Rho, Tyrosine Kinase, Ca\textsuperscript{2+}, and Junctions in Endothelial Hyperpermeability

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Increased microvascular permeability is a central hallmark of inflammation and the basis of edematous tissue injury in many acute and chronic pathological conditions, including ischemia-reperfusion injury, sepsis, and acute respiratory distress syndrome.\textsuperscript{1–4} Increased microvascular leakage may occur in any region of the vasculature, but it is most familiar in the microvasculature, especially the postcapillary venule. Ultrastructurally, anatomists recognize at least 3 types of endothelial cells: continuous, fenestrated, and discontinuous, which can all exhibit altered barrier in response to environmental and chemical factors.

Early studies by Majno and Palade\textsuperscript{5} and later studies by Simionescu et al\textsuperscript{6} suggested that inflammatory mediators, such as histamine, thrombin, and serotonin, increase solute permeability in microvessels by enlarging interjunctional spaces and allowing the extravasation of fluid, protein, and leukocytes into the tissues. It is widely held that many inflammatory mediators (eg, thrombin, histamine, and bradykinin) use a common mechanism to control junctional exchange through a Ca\textsuperscript{2+}/calmodulin (CaM) and myosin light chain kinase (MLCK)–regulated actomyosin contraction, which generates cytoskeletal tension. In theory, this tension promotes separation of junctional clefts and allows equilibration of solutes into the interstitium.\textsuperscript{7–10} Many recent studies have demonstrated that this contractile response is regulated to a large extent through the activity of several members of the small GTPase family that includes Rho, Rac, and Cdc42.\textsuperscript{7,8,11,12}

The study by van Nieuw Amerongen et al\textsuperscript{13} in this issue of Circulation Research is the most recent of several studies that describe features of Rho-dependent endothelial permeability produced by mediators, especially thrombin. While Rho is thought to play a critical role in altered permeability, van Nieuw Amerongen et al\textsuperscript{13} propose 4 main targets in barrier disruption: RhoA, calcium, tyrosine kinase, and cell junctions. The present study shows that thrombin increases endothelial permeability by activating RhoA and Rho kinase to increase MLC phosphorylation. This simultaneously promotes the organization of actin stress fibers in the cell necessary for producing tension and the loss of cell-cell apposition and initiates actomyosin contraction. These effects were significantly reduced by inhibition of Rho kinase with Y-27632, which blocked stress fiber organization, contraction, and permeability. These events were additionally reduced by chelation of intracellular calcium with BAPTA. Importantly, the authors demonstrate that Rac, an important regulator of cell endothelial shape and motility,\textsuperscript{14,15} does not appear to be activated in this model.\textsuperscript{13}

Rho Activity and Targets

Several previous studies have described the role of Rho, especially in thrombin-mediated permeability.\textsuperscript{9,11,14,16} Previously, van Nieuw Amerongen et al\textsuperscript{9} suggested that thrombin caused a tyrosine kinase–dependent activation of Rho. Activated Rho activates Rho kinase to increase MLC phosphorylation by inhibiting MLC dephosphorylation and possibly by direct phosphorylation of MLCs.\textsuperscript{17} Thrombin also increases intracellular calcium (as a result of phospholipase C\gamma), binds CaM, and activates MLCK. Fully phosphorylated MLCs then initiate cytoskeletal contraction with a loss of junctional barrier.\textsuperscript{8,18} Whereas virtually all studies agree that the extent of MLC phosphorylation is critical, there are many factors that will determine the magnitude of MLC phosphorylation.

MLC Phosphorylation

Endothelial MLCs can be phosphorylated by at least 3 possible kinases: Rho kinases,\textsuperscript{17} 214-kDa endothelial cell MLCs,\textsuperscript{19} and 130- to 150-kDa nonmuscle MLCs,\textsuperscript{20} although the dominant role of the 214-kDa form in endothelial permeability is now well established. Thrombin promotes a Ca\textsuperscript{2+}/CaM-dependent activation and translocation of MLCK to the actin cytoskeleton\textsuperscript{10} in a complex that also appears to contain the kinase p60src and cortactin.\textsuperscript{21} Although thrombin does promote p60src association with the cytoskeleton in a complex with MLCK and cortactin, there is presently no direct evidence that tyrosine phosphorylation of MLCK by p60src promotes cell contraction by this pathway. However, several studies with tyrosine kinase inhibitors indicate that tyrosine phosphorylation is still somehow permissive for MLCK activation.\textsuperscript{22} MLCK activity is also downregulated by protein kinase A–mediated phosphorylation.\textsuperscript{10} Similarly, a new member of the Rho family, PAK, has also been shown capable of directly phosphorylating MLCK and inhibiting its activity.\textsuperscript{23}

Myosin Phosphatase Activity

The magnitude of MLC phosphorylation also depends on the level of myosin phosphatase activity.\textsuperscript{24–26} mainly via type 1 phosphatase (PP1) and PP2B in endothelium. MLC dephos-
phorylation is controlled by Rho-mediated inactivation of myosin phosphatase, which regulates and sustains the contractile response initiated by MLCK. This dephosphorylation is mediated by PP1, which is thought to be the dominant myosin phosphatase in human endothelium.\textsuperscript{25} Whereas Rho kinase will directly phosphorylate and inhibit myosin phosphatase,\textsuperscript{27} myosin phosphatases can also be regulated by activity by regulating their binding to the myosin complex or by levels of intracellular Ca\textsuperscript{2+}.\textsuperscript{25}

**Calcium**

Calcium levels in the cell also critically regulate permeability in this model. Chelation of calcium in using BAPTA (an intracellular calcium chelator) attenuated thrombin-dependent permeability but did not alter Rho activation, presumably by attenuating Ca\textsuperscript{2+}/CaM-dependent MLCK activation. Although the present study indicates parallel rather than cooperative regulation of calcium and tyrosine kinases, other studies have suggested that tyrosine kinases may regulate calcium levels.\textsuperscript{28,29} Thrombin contributes significantly to calcium influx and release of calcium stores,\textsuperscript{28} which may regulate Rho and, hence, MLCK.\textsuperscript{29} These studies support the concept that Rho induces a calcium sensitization of myosin by maintaining active phosphorylated MLCs through phosphatase inhibition. This sensitization attributable to Rho may help explain the increased response to histamine in the presence of serum. Serum, unlike plasma, contains platelet-derived lipid mediators, including lysophosphatidic acid and sphingosine-1-phosphate, which activate Rho and produce extensive responses to mediators.\textsuperscript{9,30} Although these studies all document increased solute permeability through a contractile process, there is evidence that permeability changes can also occur through at least one myosin-independent pathway,\textsuperscript{16,31} which may involve actomyosin-independent changes in junctional organization.

**Role of Junctional Proteins**

The present model also proposes an additional step in the regulation of barrier, namely the reorganization of cell-cell junctions. Adherens and tight junctions (which are opened by Rho-regulated cytoskeletal tension) must either passively detach from one another or actively open to permit cleft formation and permeability. Whereas Rho and Rac can regulate E- and P-cadherin distribution to the junction in nonendothelial cells,\textsuperscript{32} vascular endothelial (VE) cadherin distribution and function is thought to be independent of Rho in human endothelium.\textsuperscript{15,33} However, VE-cadherin transfected in Chinese hamster ovary cells does exhibit Rho sensitivity, and Rho will redistribute VE-cadherin from junctions. Therefore, VE-cadherin junctional sensitivity to Rho modulation must depend on Rho-specific cadherin binding partners that are expressed in nonendothelial cells, such as epithelia, but not in all endothelial cells. Curiously, in adherens junction, Rho activation leads to cadherin disorganization; however, Rho activation may actually stabilize components of the tight junction.\textsuperscript{34} Clearly, junctional proteins must play a role in the regulation of barrier in response to thrombin; however, the exact role of Rho in the regulation of endothelial junctions has not yet been demonstrated. Although we have described a contractile mechanism to explain thrombin-mediated permeability, it has been suggested that some mediators, such as histamine, can promote barrier changes solely by junctional reorganization without invoking myosin.\textsuperscript{35} Future studies will be needed to determine how different junctions cooperate with cytoskeletal tension to coordinate permeability responses to different mediators.

**Leukocyte Extravasation**

Whereas Rho, calcium, and tyrosine kinases play roles in the junctional barrier to small solutes, there is evidence that also supports a role for Rho in leukocyte extravasation. In acute models, at least 2 groups have now demonstrated that adherent neutrophils increase MLC phosphorylation to open junctions and permit extravasation.\textsuperscript{36,37} In a related model, the engagement of intercellular adhesion molecule-1 by lymphocytes on the endothelial surface has been shown to trigger Rho and may reorganize junctions in preparation for or during diapedesis.\textsuperscript{38,39} It is not presently known if intercellular adhesion molecule-1 engagement and Rho activation also mediate neutrophil diapedesis.

**Junction-Independent Permeability and Leukocyte Extravasation?**

Despite many studies in this area that support a predominantly junctional basis for increased solute exchange and leukocyte extravasation, extrajunctional mechanisms responsible for these forms of transvascular leakage are again being reexamined. Using serial ultrastructural analysis, several reports\textsuperscript{40–42} have proposed that clusters of connected vesicles, termed vesiculo-vacuolar organelles, provide channels for exchange of solutes that are controlled by inflammatory mediators. Similarly, ultrastructural studies have also suggested that neutrophils pass directly through the endothelium independently of junctions.\textsuperscript{44} It is not clear if these forms of exchange, which appear to be independent of this contraction and junctions, are Rho-mediated. How and whether this type of system interacts with contractility mediated alterations in junctional barrier must be answered by future studies.

**Tissue and Species Specificity of Rho Effects**

Although Rho appears to regulate responses to thrombin (and perhaps other mediators), there may be considerable species and tissue heterogeneity for this response. Carbajal and Schaeffer\textsuperscript{11} reported in bovine pulmonary artery endothelium that RhoA inactivation (using C3 toxin) did enhance endothelial barrier but did not significantly protect against thrombin-mediated permeability. Most studies that used human umbilical vein endothelial cells (like the present study) have shown that RhoA does mediate thrombin effects.\textsuperscript{9,21,24} One possible explanation for these differences might be protein kinase C. Although not specifically addressed in the present study, protein kinase C activation (by phorbol 12-myristate 13-acetate) did not alter MLCK activity in human cells but did enhance bovine MLCK and might account for some of the observed differences.\textsuperscript{7}
Atherosclerosis

The events described for the regulation of junctional organization in the present study may also contribute to the pathogenesis of atherosclerosis. Oxidized LDL is a significant risk factor in atherosclerosis and may enhance monocyte and macrophage binding and extravasation by Rho activation of endothelial cells.44 The enhanced interaction of platelets with the endothelium associated with atherosclerosis can also promote the release of platelet lipid mediators, such as lysophosphatidic acid and sphingosine-1-phosphate, which are potent Rho activators.45 Rho may also contribute to the development of atherosclerosis by downregulating the expression of endothelial nitric oxide synthase (eNOS). Both Rho and eNOS are localized to caveolae in endothelial cells.46,47 Essig et al48 have shown that the statins may be useful in preventing tumor neovascularization and metastasis. Although there is general agreement now that chronic inflammation, there is considerable endothelial heterogeneity within these responses that may be tissue-, species-, or model-dependent.11 Additional studies are clearly required to determine the contribution of Rho-mediated signals in these disease processes, which in turn may provide important targets for therapeutic intervention.

Cancer

Because endothelial motility and monolayer remodeling are clearly related to Rho-regulated cell retraction,43 there is a possibility that many events in cancer progression, such as angiogenesis and tumor metastasis,49 may also be Rho-dependent. Therefore, Rho-targeting therapies may also be useful in preventing tumor neoangiogenesis and metastasis.

Future Directions

In the future, Rho inhibitors may play an important role in the treatment of acute inflammatory processes in the microvasculature as well as chronic conditions, including atherosclerosis, pulmonary hypertension, and tumor vascularization and metastasis. Although there is general agreement now that Rho, calcium, and myosin-dependent processes clearly contribute to the development of several forms of acute and chronic inflammation, there is considerable endothelial heterogeneity within these responses that may be tissue-, species-, or model-dependent.11 Additional studies are clearly required to determine the contribution of Rho-mediated signals in these disease processes, which in turn may provide important targets for therapeutic intervention.

References


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