 Crosstalk of Rac1 and RhōA in endothelial barrier regulation

Interestingly, in many conditions in which cAMP increase and Rac1 activation were found to cause barrier enhancement, activity of RhōA was reduced. For example, HGF and ANP via Rac1 blocked thrombin-induced RhōA activation by interference of RhōA interaction with p115RhōGEP. Moreover, data from fibroblasts suggest that Rac1 inhibits RhōA via p190RhōGAP. Similarly, increased cAMP blocked thrombin-mediated RhōA activation without reducing basal RhōA activity and abrogated thrombin-induced endothelial barrier breakdown in an Rac1-dependent manner. These findings support the hypothesis that a hierarchy exists in which Rac1 controls the activity of RhōA. Direct cAMP-mediated inhibition of RhōA via PKA, which would be expected to reduce basal RhōA activity as well, may be less important. Accordingly, in macrovascular endothelial cells, hypoxia was shown to cause inactivation of Rac1 followed by activation of RhōA. Whether inactivation of Rac1 is sufficient for activation of RhōA or whether other mechanisms such as receptor-mediated activation of RhōA are also required needs to be clarified.
MLC phosphorylation. However, as discussed earlier, the relevance in vivo of this pathway for barrier impairment remains elusive.

Figure 3 Involvement of cAMP and small GTPases in signalling induced by barrier-compromising mediators. The barrier-protective cAMP/Rac1 signalling pathway is targeted by LPS, TNF-α, and thrombin. The former two lead to delayed activation of RhoA, suggesting an aggravating role of RhoA which may be downstream of Rac1. Thrombin leads to impaired cAMP signalling following intracellular Ca²⁺ increase which causes both Rac1 inactivation and activation of RhoA (for example via p115RhoGEF). In contrast, VEGF may increase permeability by Rac1 activation and mechanisms that may involve ROS generation and VE-cadherin endocytosis. Alternatively, activation of the RhoA/Rho kinase pathway was proposed for VEGF-induced barrier breakdown.

thrombin-induced inactivation of Rac1 in a cAMP-dependent manner. Interestingly, thrombin-induced cAMP decrease was mediated by rapid PAR-1-induced Ca²⁺ influx and AC6 inhibition. Similar to RhoA and Rho kinase inhibition, increased cAMP completely prevented thrombin-mediated barrier breakdown but was only effective when Rac1 activation was not blocked by NSC-23766. These data can be reconciled by a mechanism in which Rac1 controls RhoA activity as outlined earlier (Figure 3). In addition, RhoA may promote a sustained thrombin-induced Ca²⁺ influx via Ca²⁺ channels of the TRPC family, thereby enhancing endothelial permeability increase.

Various other inflammatory mediators such as VEGF, PAF, histamin, and bradykinin augment intracellular Ca²⁺ to increase permeability in vivo. In addition, for VEGF, participation of both RhoA and Rac1 signalling was described (Figure 3). In one study, RhoA-mediated increase of permeability was observed in intact coronary venules in vivo and in vitro. Others found that RhoA was not required in this process but, based on studies carried out in cultured endothelium only, argued for a central role for Rac1 activation, finally leading to VE-cadherin endocytosis or PAK- and Erk-dependent MLC phosphorylation. However, as discussed earlier, the relevance of these mechanisms for inflammatory endothelial barrier regulation in vivo is difficult to interpret.

Similarly, a recent study suggested that activation of Rac1 may be responsible for PAF-induced permeability increase in vitro because Tiam1-dependent Rac1 activation following PAF treatment was detected under conditions of increased permeability. Unfortunately, the relevance of this pathway for barrier impairment remains unclear because barrier functions under conditions of reduced Tiam1 expression were not evaluated. Alternatively, it is possible that Rac1 activation might be part of the mechanisms required for barrier restoration. This is more likely because cAMP signalling via Epac/Rap1 as well as Rac1 activation blunted the PAF-mediated permeability response in vivo. These data suggest an involvement of the cAMP/Rap1/Rac1 axis in PAF-mediated barrier breakdown. This principle is also conceivable for other inflammatory mediators which, by different mechanisms downstream of intracellular Ca²⁺ signalling, appear to reduce cAMP levels. However, contribution of Rho GTPases for permeability regulation as a general mechanism remains elusive.

6. Outlook: therapeutic implications

The advanced understanding of endothelial barrier regulation in recent years may enable to improve the therapeutic approaches to plug leaky endothelium in inflammation. In principle, direct targeting of Rho GTPases to alter Rac1- or RhoA-mediated signalling would be a desirable approach. In this respect, the Rho kinase inhibitor fasudil (HA-1077) is in clinical use for the treatment of a number of vascular diseases but not for the stabilization of endothelial barrier functions under inflammatory conditions. Given the important role of cAMP-dependent Rac1 activation for the stabilization of the endothelial barrier and the absence of specific agents to directly modulate function of Rho GTPases, the possibility to target Rho GTPases by increasing endothelial cAMP levels should be evaluated. In support of this hypothesis, cAMP-increasing agents in sepsis were shown to have protective effects on the microcirculation in clinical studies.

Acknowledgements
We are grateful to Michael Christof for figure illustration.

Conflict of interest. none declared.

Funding
The studies from our laboratory were supported by grants from Deutsche Forschungsgemeinschaft (SFB 688, TP A4 and SFB 487, TP B5) and the University of Würzburg (IZKF Z3/2).

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