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Endothelial cell junctions and the regulation of vascular permeability and leukocyte transmigration

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Summary

The endothelial lining of the vasculature forms the physical barrier between the blood and underlying tissues. Junctions between adjacent endothelial cells are dynamically modulated to sustain vascular homeostasis and to support the transendothelial migration of leukocytes during inflammation. A variety of factors initiate intracellular signaling pathways which regulate the opening and resealing of junctional complexes. This review focuses on three primary signaling pathways initiated within endothelial cells by the binding of vasoactive factors and leukocyte adhesion: Rho GTPases, reactive oxygen species, and tyrosine phosphorylation of junctional proteins. These pathways converge to regulate junctional permeability, either by affecting the stability of junctional proteins or by modulating their interactions. Although much progress has been made in understanding the relationships of these pathways, many questions remain to be answered. A full understanding of the signaling cascades that affect endothelial junctions should identify novel therapeutic targets for diseases that involve excessive permeability or inappropriate leukocyte infiltration into tissues.

Keywords

cell-cell junctions; leukocyte transmigration; reactive oxygen species; Rho GTPases; tyrosine phosphorylation; vascular permeability

Introduction

The vascular system is a network of vessels that serve as conduits for the transport of nutrients, macromolecules and blood cells throughout the body. The luminal layer of the blood vessel wall consists of a single sheet of endothelial cells (ECs) which provide the primary physical barrier between the blood circulation and underlying tissues. Cell junctions linking adjacent ECs are important regulators of the permeability characteristics of the endothelial vessel wall and are modulated to allow selective and specific passage of blood cells and macromolecules. Opening and resealing of the junctional barrier must occur during normal physiological processes such as immune surveillance, antigen recognition, and acute inflammatory responses. Conversely, dysregulation of cell junctions can lead to pathological situations, including many chronic inflammatory diseases and edema. As such, the endothelium and its junctions play a critical role in regulating vascular function during both physiological and pathological processes. Vascular permeability is the sum of mechanisms which balanced with mechanisms which stabilize barrier function [1,2]. In this review, we will focus on those

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pathways which mediate reversible, transient increases in permeability due to junctional disruption.

Vascular barrier function is not only differentially modulated in response to physiological stimuli within a given vessel, but additionally, different vascular beds have unique permeability and barrier characteristics due to morphologically heterogeneous endothelial junctions [3]. At two extremes of the continuum are the relatively impermeable endothelium forming the blood brain barrier, and the high endothelial venules which are sites of constitutive leukocyte transendothelial migration (TEM).

Regulation of EC junctions, and by extension, vascular permeability is provided by a variety of signaling cues. These signals function to promote normal homeostasis but are also involved in pathological changes in permeability. In this review we discuss regulation of EC junctions, first in the context of permeability to fluid and small macromolecules; and second how these junctions are modified to allow leukocyte TEM during inflammation. Common pathways responsible for regulation of junctional permeability exist despite differences in the initiating signaling event. These pathways converge to increase junctional permeability by a variety of mechanisms.

1. Endothelial Junctions and the Cytoskeleton

Tight Junctions—Compared to the well-developed and spatially distinct tight junctions (TJs) of epithelial cells, in ECs TJs are less structured and more intermixed with the adherens junctions. Membrane-spanning junctional proteins contain extracellular domains for adhesion via homophilic interactions with adjacent cells, forming a physical barrier. Occludin was the first transmembrane protein to be identified at the TJs of both epithelia and endothelia [4]. Numerous lines of evidence point to a central role for occludin in regulating paracellular permeability of blood vessels [5]. The claudin family of transmembrane proteins are also a major component of TJs. The over 20 proteins which make up the claudin family have a wide distribution, although claudin-5 appears to be specific to ECs [6]. The junctional adhesion molecule (JAM) proteins uniquely contribute to endothelial permeability because they can participate directly in barrier function, but can also act as receptors that interact with leukocyte integrins to regulate and direct leukocyte transmigration. Furthermore, the heterogeneity of EC junctions is reflected by differential expression of specific JAM isoforms (reviewed in Weber et al., 2007) [7]. In addition to these transmembrane proteins, there are also proteins that form a complex on the cytoplasmic face of the TJ, including ZO-1 and ZO-2. These scaffold proteins link the cytoplasmic domains of the transmembrane proteins to the actin cytoskeleton [8], as illustrated in Figure 1.

Adherens Junctions—ECs also contain adherens junctions (AJs), which mediate strong adhesion between adjacent cells predominantly via the transmembrane protein VE-cadherin. The importance of VE-cadherin for barrier maintenance in ECs is underscored by the observation that injection of VE-cadherin function-blocking antibodies induces neutrophil infiltration in an *in vivo* peritonitis model [9], and vascular hyper-permeability in lung interstitium [10]. The intracellular region of VE-cadherin binds to cytoplasmic AJ-associated proteins, particularly the catenins: α-, β-, and p120 catenin. Since their discovery, α- and β-catenin have been considered as critical linkages between cadherin cytoplasmic domains and the actin cytoskeleton. However, the dogma that this is a stable, quaternary interaction has been challenged by experiments showing that α-catenin cannot interact simultaneously with the cadherin/β-catenin complex and F-actin [11], indicating that linkage to actin may require other components [12]. Finally, p120 catenin is important during barrier maintenance not by acting as a link to the actin cytoskeleton, but by regulating the stability of the cadherin complex, maintaining VE-cadherin's surface expression [13].

Actin Cytoskeleton—Many studies have shown that an interaction with the actin cytoskeleton is required for EC junctional integrity to anchor and reinforce junctions in ECs [14,15]. Actin-based structures in the cell periphery (cortical actin bundles) are thought to promote junctional stability and barrier function [15]. Conversely, under conditions of increased permeability and junctional instability, cortical actin bundles become less prominent and induction of cytoplasmic stress fibers occurs. Presumably, tension forces imparted by these stress fibers provide a mechanism for inducing permeability [16]. The formation of these actin structures is largely regulated by Rho family GTPases, as discussed below.

2. Mechanisms Affecting Vascular Permeability

Vasoactive compounds such as thrombin, histamine and vascular endothelial growth factor (VEGF) can regulate endothelial junctions by multiple mechanisms. They can alter the architecture of the endothelial cleft by affecting junctional protein expression, localization and stability. Sometimes this occurs via protein phosphorylation. They may also activate downstream signaling pathways such as the production of reactive oxygen species (ROS) and alter the activity of Rho and Rac small GTPases, leading to changes impacting the cytoskeleton.

Expression and Stability of Junctional Proteins—There are several examples where loss of a junctional protein has been demonstrated to negatively impact barrier function. For example, the TJ protein claudin-5 leaves morphologically normal blood vessels, but they become selectively permeable to small molecules [17]. In certain physiological instances, exposure to vasoactive compounds also causes loss of a junctional protein. For instance, histamine markedly reduces ZO-1 expression in cultured retinal ECs [18]. VEGF increases brain microvascular EC permeability by affecting occludin and ZO-1 localization at TJs, and decreasing levels of occludin expression [19]. In some situations, junctional components may be removed by proteolysis. For example, the cytokine TGF-β upgregulates expression of matrix metalloprotease-9 (MMP-9), which results in subsequent degradation of occludin in retinal ECs [20].

One final illustration of the need for appropriate expression of junction components is provided by the interplay between p120 catenin and VE-cadherin stability and surface expression. Xiao and colleagues demonstrated that p120 catenin is required for stability and regulation of cellular VE-cadherin content [13]. Reducing levels of p120 catenin caused a dramatic dose-dependent reduction of VE-cadherin in the cell. In contrast, overexpression of p120 catenin increased surface expression of VE-cadherin by inhibiting endocytosis and endosomal degradation of cell surface VE-cadherin. Subsequent work confirmed that the interaction of p120 with VE-cadherin is required for the maintenance of endothelial barrier function [21]. This and earlier observations that vasoactive compounds such as thrombin can alter p120 catenin phosphorylation [22], suggest a mechanism linking p120 phosphorylation status, association with VE-cadherin, and stabilization of endothelial barrier function.

Phosphorylation and Dephosphorylation—Phosphorylation is a common post-translational modification for regulating intracellular signaling. The importance of tightly regulating junctional protein phosphorylation is underscored by the observation that certain phosphatases constitutively associate with cell-cell junctions in quiescent ECs, presumably to maintain the low level of phosphorylation required for junctional integrity [22].

Occludin phosphorylation is a major mechanism for regulating TJ permeability. Antonetti and colleagues showed that VEGF induces rapid phosphorylation of occludin and ZO-1 under conditions where permeability was also increased [23]. However, there are also observations that show *in vitro* VEGF treatment leaves junctions morphologically intact, suggesting that VEGF enhances transcellular permeability [24]. Therefore, regulation of EC permeability by

VEGF is complex and possibly involves two or more distinct modes of permeability. Other vasoactive compounds such as histamine and lysophosphatidic acid (LPA) also induce phosphorylation of occludin in EC lines [25]. While these studies linked phosphorylation of occludin with increased vascular permeability, a recent study proposed that occludin dephosphorylation may also increase permeability [26]. This might indicate that phosphorylation at particular residues may either promote or inhibit permeability, and that a basal level of phosphorylation on specific residues may be required for occludin's barrier enhancing role.

Numerous studies have demonstrated the role of tyrosine phosphorylation of adherens complex proteins in the disassembly of junctions [27,28]. The catenins are frequent targets of phosphorylation, which promotes their disassociation from VE-cadherin. ECs lacking the adhesion protein PECAM-1 have elevated levels of serinephosphorylated β-catenin, constitutive association of this pool with activated GSK-3β, and a resulting increase in proteasomal degradation of β-catenin [29]. A model is suggested in which tyrosine phosphorylation of PECAM-1 enables it to act as a scaffold, promoting recruitment of both the phosphatase SHP-2 and phosphorylated β-catenin. This facilitates dephosphorylation of β-catenin, allowing it to bind to VE-cadherin and thus promoting AJ complex reassembly.

VE-cadherin itself is also a target for phosphorylation downstream of vasoactive agents. Phosphorylation on two tyrosine residues present on the cytoplasmic C-terminal region of VE-cadherin, Y658 and Y731, was found to be critical to the binding of p120- and β-catenin respectively to this region [30]. Furthermore, expression of phospho-mimetic VE-cadherin mutants in an artificial cell system (CHO cells) resulted in increased permeability to HRP-IgG. However, another residue (Y685) has been recently discovered to be phosphorylated by Src [31]. In this second study, treatment with VEGF was the stimulus, so it is possible that different tyrosines are phosphorylated in different conditions. Adding another layer of complexity, VE-cadherin can also be phosphorylated on serine residues. Gavard and colleagues found that Ser665 is phosphorylated by p21-activated kinase (PAK) upon VEGF treatment and this results in β-arrestin-dependent endocytosis of VE-cadherin and promotion of EC permeability [32]. The process is initiated by Src-dependent phosphorylation and activation of the Rac1 GEF Vav2, followed by Rac1-mediated activation of its downstream effector PAK.

Rho GTPases—Rho GTPases, particularly RhoA, Rac1, and Cdc42 have a long history of being implicated in cytoskeleton regulation [33]. In the context of ECs, Rho signaling regulates cell junctions and vascular permeability by influencing actin cytoskeleton dynamics [16]. In turn, engagement of adhesion proteins influences activity of the GTPases, providing a two-way feedback mechanism [34,35]. This review focuses on RhoA and Rac1 signaling, however, it should be mentioned that Cdc42 has also been shown to have a role in regulating vascular permeability by promoting the reassembly of AJs [36].

The development of isometric tension in ECs has been suggested as a primary mechanism of regulating endothelial barrier function [37]. Very often, agents that increase vascular permeability activate RhoA. In turn, active RhoA stimulates Rho kinase which leads to actomyosin contractility by promoting the phosphorylation of myosin light chain (MLC) directly or indirectly through inhibition of MLC phosphatase [33]. This contractility drives the formation of stress fibers and assembly of focal adhesions [38]. Rho-mediated contractility has been suggested to be a primary mechanism leading to increased permeability downstream of thrombin binding [39]. It should be noted, however, that Rho kinase also phosphorylates occludin and claudin-5, affecting their functions [40].

The case for Rac GTPase is much more controversial. Whereas activated Rac1 enhances cell–cell adhesion in epithelial cells [41], in ECs the situation is contradictory. The earliest data

showed that both dominant negative and constitutively active Rac induced permeability, consistent with the level of Rac needing to be finely tuned for optimal junctional integrity [42]. Others have also demonstrated that activation of Rac, for example in response to sphingosine-1-phosphate, promoted cortical actin rearrangement and enhanced barrier function in EC monolayers [43,44]. However, other groups have shown the opposite, with Rac activation causing increased permeability [32,45]. VEGF treatment produced a similar effect, where downstream activation of the Rac effector PAK induced VE-cadherin serine phosphorylation and endocytosis, leading to junctional disruption [32]. PAK family kinase activity downstream from Rac signaling can also stimulate opening of cell junctions by direct phosphorylation of MLC and subsequent induction of actomyosin contractility [46]. That active Rac in some situations increases permeability but in others decreases permeability, suggests that the input signal may be critical and that different scaffolding proteins may direct Rac signaling pathways in seemingly opposite directions.

Reactive Oxygen Species—Historically, ROS have been studied in the context of pathogen killing by phagocytic cells of the immune system, and as potentially harmful by-products of aerobic metabolism. However, more recently ROS have been recognized as important components of cell signaling pathways [47].

Numerous studies have shown that ROS can increase endothelial permeability both *in vitro* and *in vivo*, and this permeability can be inhibited by antioxidants and free radical scavengers [48]. Interestingly, Rac1 is a major component of the vascular NADPH oxidase complex [49]. Expression of constitutively active Rac1 resulted in ROS production concomitant with disruption of VE-cadherin at cell-cell junctions, tyrosine phosphorylation of α-catenin, and ultimately increased permeability [45]. It is now known that Rac-mediated ROS production leads to activation of the tyrosine kinase Pyk2, which subsequently phosphorylates β-catenin and thus destabilizes the AJ [50]. ROS generation also occurs downstream from a variety of vasoactive factors, notably VEGF. Recently, it was confirmed that VEGF-induced junctional disruption and ROS production similarly involves Rac1 activation [49]. Moreover, VEGF-induced Rac activation requires VEGFR-2, active Src, subsequent activation of the guanine nucleotide exchange factor Vav2, and finally activation of Rac1 [51]. Ultimately, production of ROS downstream of Rac in this pathway would be one mechanism of inducing EC permeability, although other pathways have been suggested [32].

One of the mechanisms by which ROS could alter vascular permeability involves regulation of junctional protein phosphorylation, which as discussed earlier, is often associated with increased permeability. ROS can strongly inactivate protein phosphatases by oxidation of a critical cysteine residue in the catalytic site [52]. The prevalence of phosphatases (VE-PTP, DEP-1, PTPμ, SHP-2) at EC junctions suggests they are critical for maintaining the low basal phosphorylation levels conducive for junctional integrity [53]. Localized production of ROS and inactivation of phosphatases at these sites would therefore contribute to elevated phosphorylation and junctional disruption.

ROS may also regulate junctional permeability by affecting the organization of the actin cytoskeleton [54]. Additionally, ROS production in ECs has been associated with changes in the activities of RhoA and Rac1 [55].

3. Mechanisms Affecting Leukocyte Transendothelial Migration

The regulated transmigration of leukocytes across the endothelial lining of the vasculature is critical to the inflammatory response. However, when TEM is excessive or inappropriately localized, it can initiate many pathological processes. For example, the transmigration of leukocytes is an early step in the development of atherosclerotic plaques [56]. Also, the

pathogenesis of multiple sclerosis involves a massive influx of leukocytes transmigrating into the brain, leading to tissue damage and neurological disability [57].

Leukocyte TEM, can occur by two routes; the most prevalent mechanism *in vitro* is "paracellular" TEM, which requires transient junctional disruption as leukocytes migrate between adjacent cells. TEM can also occur by a "transcellular" route in which a leukocyte moves through the body of an EC, probably via the transient formation of a pore. The factors which affect the route preference for TEM are still largely unknown. Here, we will focus on signals initiated by leukocyte engagement of EC adhesion molecules that result in regulation of junctions and thus the paracellular mode of TEM.

TEM occurs in sequential steps, each of which initiates signaling that facilitates progression to the next step [58]. Rolling adhesion occurs when leukocytes are first captured from the bloodstream, and become loosely associated with the ECs lining the blood vessel. Next, firm adhesion of leukocytes to the surface of ECs occurs through the interaction of EC adhesion molecules and activated leukocyte integrins. This interaction triggers a variety of signals within the EC that ultimately initiate TEM.

Adhesion Molecules that Initiate Signaling – ICAM and VCAM—Intercellular adhesion molecule 1 (ICAM-1) and vascular endothelial cell adhesion molecule 1 (VCAM-1) are particularly important for firm, integrin-mediated adhesion of leukocytes to ECs and subsequent TEM [59]. The role of ICAM-1 and VCAM-1 has been extensively reviewed [60]. The importance of ICAM-1-mediated signaling is demonstrated by the deficient immune response exhibited by ICAM-1-null mice. Furthermore, antibody blockade of ICAM-1 inhibits leukocyte TEM. Similarly, blocking VCAM signaling with function blocking antibodies against VCAM-1 or its integrin ligand VLA-4 inhibits leukocyte adhesion to ECs and decreases TEM. Both ICAM-1 and VCAM-1 are important components of cup-like docking structures which form at the site of leukocyte-EC interaction [61,62]. These structures have been described as the origin of leukocyte pseudopods which extend through EC junctions to facilitate paracellular TEM [63]. Figure 2 illustrates three potential mechanisms downstream of VCAM-1 and ICAM-1 engagement which lead to paracellular TEM of leukocytes: Rho GTPase signaling, ROS-mediated regulation, and tyrosine phosphorylation of junctional components.

Rho GTPases—One well-studied outcome of ICAM-engagement is RhoA activation. Artificially simulating leukocyte adhesion by crosslinking ICAM-1 with antibodies results in stress fiber formation downstream of Rho GTP-loading [64,65]. By using C3 transferase, a bacterially-derived Rho inhibitor, Adamson and colleagues showed that ICAM-1-stimulated cytoskeletal rearrangements required for efficient T-lymphocyte TEM were dependent on RhoA activation [66]. Treatment of ECs with Y-27632, an inhibitor of Rho kinase, or inhibitors of myosin light chain kinase also reduced neutrophil TEM [67,68]. Our laboratory has recently shown that ICAM-1 engagement also activates RhoG [69]. This activation was critical for formation of the apical membrane protrusions which extend from ECs to partially engulf adherent leukocytes. Depletion of endothelial RhoG decreased cup formation and also inhibited leukocyte TEM. Further investigation revealed that the cytoplasmic tail of ICAM-1 binds SGEF, a RhoG guanine nucleotide exchange factor (GEF), which provides a mechanism of RhoG activation downstream of ICAM-1.

VCAM-1 has also been placed upstream of Rho family GTPases. Antibody-mediated VCAM-1 crosslinking on HUVECs results in activation of Rac1, induction of stress fibers, and formation of gaps in the EC monolayer leading to a decrease in electrical resistance [70]. The junctional disorganization induced by VCAM-1 engagement was dependent on ROS.

Reactive Oxygen Species—ROS have been implicated as an important mechanism regulating leukocyte TEM. By activating Rac, VCAM-1 crosslinking results in NADPH oxidase activation and ROS production in ECs [71]. Generation of low levels of ROS upon VCAM-1 engagement is required for stereotypical actin cytoskeleton changes to occur. Extracellular release of ROS due to VCAM signaling results in the activation of EC-associated matrix metalloproteases (MMPs) [72], and this ROS-mediated activation of MMPs was required for efficient lymphocyte migration. This could potentially regulate TEM by MMP-mediated degradation of junctional components such as VE-cadherin. Evidence has been presented that ROS activates the redox-sensitive kinase Pyk2 [50]. Pyk2 phosphorylates β-catenin promoting its dissociation from VE-cadherin. Another kinase, PKC-α, was shown to be activated by oxidation downstream of VCAM. This activation was DAG-independent, and was required for efficient TEM of splenic lymphocytes [73]. Subsequently, the downstream target of PKC-α in this pathway was discovered to be protein tyrosine phosphatase 1B (PTP1B) [74]. The exact mechanism of how PTP1B activity promotes lymphocyte TEM remains to be determined; however this is yet another indication that a balance of phosphorylation is important for the maintenance of junctional integrity.

Phagocytic cells generate high concentrations of extracellular ROS in a respiratory burst event mediated by the NADPH oxidase complex [75]. Exogenous ROS have been shown to enhance neutrophil binding to ECs due to increased expression of EC cell surface adhesion molecules [76]. Additionally, ROS released from fMLP-stimulated neutrophils was found to increase vascular permeability [77]. It is intriguing to consider that leukocyte-generated ROS may act locally on ECs to trigger intracellular signaling that facilitates TEM. This mechanism of signaling may amplify existing signals initiated by EC-generated ROS or initiate separate pathways.

Tyrosine phosphorylation of junctional proteins—VE-cadherin plays a critical role in regulating paracellular permeability during TEM. Adhesion of leukocytes to ECs has been shown to induce a localized, transient disruption of VE-cadherin which is required for efficient TEM [78]. Tyrosine phosphorylation of junctional components has been implicated as an important mechanism for regulating junctional integrity during TEM. Inhibition of protein tyrosine phosphatases in ECs increases tyrosine phosphorylation of AJ components and promotes neutrophil TEM [28,79]. ICAM-1 engagement results in tyrosine phosphorylation of VE-cadherin through activation of Src and Pyk2 kinases [80]. Phosphorylation on tyrosines 658 and 731 inhibits binding of p120-catenin and β-catenin [30], and increases TEM of neutrophils [80]. Similarly, ICAM-mediated VE-cadherin phosphorylation in brain microvascular cells increased paracellular TEM of lymphocytes [81]. Src activation due to ICAM signaling has been reported previously to induce tyrosine phosphorylation of a number of proteins including cortactin [82]. A pathway has been defined linking cortactin phosphorylation to TEM of neutrophils [83]. It was concluded that cortactin phosphorylation by Src serves to link ICAM-1 to the actin cytoskeleton, aiding ICAM-1 clustering at sites of leukocyte adhesion.

Concluding Remarks

The permeability of endothelial junctions is affected by vasoactive agents as well as by leukocytes during inflammation. Although the receptors involved are often different, many of the signaling pathways overlap and converge on the same targets. Typically, many of the downstream signals impact the activities of Rho proteins and in some cases have also been shown to involve ROS. Ultimately, there is an increase in the tyrosine phosphorylation of junctional components, which affects protein stability and binding interactions. The complex interplay between Rho GTPases, ROS and tyrosine phosphorylation of junctional components is beginning to be elucidated but we are confident that many more interconnections remain to

be discovered. Identifying these pathways should reveal new targets for therapeutic intervention that will be relevant for treating inflammatory diseases and the many pathological situations where vascular permeability is adversely affected.

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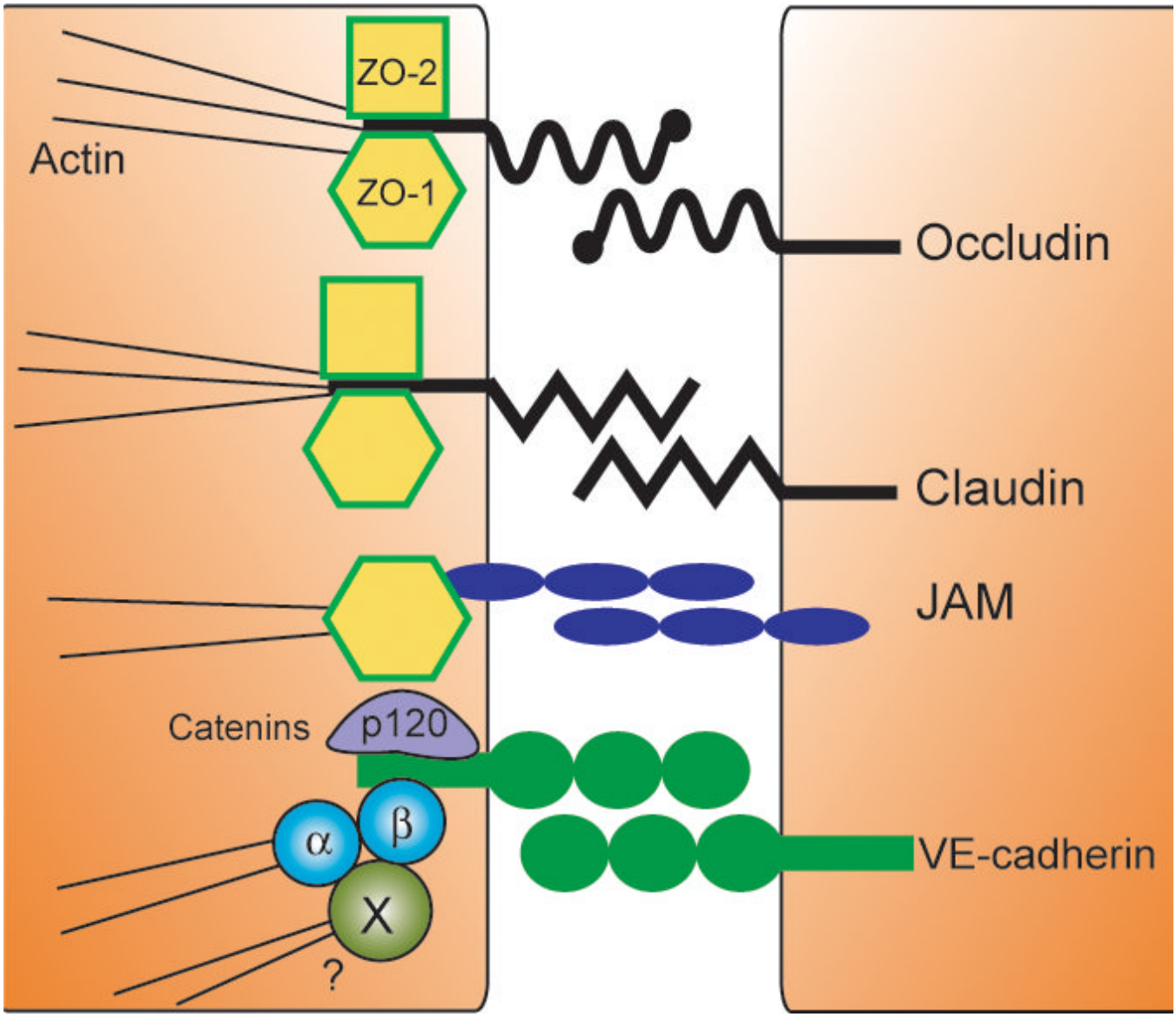

Figure 1. Components of endothelial junctions and their association with the cytoskeleton

Tight junctions and adherens junctions are intermixed along the lateral surface of ECs. Extracellular domains of occludin, claudins, and JAM proteins form homophilic interactions to create tight junctions. The intracellular domains of these proteins associate with the actin cytoskeleton through ZO proteins (ZO-1/ZO-2) and other components (not shown). The major transmembrane component of adherens junctions is VE-cadherin. The cytoplasmic domain associates with the cytoskeleton via α and β-catenin and likely other unidentified components (X). p120 catenin serves a supporting role to maintain stability of the cadherin complex.

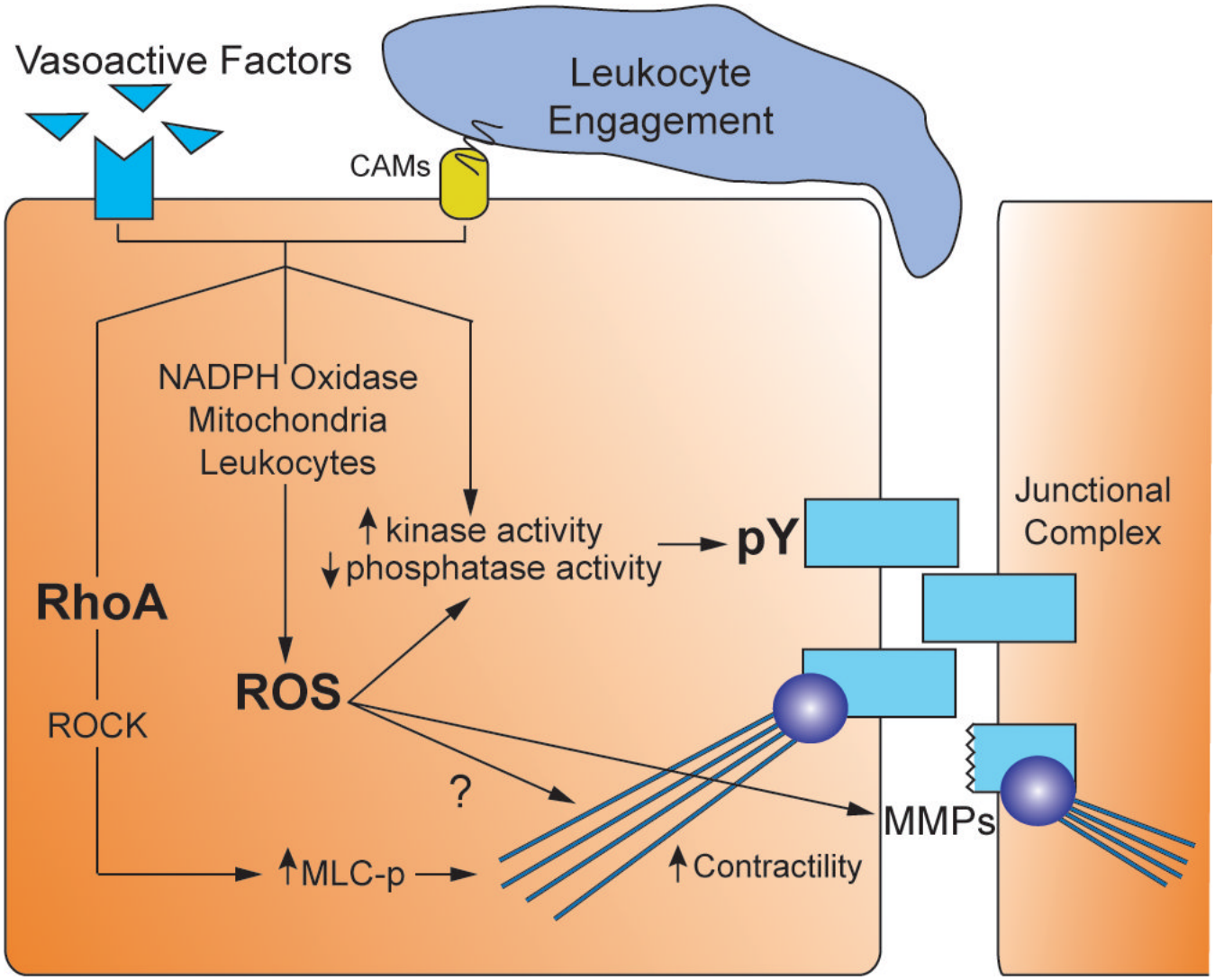

Figure 2. Key signaling pathways involved in regulating vascular permeability and leukocyte TEM

The binding of vasoactive factors or the adhesion of leukocytes to cell adhesion molecules (CAMs) initiates intracellular signaling pathways that modulate junctional permeability. Tyrosine phosphorylation (pY) is a primary mechanism for regulating EC junctions and is mediated by the balance of kinase and phosphatase activities. Rho GTPases play a major role in regulating junctional integrity through their respective effectors. Phosphorylation of myosin light chain (MLC-p) and actomyosin contractility downstream of RhoA and Rho kinase (ROCK) is a major contributor to increased junctional permeability. ROS can affect junctions by indirectly modifying tyrosine phosphorylation or by the activation of matrix metalloproteases (MMPs) which degrade junctional components. ROS may also affect the actin cytoskeleton.