

Phosphorylation at endothelial cell–cell junctions: Implications for VE-cadherin function

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Abstract: Endothelial cell–cell junctions are strictly regulated in order to control the barrier function of endothelium. Vascular endothelial (VE)-cadherin is one of the proteins that is crucial in this process. It has been reported that phosphorylation events control the function of VE-cadherin. This review summarizes the role of VE-cadherin phosphorylation in the regulation of endothelial cell–cell junctions and highlights how this affects vascular permeability and leukocyte extravasation.

Keywords: endothelium, VE-cadherin, junctions, barrier, phosphorylation

Introduction

The vascular endothelium is the inner lining of blood vessels and forms a physical barrier between the vessel lumen and surrounding tissue; controlling the extravasation of fluids, plasma proteins and leukocytes. Changes in the permeability of the endothelium are tightly regulated. Under basal physiological conditions, there is a continuous transfer of substances across the capillary beds.¹ In addition the endothelium can mediate inducible, transient hyperpermeability in response to stimulation with inflammatory mediators, which takes place primarily in postcapillary venules. However, when severe, inflammation may result in dysfunction of the endothelial barrier in various parts of the vascular tree, including large veins, arterioles and capillaries.^{1,2} Dysregulated permeability is observed in various pathological conditions, such as tumor-induced angiogenesis, cerebrovascular accident and atherosclerosis. Two fundamentally different pathways regulate endothelial permeability, the transcellular and paracellular pathways. Solutes and cells can pass through the body of endothelial cells via the transcellular pathway, which includes vesicular transport systems, fenestrae, and biochemical transporters.³ The paracellular route is controlled by the coordinated opening and closing of endothelial junctions and thereby regulates traffic across the intercellular spaces between endothelial cells.^{4,5}

Endothelial cells are connected by tight, gap and adherens junctions, of which the latter, and particularly the adherens junction component, vascular endothelial (VE)-cadherin, are of central importance for the initiation and stabilization of cell–cell contacts.^{6–8} Although multiple adhesion molecules are localized at endothelial junctions, blocking the adhesive function of VE-cadherin using antibodies is sufficient to disrupt endothelial junctions and to increase endothelial monolayer permeability both *in vitro* and *in vivo*.^{9–11} Like other cadherins, VE-cadherin mediates adhesion via homophilic, calcium-dependent interactions.⁶

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This cell–cell adhesion is strengthened by binding of cytoplasmic proteins, the catenins, to the C-terminus of VE-cadherin.¹² VE-cadherin can directly bind β -catenin and plakoglobin, which both associate with the actin binding protein α -catenin.¹³ Initially, α -catenin was thought to directly anchor cadherins to the actin cytoskeleton, but recently it became clear that α -catenin cannot bind to both β -catenin and actin simultaneously.^{14,15} Data using purified proteins show that monomeric α -catenin binds strongly to cadherin-bound β -catenin; in contrast to the dimer which has a higher affinity for actin filaments, indicating that α -catenin might function as a molecular switch regulating cadherin-mediated cell–cell adhesion and actin assembly.^{14,15} Thus, interactions between the cadherin complex and the actin cytoskeleton are more complex than previously thought. Recently, Takeichi and colleagues reported that the actin binding protein EPLIN (epithelial protein lost in neoplasm) can associate with α -catenin and thereby link the E-cadherin–catenin complex to the actin cytoskeleton.¹⁶ Although this study was performed in epithelial cells, an EPLIN-like molecule might serve as a bridge between the cadherin–catenin complex and the actin cytoskeleton in endothelial cells.

Next to β -catenin and plakoglobin, p120-catenin also binds directly to the intracellular tail of VE-cadherin. Numerous lines of evidence indicate that p120-catenin promotes VE-cadherin surface expression and stability at the plasma membrane.^{17,18} Different models are proposed that describe how p120-catenin regulates cadherin membrane dynamics, including the hypothesis that p120-catenin functions as a ‘cap’ that prevents the interaction of VE-cadherin with the endocytic membrane trafficking machinery.¹⁹ In addition, p120-catenin might regulate VE-cadherin internalization through interactions with small GTPases.¹⁹ Cytoplasmic p120-catenin, which is not bound to VE-cadherin, has been shown to decrease RhoA activity, elevate active Rac1 and Cdc42, and thereby is thought to regulate actin cytoskeleton organization and membrane trafficking.^{20,21}

The intact cadherin–catenin complex is required for proper functioning of the adherens junction. Mutant forms of VE-cadherin which lack either the β -catenin, plakoglobin or p120 binding regions reduce the strength of cell–cell adhesion.^{13,22} Moreover, our own results showed that interfering with the interaction between α -catenin and β -catenin, using a cell-permeable peptide which encodes the binding site in α -catenin for β -catenin, resulted in an increased permeability of the endothelial monolayer.²³ Several mechanisms may be involved in the regulation of the

organization and function of the cadherin–catenin complex, including endocytosis of the complex, VE-cadherin cleavage and actin cytoskeleton reorganization. The remainder of this review primarily focuses on the role of tyrosine phosphorylation in the control of VE-cadherin-mediated cell–cell adhesion.

Regulation of the adhesive function of VE-cadherin by tyrosine phosphorylation

It is a widely accepted concept that tyrosine phosphorylation of components of the VE–cadherin–catenin complex correlates with the weakening of cell–cell adhesion.^{4,24} One of the first reports that supported this idea showed that the level of phosphorylation of VE-cadherin was high in loosely confluent endothelial cells, but low in tightly confluent monolayers, when intercellular junctions are stabilized.²⁵ In addition, several conditions that induce tyrosine phosphorylation of adherens junction components, like v-Src transformation and inhibition of phosphatase activity by pervanadate, have been shown to shift cell–cell adhesion from a strong to a weak state.^{26–28} More physiologically relevant; permeability-increasing agents such as histamine, tumor necrosis factor- α (TNF- α), thrombin, platelet-activating factor (PAF) and vascular endothelial growth factor (VEGF) increase tyrosine phosphorylation of various components of the cadherin–catenin complex.^{29–34}

Although most of the data showing a correlation between tyrosine phosphorylation and the weakening of cell–cell adhesion are obtained using cultured cells, some studies have shown that VE-cadherin can also be phosphorylated *in vivo*. Systemic VEGF administration into healthy, but not in Src kinase-deficient mice, was shown to induce an increased permeability of the vasculature in the heart.³⁵ According to the authors, this increased vascular permeability was associated with higher tyrosine phosphorylation levels of VE-cadherin and β -catenin and a disruption of the VE-cadherin– β -catenin association.³⁵ Others detected phosphorylated VE-cadherin in mouse lung, uterus and to a lesser extent in ovary extracts.³⁶ While VE-cadherin was only weakly phosphorylated in resting vasculature in the female reproductive organs, higher phosphorylation states were detected during hormonally induced angiogenesis.³⁶

A general idea has emerged that tyrosine phosphorylation of the VE-cadherin complex leads to the uncoupling of VE-cadherin from the actin cytoskeleton through dissociation of catenins from the cadherin. However,

partly contradictory results were obtained *in vitro* for the effect of tyrosine phosphorylation on the molecular organization of the VE-cadherin–catenin complex. Cell density-dependent and pervanadate-induced increases in tyrosine phosphorylation were both found to go along with increased binding of p120- and β -catenin to VE-cadherin. These two catenins were largely replaced by plakoglobin when the phosphotyrosine content of VE-cadherin was reduced.²⁵ Increased tyrosine phosphorylation correlated with a reduced binding of p120- and β -catenin to VE-cadherin in response to both thrombin and VEGF stimulation in studies of Rabiet et al³⁴ and Monaghan-Benson et al³⁷ whereas others have reported that the composition of the cadherin–catenin complex remained unaltered during stimulation with various permeability-inducing agents.^{29–31} Potter and colleagues studied the effect of phosphorylation of specific VE-cadherin tyrosine residues on cadherin–catenin complex organization.³⁸ They showed that replacement of the tyrosines 658 and 731 of VE-cadherin by glutamate, which is used to mimic the charge effect of phosphorylation, results in a loss of binding of p120 and β -catenin, respectively. Expression of these mutants of VE-cadherin in Chinese hamster ovary (CHO) cells led to an inhibition of cell barrier function. Interestingly, the authors also showed, (by using immobilized VE-cadherin-Fc fusion proteins) that these mutants did not affect the adhesive function of VE-cadherin. However, based on this study, tyrosine phosphorylation of residues Y658 and Y731 is thought to negatively regulate VE-cadherin-mediated cell–cell adhesion.³⁸ In line with this, phosphorylation of VE-cadherin at both Y658 and Y731 was recently found to be increased in response to VEGF stimulation in human pulmonary microvessel endothelial cells (HMVECs), which was critical for the VEGF-induced vascular permeability.³⁷ A schematic overview of the signals leading to VE-cadherin phosphorylation is shown in Figure 1A.

The functional significance of the Y658 and Y731 residues of VE-cadherin is further underscored by Allingham and colleagues,³⁹ who reported that the adhesion of leukocytes to endothelial cells via intercellular adhesion molecule-1 (ICAM-1) induced VE-cadherin tyrosine phosphorylation at these specific tyrosine residues. In addition, human umbilical vein endothelial cells (HUVECs) overexpressing a mutated form of VE-cadherin, (in which a single tyrosine was substituted for phenylalanine at either residue 658 or 731) showed reduced migration of leukocytes across the endothelial monolayer.³⁹ These findings were extended by a study which showed

that overexpression of p120-catenin resulted in decreased transmigration of leukocytes. The authors suggested that this was caused by a p120-catenin-induced repression of VE-cadherin phosphorylation at Y658,⁴⁰ -the residue corresponding to the p120-catenin binding site.³⁸ In a similar study, expression of nonphosphorylatable mutants of VE-cadherin for either tyrosine 645, 731 or 733 in VE-cadherin-null endothelial cells resulted in less efficient transendothelial migration (TEM) of lymphocytes compared to conditions when wild-type VE cadherin was re-expressed.⁴¹ In contrast to the reports of Allingham et al³⁹ and Alcaide et al,⁴⁰ these authors demonstrated that the expression of the VE-cadherin construct with a single point mutation in Y658 did not have any effect on lymphocyte TEM. These discrepancies concerning the importance of different tyrosine residues for TEM might, in part, reflect differences in experimental conditions, such as different types of endothelial cells (HUVEC versus microvascular endothelial cells (MVEC) and VE-cadherin-null EC) and leukocytes (neutrophils versus lymphocytes). However, the results demonstrate that tyrosine phosphorylation of VE-cadherin is required for efficient transmigration of leukocytes. This suggests that VE-cadherin-mediated cell–cell contacts are not just pushed open by the migrating leukocytes, but play a more active role in the transmigration process. A schematic overview of leukocyte adhesion-induced signals leading to VE-cadherin phosphorylation is shown in Figure 1B.

Tyrosine phosphorylation of VE-cadherin-associated catenins

Changes in VE-cadherin-mediated cell–cell adhesion are often not only correlated with alterations in tyrosine phosphorylation of the cadherin itself, but of VE-cadherin-associated catenins as well. β -catenin, p120-catenin and plakoglobin can be tyrosine phosphorylated by the same permeability-increasing agents that induce VE-cadherin phosphorylation.^{29–31} Therefore, catenin phosphorylation is also generally associated with the disruption of cell–cell junctions; however, the direct relationship between phosphorylation of the catenins and reduced VE-cadherin-mediated cell–cell adhesion has remained elusive. Increased tyrosine phosphorylation of catenins has been correlated with a reduction in their affinity for the cadherin cytoplasmic tail,^{34,37} while other data indicate that phosphorylation of catenins on the tyrosines did not result in disassembly of the cadherin–catenin complex.^{25,29–31} However, in these studies phosphorylation of the catenins was induced in parallel with the phosphorylation of VE-cadherin. Therefore, it is difficult to conclude that; even if there was a

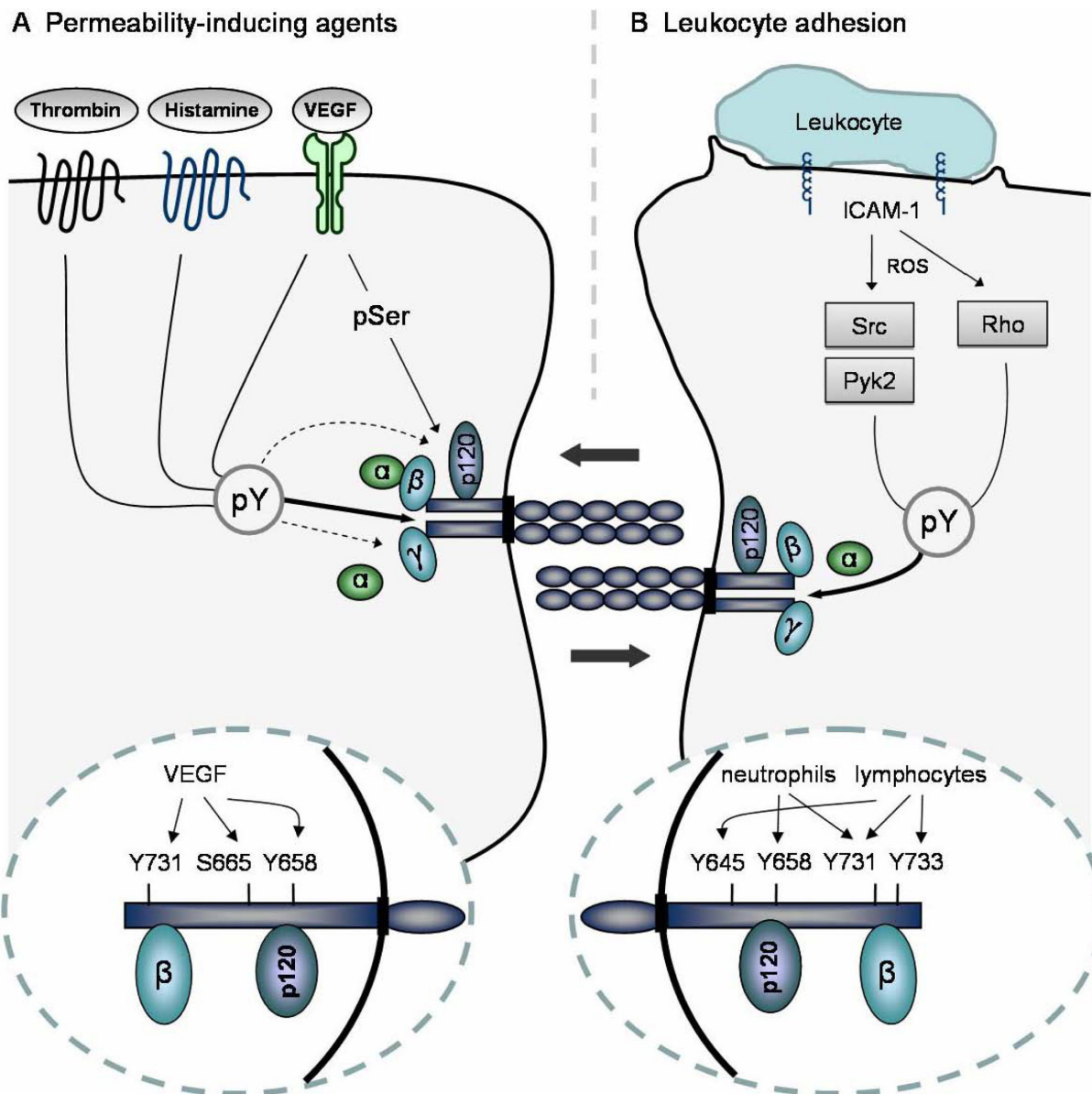


Figure 1 Regulation of the integrity of endothelial cell–cell contacts by phosphorylation of VE-cadherin.

Notes: **A)** Permeability-inducing agents such as thrombin, histamine and VEGF, induce tyrosine phosphorylation (pY) of VE-cadherin and the associated catenins. Although the specific consequences of catenin tyrosine phosphorylation in endothelial cells are still unknown, VE-cadherin tyrosine phosphorylation results in opening of the cell–cell junctions (indicated by arrows) and enhanced vascular permeability. How tyrosine phosphorylation affects VE-cadherin adhesiveness is not yet well understood; disrupted binding of catenins, which link the cadherin to the actin cytoskeleton, may be involved. VEGF induces phosphorylation of VE-cadherin at specific residues, Y658 and Y731, which have been reported to regulate p120-catenin and β -catenin binding, respectively. Moreover, VEGF stimulation results in serine phosphorylation (pSer) of VE-cadherin, specifically at residue S665, which leads to its endocytosis. **B)** Adhesion of leukocytes to endothelial cells via ICAM-1 increases endothelial permeability by inducing phosphorylation of VE-cadherin on tyrosine residues. Essential mediators, such as the kinases Pyk2 and Src, and signaling routes involving reactive oxygen species (ROS) and Rho, have been shown to act downstream of ICAM-1. Different tyrosine residues within the cytoplasmic domain of VE-cadherin are involved in the extravasation of neutrophils and lymphocytes, including Y658 and Y731. (β : β -catenin, α : α -catenin, γ : γ -catenin/plakoglobin).

dissociation of catenins from VE-cadherin, phosphorylation of catenins is the initiating event.

The consequences of catenin phosphorylation on E-cadherin function have been studied in more detail. Overall, most of the studies in epithelial cells suggest that tyrosine phosphorylation of β -catenin results in its dissociation from E-cadherin.^{42–45} Several specific tyrosine residues in β -catenin

that are relevant for the regulation of cell–cell contacts are identified. The binding of β -catenin to E-cadherin was found to be disrupted by phosphorylation of β -catenin on Y489 or Y654.^{43,44} In addition, phosphorylation of β -catenin on Y142 negatively regulates binding to α -catenin.^{46,47} For p120-catenin, more contradictory studies have been published.⁴⁸ Phosphorylation of the p120-Y217 residue has

been reported to negatively regulate E-cadherin function, since v-Src kinase induced effects on cadherin function in L-cells were partially reversed by the mutation of this tyrosine residue to phenylalanine.⁴⁹ In a similar study in A431 cells, expression of a non-phosphorylatable p120-catenin construct, in which seven other tyrosine residues were mutated, did not affect E-cadherin adhesiveness.⁵⁰ Interestingly, several studies even demonstrate that tyrosine phosphorylation of p120-catenin correlates with an increased affinity for E-cadherin.^{27,44,47,51} These contrasting results may reflect differences in experimental conditions; eg, the data were obtained in different cell lines. Moreover, the discrepancies might also be the result of a complex regulation of p120-catenin phosphorylation, in which phosphorylation of specific tyrosine residues of p120-catenin may have varying effects on the association of p120-catenin with the cadherin.

In conclusion, the precise consequences of catenin phosphorylation for vascular permeability are still unknown; however, the data from studies using epithelial cells thus far suggest that tyrosine phosphorylation of catenins can influence the adhesiveness of E-cadherin. While regulation of the affinity between cadherins and catenins by tyrosine phosphorylation can of course differ, depending on cell type and cadherin specificities, these data suggest that tyrosine phosphorylation of catenins may also play an important role in regulation of VE-cadherin-mediated cell–cell adhesion.

Regulation of VE-cadherin function by serine phosphorylation

In addition to tyrosine phosphorylation, serine phosphorylation of VE-cadherin has been reported to be involved in the regulation of the endothelial barrier function.⁵² Phosphorylation of a specific serine residue within the intracellular domain of VE-cadherin, Ser665, leads to the endocytosis of VE-cadherin.⁵² Phosphorylation of residue Ser665 was induced upon VEGF stimulation, through a signaling pathway that involves the Src-dependent activation of the guanine exchange factor (GEF) Vav2 and the consequent activation of Rac1. Subsequently, phosphorylation of VE-cadherin at the serine residue recruits β -arrestin 2, thereby promoting clathrin-dependent internalization of VE-cadherin. Interestingly, the Ser665 site is adjacent to the binding region of p120-catenin, which has been shown to stabilize VE-cadherin expression at the plasma membrane by preventing its clathrin-mediated endocytosis.^{52,53} Therefore, Gavard and Gutkind suggest that the association of p120-catenin with VE-cadherin

may regulate VE-cadherin phosphorylation at the serine residue and its interaction with β -arrestin 2.⁵² Thus, serine phosphorylation and tyrosine phosphorylation may act in concert to coordinate the dynamic disassembly and reassembly of adherens junctions (Figure 1A).

VE-cadherin regulation by protein tyrosine phosphatases (PTP)

Phosphorylation of adherens junctions is regulated by a dynamic balance between the activity of kinases and phosphatases.⁵⁴ Data from our studies, and others, show that Src-kinase and proline-rich tyrosine kinase (Pyk) 2 are both able to increase tyrosine phosphorylation levels of junctional proteins, including VE-cadherin and β -catenin, resulting in weakening of the endothelial junctions.^{35,39,55} In addition, the reduced activity of tyrosine phosphatases may promote tyrosine phosphorylation.⁵⁶ Several phosphatases have been described to play an important role in the regulation of VE-cadherin function.

Density-enhanced phosphatase-1 (DEP-1)/CD148 regulates VE-cadherin-mediated cell–cell contacts through the VEGF receptor 2 (VEGFR-2).⁵⁷ In epithelial cells, DEP-1 is involved in the maintenance of the barrier function through the regulation of tight junctions.⁵⁸ The vascular endothelial protein tyrosine phosphatase (VE-PTP) interacts with the extracellular domain of VE-cadherin and supports VE-cadherin function in protecting endothelial integrity.^{59,60} Interestingly, leukocyte adhesion or VEGF treatment induces a rapid dissociation of VE-PTP from VE-cadherin, followed by an increased leukocyte migration across the endothelial monolayer and increased permeability. The dissociation of VE-PTP from VE-cadherin results in increased levels of tyrosine phosphorylation of not only VE-cadherin, but also of β -catenin and plakoglobin. Again, these studies show that tyrosine phosphorylation of VE-cadherin results in an inhibition of endothelial barrier function.

The SH2 domain-containing protein tyrosine phosphatase 2 (SHP2) has been implicated in the regulation of endothelial cell–cell junctions upon thrombin stimulation.⁶¹ According to the authors, thrombin stimulation results in a dissociation of SHP2 from β -catenin. This would result in increased phosphotyrosine levels of β -catenin, followed by a dissociation of β -catenin from VE-cadherin and a loss of barrier function. Thus, just as several tyrosine kinases are involved in the regulation of VE-cadherin phosphorylation, different phosphatases are important as well. This suggests that various signaling

pathways exist that control VE-cadherin phosphorylation and endothelial barrier integrity.

ROS-mediated VE-cadherin phosphorylation

Although different protein phosphatases and kinases regulate VE-cadherin function by affecting its phosphorylation, it is less clear how these proteins are activated or triggered.

Several studies have shown that reactive oxygen species (ROS) can effectively inhibit phosphatase activity.⁶² By oxidation of crucial cysteine residues, the phosphatases lose their active conformation and return to their inactive state.^{56,63,64} ROS are also generated in endothelial cells and can serve as potential signaling mediators.⁶⁵ Introducing active mutants of Rac1 increases the production of ROS in endothelial cells.⁶⁶ This results in a loss of VE-cadherin-mediated cell–cell junctions and an increase in the permeability of the endothelial monolayer. Moreover, disrupting VE-cadherin-mediated cell–cell junctions by treating the endothelial monolayer with an anti-VE-cadherin antibody also induced the production of ROS.⁵⁵ Detailed analysis of this action by high resolution confocal microscopy showed that ROS production precedes the loss of cell–cell contacts. The induction of ROS by antibody-induced loss of VE-cadherin-mediated cell–cell contacts increased the tyrosine phosphorylation levels of Pyk2. The activation of this kinase has been shown to be redox-sensitive.⁶⁷ In addition, β -catenin tyrosine phosphorylation was increased upon the loss of VE-cadherin-mediated cell–cell junctions, which was prevented by the overexpression of a dominant negative mutant of Pyk2.⁵⁵ In line with this, the dominant, negative mutant of Pyk2 prevented the antibody-induced loss of VE-cadherin-mediated cell–cell contacts. However, these data do not exclude that ROS can directly act on a phosphatase by inhibiting its activity and, as a result, shift the balance to increased

phosphorylation. Interestingly, scavenging ROS resulted in increased barrier function in non-stimulated endothelial monolayers.⁵⁵ In addition, overall tyrosine phosphorylation levels were reduced when ROS was scavenged (JDvB, unpublished results). Thus, Rac1-induced ROS production may indirectly increase kinase activity by inhibiting phosphatases. This hypothesis is recently underscored by a publication by Monaghan-Benson and Burrige. They showed that VEGF leads to a Rac1-mediated generation of ROS, which, in turn, elevates the tyrosine phosphorylation of VE-cadherin and β -catenin, negatively regulating adherens junction integrity.³⁷ It is interesting to note that Rac1-wt and VE-cadherin co-localize at cell–cell junctions in endothelial cells and therefore are in close proximity to each other (Figure 2). Whether ROS is generated at or near cell–cell junctions is unknown and requires further investigation.

The generation of ROS is controlled by the NADPH-oxidase (NOX) protein complex in many different cell types.⁶⁸ ROS levels in endothelial cells are more than 100-fold less than the levels of neutrophils.⁶⁹ Nevertheless, all major components of the NADPH-complex, including the NADPH-oxidase complex catalytic units NOX2 and NOX4, are expressed in endothelial cells.⁷⁰ Interestingly, Chen and co-workers showed that NOX4 is critical in the regulation of protein tyrosine phosphatase 1B in endothelial cells.⁷¹ Nevertheless, for many pathways that involve the generation of ROS, it is not clear which of these NOX-proteins are responsible for the ROS production in endothelial cells. Future research should clarify this point.

Concluding remarks

VE-cadherin is an endothelial-specific adhesion molecule, which is crucial for the maintenance and control of endothelial cell–cell contacts. Much progress has been

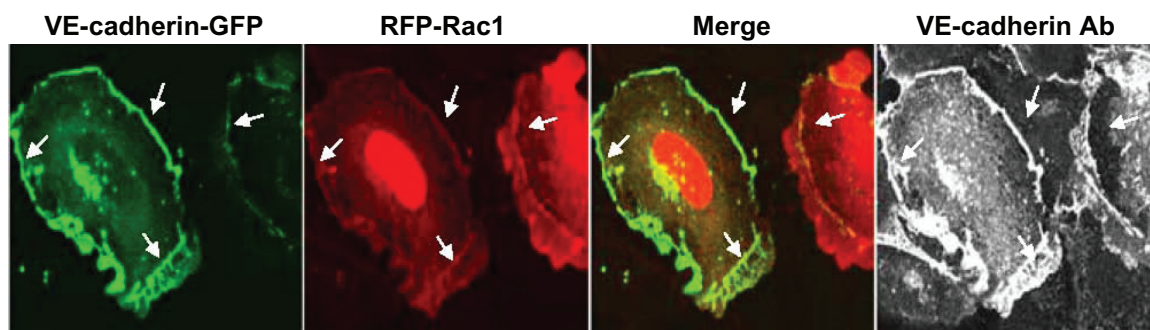


Figure 2 Rac1 co-localizes with VE-cadherin at endothelial cell–cell junctions.

Notes: HUVECs were transiently transfected with VE-cadherin-GFP (green) and RFP-Rac1-wildtype (red). Merge shows the co-localization of the green and red signal. Most right image shows endogenous VE-cadherin at cell–cell junctions (white).

made in our understanding of the molecular mechanisms regulating VE-cadherin function. Recent studies have identified specific tyrosine residues within the cytoplasmic tail of VE-cadherin that regulate vascular permeability and extravasation of leukocytes. Moreover, several kinases and phosphatases were found to be involved in the regulation of the adhesive strength of VE-cadherin. However, further work is required to address important questions such as; how tyrosine phosphorylation of the VE-cadherin-catenin complex leads to changes in the integrity of cell–cell junctions. Furthermore, the relevance of tyrosine phosphorylation of VE-cadherin *in vivo* remains elusive. While VE-cadherin can be phosphorylated in angiogenic and ischemic conditions in mouse tissues,^{35,36} it is still not clear whether VE-cadherin phosphorylation is involved in the basal, dynamic regulation of the endothelial barrier *in vivo* or whether it only plays a role in pathological conditions.

Interestingly, VE-cadherin has been recently shown to indirectly control tight junction organization.⁷² VE-cadherin expression and clustering was demonstrated to be required for transcriptional upregulation of the tight junction adhesive protein claudin-5. This direct positive relationship between adherens junctions and tight junctions may have important functional consequences and may further explain why interfering with only VE-cadherin function has marked effects on junctional organization and vascular permeability in general.

Ultimately, elucidating the molecular mechanisms by which VE-cadherin function is regulated in endothelial cell–cell junctions will help to identify new therapeutic targets for the treatment of many diseases that are accompanied by increased vascular permeability.

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Disclosures

The authors report no conflicts of interest relevant to this research.

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