Cardiovascular Research (2010) **87**, 272–280
doi:10.1093/cvr/cvq144

**SPOTLIGHT REVIEW**

# Myosin light chain kinase in microvascular endothelial barrier function

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*Received 9 December 2009; revised 8 April 2010; accepted 11 May 2010; online publish-ahead-of-print 17 May 2010*

Microvascular barrier dysfunction is implicated in the initiation and progression of inflammation, posttraumatic complications, sepsis, ischaemia–reperfusion injury, atherosclerosis, and diabetes. Under physiological conditions, a precise equilibrium between endothelial cell–cell adhesion and actin–myosin-based centripetal tension tightly controls the semi-permeability of microvascular barriers. Myosin light chain kinase (MLCK) plays an important role in maintaining the equilibrium by phosphorylating myosin light chain (MLC), thereby inducing actomyosin contractility and weakening endothelial cell–cell adhesion. MLCK is activated by numerous physiological factors and inflammatory or angiogenic mediators, causing vascular hyperpermeability. In this review, we discuss experimental evidence supporting the crucial role of MLCK in the hyperpermeability response to key cell signalling events during inflammation. At the cellular level, *in vitro* studies of cultured endothelial monolayers treated with MLCK inhibitors or transfected with specific inhibiting peptides have demonstrated that induction of endothelial MLCK activity is necessary for hyperpermeability. *Ex vivo* studies of live microvessels, enabled by development of the isolated, perfused venule method, support the importance of MLCK in endothelial permeability regulation in an environment that more closely resembles *in vivo* tissues. Finally, the role of MLCK in vascular hyperpermeability has been confirmed with *in vivo* studies of animal disease models and the use of transgenic MLCK210 knockout mice. These approaches provide a more complete view of the role of MLCK in vascular barrier dysfunction.

**Keywords** MLCK • MLC • Contractile cytoskeleton • Endothelial barrier function • Microvascular permeability

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

## 1. Introduction

Myosin light chain kinases (MLCK) are a family of soluble protein kinases that function principally to phosphorylate the 20 kDa regulatory myosin light chain (MLC-2) and thereby induce ATPase driven actin–myosin contraction.[1,2] In most cells, MLCK is a transducer for signalling MLC phosphorylation in response to $Ca^{2+}$ binding to MLCK-associated calmodulin. MLCK-mediated MLC phosphorylation and actomyosin contractility is important in muscle contraction, cell migration, and endo/exocytic processes, and is recognized for its central role in signalling endothelial cell–cell adhesion and barrier function. In this review, we discuss the molecular physiology of MLCK, and the biochemical basis for actin–myosin contraction in the context of vascular endothelial permeability. We use an experimental approach that incorporates molecular information from cultured endothelial cell monolayers into physiological responses in intact microvessels, *ex vivo* and *in vivo*, providing a more complete understanding of the control of endothelial permeability by MLCK in pathophysiological conditions.

## 2. MLCK structure and molecular physiology

MLCK contains a C2 immunoglobin domain that binds to unphosphorylated MLC, a catalytic site for kinase activity,[3] and a calmodulin-binding regulatory domain that functions as an autoinhibitory domain to suppress constitutive activity in the absence of calmodulin.[4,5] By way of binding to calmodulin, $Ca^{2+}$ is an essential regulator of MLCK activity. While the structures required for MLCK activity and binding to MLC are conserved across species and tissue types,[6] there are differences in the components required for regulation of MLCK variants in different cell types. For example, in smooth muscle cells, $Ca^{2+}$ binding to calmodulin is sufficient to activate MLCK and induce actin–myosin contraction. In other cell types, $Ca^{2+}$ binding to calmodulin is necessary for MLCK activity, but alone is not sufficient to elicit an actin–myosin contractile response.[7] Differences in regulation are pronounced in muscle vs. non-muscle MLCK.

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## 2.1 Muscle MLCK isoforms

There are three types of muscle MLCK: skeletal (skMLCK), cardiac (cMLCK), and smooth muscle (smMLCK), products of genes *mylk*2, *mylk*3, and *mylk*1, respectively.[1,8,9] MLCK is activated in response to $Ca^{2+}$ released from intracellular stores. $Ca^{2+}$ binding to calmodulin induces MLCK to phosphorylate MLC on serine 19, which increases the actomyosin contractile response. In skeletal or cardiac myocytes, $Ca^{2+}$-binding troponin triggers actomyosin contraction, and MLCK activation increases contractile strength.[1,10] In smooth muscle, smMLCK induces actin–myosin contraction. The smMLCK isoform differs markedly in structure from skMLCK or cMLCK, having greater similarity to non-muscle MLCK (discussed below).[11] In contrast to skMLCK or cMLCK, smMLCK bears an additional C-terminal insert, and a long N-terminus that contains a fibronectin domain, two immunoglobin domains, and numerous putative phosphorylation sites.[1,6,10] The smMLCK has 1147 amino acids [12] and is also called MLCK108 based on its predicted molecular weight of 108 kDa. MLCK108 shows apparent weights ranging 125–155 kDa,[4] compared with 77–103 kDa for skMLCK.[13]

## 2.2 Non-muscle MLCK isoforms

Earlier work by Garcia's group has identified and cloned a single gene of human non-muscle MLCK (nmMLCK) that encodes four high molecular weight MLCK isoforms (MLCK1-4).[6] Non-muscle MLCK differs from smMLCK mainly in that nmMLCK contains an additional stretch of 922 amino acids spliced into the N-terminus, with multiple sites for protein–protein interactions including p60Src-mediated tyrosine phosphorylation.[14,15] All nmMLCK isoforms are splice variants derived from the *mylk*1 gene on human chromosome 3 (3qcen-q21).[9,16,17] MLCK1, previously known as endothelial MLCK (eMLCK), was originally cloned from human endothelial cells,[7] and believed to have a predominantly endothelial tissue distribution. Recent evidence shows that MLCK1 is also expressed in other tissues including gut epithelium, and more recently was observed in neutrophils. MLCK isoforms 1 and 2 are the most highly expressed in endothelium.[6] MLCK1 is the highest molecular weight MLCK variant with 1914 amino acids (predicted 210 kDa), also known as MLCK210.[6] MLCK2 is identical to MLCK1, but lacks a stretch of 69-amino acid containing two tyrosine residues necessary for phosphorylation and activation of MLCK1 by p60Src.[6,14] MLCK 3a and 3b are identical to MLCK1 and 2, respectively, but bearing an additional distal deletion (exon 30) corresponding to a stretch of 51 amino acids. The complete sequence information for MLCK4 is not yet known, but has similar tissue distribution to other nmMLCK isoforms: human lung, liver, brain, and kidney tissues, as well as endothelial cells. The remainder of this review is dedicated to the role of MLCK in regulating permeability of vascular endothelial barriers.

# 3. Endothelial barriers

The microvascular endothelium consists of a layer of closely apposed endothelial cells, forming a semi-permeable barrier between blood and tissue to control exchange of fluids, electrolytes, and proteins.[18] The integrity of this barrier is crucial in maintaining circulatory homeostasis and physiological organ function. Pathological aberration of endothelial barrier function leads to microvascular hyperpermeability and plasma extravagation, resulting in tissue oedema and organ dysfunction.[18–21] The problem is associated with inflammatory disease, traumatic or thermal injury, diabetes mellitus, myocardial infarction, and tumorigenesis.[21–24] Although transcellular transport of albumin does occur, it is now clear that leakage of fluid and macromolecules across the endothelial barrier during the aforementioned disease processes occurs in a largely paracellular fashion via cell–cell junctions.[25–27]

The cell–cell junctional structures of the vascular endothelium include tight junctions and adherens junctions. Tight junctions are detected primarily in blood-brain, blood-retinal, or blood-testis barrier microvasculature.[26,28] Tight junctions are zipper-like structures formed of homophilic interactions between occludin, claudins, and junction adhesion molecule (JAM) A. *In vivo*, tight junctions exhibit high transendothelial electrical resistance (TER)[29,30] and are exceptionally impermeant to the passage of solutes.[31] Adherens junctions are found in nearly all vascular beds, especially in the peripheral microvasculature.[25,26,32] Adherens junctions are mainly composed of homophilic interactions of the junctional adhesion protein VE-cadherin, as well as JAM A, B, and C.

Intracellularly, tight junction proteins are connected to actin filaments via zona occludens-1/2 (ZO-1/2); adherens junction proteins are connected via catenins ($\alpha$, $\beta$, $\gamma$, and p120).[32–35] By way of these connections, endothelial cytoskeletal contractile forces strongly influence cell–cell junctions and thus paracellular permeability. In addition, the vascular endothelium is anchored to extracellular matrix through focal adhesions, mediated by transmembrane integrins and actin-linking proteins, e.g. focal adhesion kinase, talin, paxillin, and vinculin.[35,36] Cell–cell and cell–matrix adhesion structures act coordinately with cytoskeleton proteins to maintain the integrity of the endothelial barrier and a low basal permeability.[33]

# 4. Actomyosin contractile machinery

The contractile machinery of cells is driven by a mechanochemical interaction between actin and myosin.[37] In vascular endothelial cells, the actin–myosin interaction is regulated by the phosphorylation status of MLC, and activation of MLCK is a key step in the development of actomyosin-based contractile forces.[38,39] Upon activation by $Ca^{2+}$/calmodulin or by tyrosine kinase-mediated phosphorylation at Tyr-464 and Tyr-471, MLCK phosphorylates MLC at Ser-19 and subsequently at Thr-18, resulting in a change in the myosin tertiary structure favouring contractile movement against actin (*Figure 1*).[40–42] Opposing MLCK, myosin light chain phosphatase (MLCP) dephosphorylates MLC, decreasing tension and relaxing the cytoskeleton.[43] Therefore, optimal control of the contractile status arises from the balance between MLCK and MLCP activity. RhoA, a member of Rho family small GTPases, plays a critical role in regulating MLCP activity. Once activated in a GTP-bound form, RhoA can activate its downstream Rho kinase (ROCK) that subsequently phosphorylates and inhibits MLCP, resulting in increased MLC phosphorylation and actomyosin contraction (*Figure 1*).[32,44,45] There is also evidence that ROCK directly phosphorylates MLC *in vitro*,[46] or increases endothelial permeability by inducing VE-cadherin phosphorylation, though the relative importance of these events is unclear.[46,47] In many cases, endothelial paracellular permeability is controlled by MLCK-dependent processes; however, endothelial hyperpermeability can also occur through MLCK-independent mechanisms.[48,49] This review is focused on control of MLCK activity and actomyosin

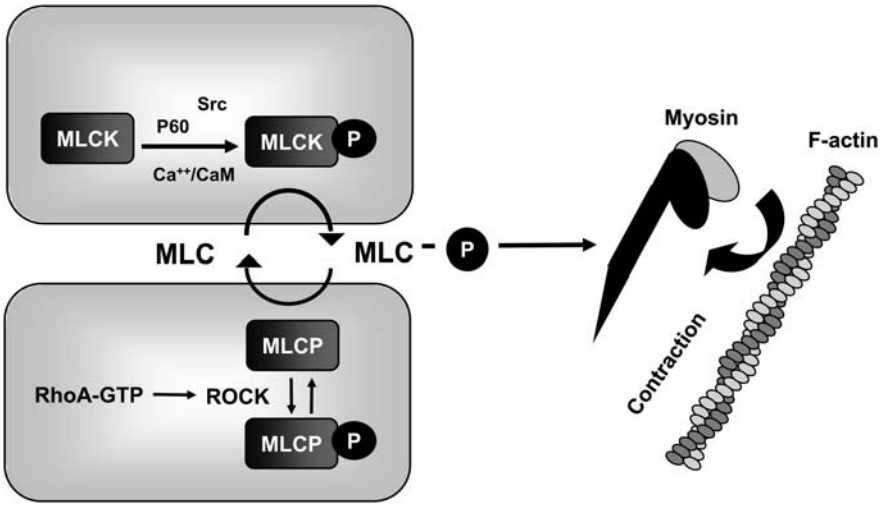


**Figure 1** Control of actin–myosin contraction in endothelium. Increased MLC phosphorylation in response to MLCK activation by $Ca^{2+}$-calmodulin binding and src kinase activity, or to inhibition of MLCP by ROCK activation downstream of RhoA, increases MLC ATPase-driven force generation relative to actin.

contractility in endothelial hyperpermeability, which is central to the pathophysiology of vascular barrier dysfunction.

## 5. MLCK in cultured endothelial cells

Endothelial cell monolayers are powerful tools for investigating molecular mechanisms that control cell junction structure and permeability. Many studies employ endothelial cells of macrovascular or arterial origin, such as human umbilical vein endothelial cells (HUVECs) or aortic endothelial cells. These are useful models for studying cell morphology or signalling; however, permeability responses in these cell lines may not resemble the microvascular exchange process *in vivo*, because these cells are derived from non-exchange vessels. Even cultured cells of microvascular origin do not retain all physiological barrier properties present in the microvasculature. Adamson *et al.* have pointed out that FITC-albumin leakage in intact microvessels occurs at less than 5% of endothelial cells,[49] therefore cultured endothelial cells (especially clonal populations) may not retain the complete phenotype observed in intact microvessels. Despite this limitation, however, we have gained much information about the molecular mechanisms underlying the MLCK-dependent permeability response by studying cultured cells.

Our laboratory and others have shown that the phosphorylation status of MLC is critical in the barrier response to histamine, thrombin, oxygen radicals, and activated neutrophils.[20,40,50–53] Also, the pattern of hyperpermeability response varies depending on agonists. Histamine exposure increases MLC phosphorylation and actomyosin contraction, manifest as rapid and transient (5 min) hyperpermeability in HUVEC monolayers.[53–55] Similar effects are seen in endothelial cells from carotid artery and aorta, with hyperpermeability beginning within 10 min and lasting for up to 2 h.[56] Thrombin also induces MLC phosphorylation, cellular contraction, and intercellular gap formation; however, in HUVEC monolayers, the hyperpermeability response to thrombin is sustained (up to 5.5 h) relative to the transient effect of histamine.[55,57] This indicates either that different stimuli activate different signalling processes, or that different resolution mechanisms are involved. A pattern of response similar to that of thrombin is seen with the serine/threonine phosphatase inhibitor calyculin-A, suggesting that sustained hyperpermeability may be due to the inability to dephosphorylate MLC.[40,57] Conversely, inhibition of serine/threonine MLC phosphorylation reduces the hyperpermeability elicited by these agonists.[40,50,51,53]

The necessity of activated MLCK in MLC phosphorylation and endothelial permeability was confirmed using a protein transference technique.[58,59] Briefly, proteins or engineered peptides can be transfected directly into endothelial cells using the polyamine transfection reagent transIT, without apparent toxicity, producing protein transfection efficiencies of up to 90%.[59,60] Furthermore, transIT transfection of endothelial cells with a protein kinase C (PKC)-specific inhibiting peptide dramatically reduced intracellular PKC activity to the same extent as application of the PKC inhibitor to cell lysates *in vitro*, illustrating the effectiveness of this method for perturbing specific elements of cell signalling in live cells. To determine that activated MLCK is sufficient to elicit endothelial hyperpermeability, we introduced purified, constitutively active MLCK protein into coronary venular endothelial cells (CVEC) as a polyamine-conjugated complex.[58] Transfected MLCK significantly increased the phosphorylation level of MLC (~60%), and was accompanied by an increase in transendothelial flux of albumin across the CVEC monolayer. Similar to the effect of calyculin-A on MLC, MLCK transfection exclusively induced the diphosphorylated form of MLC. This is significant in that Thr-18/Ser-19 diphosphorylation of MLC generates higher myosin ATPase activity than does monophosphorylation,[61] and also speaks to the complexity of signalling to MLC. Fluorescent microscopy studies further revealed that increased MLCK activity led to widespread intracellular gap formation in the monolayer, loss of peripheral catenin, and contractile cytoarchitecture.[58] All of these MLCK-mediated effects on MLC phosphorylation, endothelial cell

morphology, and barrier function are abrogated by inhibition of MLCK. Thus, accumulating evidence indicates that abnormally activated MLCK is a major determinant in microvascular barrier dysfunction in response to many signalling mediators and in a variety of pathophysiological processes.

PKC activation and NO production are important for the MLCK-dependent vascular endothelial hyperpermeability responses to thrombin, histamine, and vascular endothelial growth factor (VEGF).[62–65] There is significant controversy over the effects of nitric oxide (NO) on endothelial hyperpermeability, as some groups have reported barrier enhancing effects of NO and others have reported hyperpermeability-inducing effects of NO.[66] The reasons for these differences are not clear, however may be due to heterogenous responses to different experimental conditions, differential expression of nitric oxide synthase (NOS) isoforms, or to cell type-specific effects of NO on a multitude of cellular processes including impaired $Ca^{++}$ homeostasis, or interaction with reactive oxygen species.[67–70] In general, activation of endothelial nitric oxide synthase (eNOS) and NO production induce vascular endothelial permeability through activation of guanylate cyclase (GC), production of cyclic guanosine 3′,5′-cyclic monophosphate (cGMP), and activation of protein kinase G (PKG) (*Figure 2*).[20,21,60,71] In addition, the hyperpermeability response to these agents or to NO donors is prevented by MLCK inhibitors, indicating that NO-cGMP signalling lies upstream of MLCK activation. Also, NO may activate MLCK by elevating intracellular calcium levels.[70] In HUVEC cells, treatment with VEGF causes activation of NO, PKG and subsequent activation of Raf-1, mitogen-activated protein kinases, and extracellular regulated kinase (ERK1/2).[72,73] This suggests that NO- and PKG-dependent activation of MLCK is signalled through MAP/ERK (MEK) kinases (*Figure 2*). PKC is also an important signalling molecule in endothelial hyperpermeability responses. Treatment of bovine pulmonary artery endothelial monolayers with the phorbol ester 12-phorbol,13-myristate acetate (PMA) causes a dose-dependent increase in PKC activation accompanied by increased transendothelial albumin flux.[63] In HUVEC monolayers, PKC activation worked synergistically with elevated intracellular $Ca^{2+}$ to increase MLC phosphorylation and hyperpermeability,[40] demonstrating that MLCK-dependent and -independent signalling pathways contribute to MLC phosphorylation and hyperpermeability. Furthermore, both pathways may be modulated by PKC.

Endothelial hyperpermeability may also mediated by MLCK-dependent activation of stores operated $Ca^{++}$ (SOC) channels.[74,75] In pulmonary artery or brain capillary endothelial cells, MLCK activates stores-operated membrane transient receptor potential (TRPC) $Ca^{++}$ channels, thereby increasing junctional permeability.[76,77] Application of the MLCK inhibitor ML-9 prevents activation of TRPC and vascular leakage.[76,78] This suggests that activation of TRPC channels depends upon MLCK activity, and that MLCK-dependent endothelial hyperpermeability depends upon $Ca^{++}$ entry following MLC phosphorylation. On the other hand, activation of MLCK and subsequent MLC phosphorylation is also dependent upon intracellular $Ca^{++}$.[79]

The neutrophil is an important inducer of endothelial hyperpermeability.[80,81] Although the signalling events involved in neutrophil-endothelial cell interaction have been extensively studied, the molecular mechanisms by which neutrophils cause microvascular leakage have not been fully established. Conventional theories in this area emphasize neutrophil migration-mediated mechanical disruption

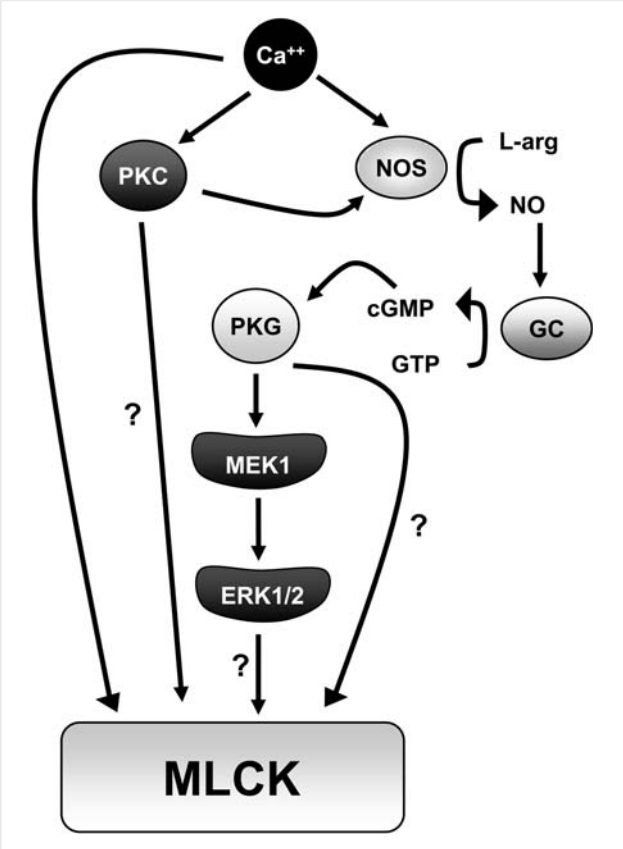


**Figure 2** MLCK activation in endothelial hyperpermeability. Endothelial MLCK is activated in response to multiple cell signalling events including elevated intracellular $Ca^{++}$, protein kinase C (PKC) activation, and signalling through nitric oxide synthase (NOS)- dependent production of NO, and guanylate cyclase (GC)-dependent production of cGMP. Activation of cGMP-dependent protein kinase (PKG) activates MLCK through activation of MEK1 and ERK1/2. Possible direct activation by PKC, PKG, or ERK1/2 is represented by question marks.

of endothelium, which is dependent on proteases released from neutrophils [82–84] during transendothelial migration via paracellular and/or transcellular routes.[85] Fluorescence microscopy images of transcellular diapedesis indicate that transendothelial pore formation only occurs at surfaces in close contact with invading leucocytes, suggesting that leucocyte transcellular migration is not accompanied by serum leakage.[86,87] In addition, convincing ultrastructural evidence by Lewis and Granger[88] demonstrates paracellular neutrophil transmigration across microvessel endothelium in the absence of serum protein extravasation. Therefore, neutrophil transmigration may not necessarily cause hyperpermeability.

Neutrophil adhesion to vascular endothelial cells (CVEC or HUVEC) induces tyrosine phosphorylation of adherens junction proteins, and increases cytosolic $Ca^{2+}$-dependent opening of intercellular junctions, and endothelial hyperpermeability.[89,90] Because $Ca^{2+}$-calmodulin activates MLCK,[10,33] we investigated MLCK-mediated MLC phosphorylation and the contractile cytoskeleton in microvascular endothelial cells in response to activated neutrophils. In CVEC monolayers, exposure to C5a-activated neutrophils induced MLCK-dependent transendothelial albumin flux.[91] Activated

neutrophils induced a concentration- and time-dependent phosphorylation of MLC at Thr-18/Ser-19 shown on urea gels, which was significantly abrogated by pre-treatment with ML-7 or by transference of an MLCK-inhibiting peptide.[91–93] Further, exposure of endothelial cells to activated neutrophils resulted in increased contractile stress fibre formation and intercellular gaps as shown by immunocytochemical staining. Both the neutrophil-induced MLC phosphorylation and stress fibre formation are substantially attenuated through inhibition of MLCK with ML-7.[91] This suggests that inhibiting MLCK-mediated MLC phosphorylation improves vascular barrier function during inflammatory injury. We have seen that increased isometric force is generated in confluent CVEC monolayers in the presence of activated neutrophils that closely parallels decreased TER, and that both effects are abrogated by ROCK inhibitors.[93] We also found that activated neutrophils potentiate the hyperpermeability effect caused by MLCK transference.[91] Therefore, mechanisms in addition to MLCK activation may be involved in neutrophil-induced microvascular barrier dysfunction, including RhoA-ROCK-mediated MLCP inhibition (*Figure 2*).[18,35]

## 6. MLCK in isolated, intact microvessels

In order to interrogate the molecular mechanisms of endothelial permeability in exchange microvessels under physiologically relevant conditions, we developed an isolated and perfused venule model.[94] Briefly, this model entails dissection of postcapillary venules 20–50 μm in diameter from living tissues. The vessel is cannulated with a pipette-in-pipette system and is perfused with a physiological salt solution containing fluorescently labelled albumin under a selected perfusion pressure and flow rate. The apparent permeability coefficient of albumin (Pa) is determined based on the ratio of albumin flux to its transmural concentration difference.[95] This approach enables quantitative assessment of the permeability of intact microvascular endothelium in its native environment, where physical forces and chemical conditions are tightly controlled, and extrinsic confounding factors are eliminated.[94] Other applications include direct observation and real-time quantification of neutrophil–endothelium interactions. This model has advantages over cultured endothelial cells in that microvessels isolated from living tissues behave with permeability characteristics that more closely resemble *in vivo* systems. On the other hand, *in vivo* studies do not provide specific information needed to understand the molecular bases for physiological phenomena. The isolated microvessel preparation is better suited to address mechanistic questions about vascular endothelium-specific processes because there is limited interference from other cell types or systemic factors as would occur *in vivo*.

Using the isolated microvessel technique, we have examined the effects of MLCK activity on endothelial barrier function in porcine coronary venules as well as microvessels from rodent skeletal muscle and mesentery.[51] An interesting finding is that the MLCK inhibitor ML-7 significantly reduced basal permeability to FITC-albumin. The inhibitory effect of ML-7 was dose-dependent and persisted in the presence of this inhibitor. In contrast, treatment with calyculin-A increased MLC phosphorylation and significantly increased the basal vascular permeability.[51,58] We postulate that MLCK-mediated actomyosin activity plays a role in maintaining basal barrier function in intact microvascular endothelium.

We have previously shown that NO production and cGMP mediate shear stress- and agonist-induced hyperpermeability responses.[94,96,97] In isolated venules, increasing intraluminal flow velocity induces hyperpermeability, which is abrogated by the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) or mimicked by the NO precursor L-arginine.[94] In a similar fashion, histamine increases coronary venular permeability through a phospholipase C (PLC)-NOS-cGMP signalling cascade (*Figure 2*).[96] VEGF also increases microvascular permeability via increased NO synthesis and subsequent PKG activation.[97] VEGF binding to its membrane receptor KDR initiates PLC-mediated cytosolic $Ca^{2+}$ elevation and PKC activation, activating eNOS and inducing venular hyperpermeability.[62] In as much as the cytoskeleton is a ubiquitous structural end point for intracellular signalling events, this indicates the possibility that MLCK activity is downstream of the NO-cGMP cascade. Using isolated microvessels, we have demonstrated that activated MLCK is a critical mediator of NO- or cGMP-induced microvascular hyperpermeability.[51] Venule hyperpermeability in response to the NO donor sodium nitroprusside (SNP), or the PKG activator 8-bromoguanosine 3′,5′-cyclic monophosphate (8Br-cGMP) is substantially attenuated by MLCK inhibition. It is not clear how PKG activates MLCK, however, in isolated coronary venules, VEGF-, histamine-, SNP-, or 8Br-cGMP-induced hyperpermeability are attenuated by treatment with U0126 or PD98059, indicating signalling through MEK1 and ERK1/2.[98]

To study the MLCK-dependent mechanism in PKC-induced endothelial dysfunction, which is known to occur in the early stages of diabetes,[99] we treated coronary venules with the PKC activator PMA.[51] PMA-induced hyperpermeability was considerably reduced by ML-7 in a dose-dependent manner, reinforcing the notion that activated MLCK is a common downstream effector in executing the hyperpermeability effects of many signalling mediators.

As indicated above, neutrophil binding to endothelium causes elevated intracellular $Ca^{2+}$, and hence $Ca^{2+}$-calmodulin *in vitro*.[89,90] Because $Ca^{2+}$-calmodulin increases MLCK activity,[10,33] we investigated the microvascular endothelial responses to activated neutrophils focusing on MLCK-mediated MLC phosphorylation and contractile cytoskeleton. In isolated coronary venules, we found that perfusion of microvessels with C5a-activated neutrophils induced a time- and concentration-dependent increase in albumin permeability.[91] Inhibition of MLC phosphorylation by treatment with ML-7 significantly attenuated neutrophil-induced hyperpermeability. Based on the endothelial cell transfection technique, we have further developed the technique with an enhanced capacity to transfect large molecules or proteins into intact microvessels.[100] We demonstrated inhibition of neutrophil-induced hyperpermeability in microvessels transfected with either an MLCK-inhibiting peptide or dominant negative (purified, inactivated by proteolysis) MLCK.[91] Taken together, these results confirm the importance of MLCK signalling in neutrophil-mediated hyperpermeability at the microvascular level.

## 7. MLCK and hyperpermeability *in vivo*

*In vivo* studies are regarded as the most realistic representations of actual biological conditions. Models used to study microvascular permeability include intravital microscopy in mesenteric tissues that

are semi-transparent, displaying well-defined vessels.[95,101] Using such preparations, it is possible to spread the tissue across the microscope visual field, to measure fluorescent tracer flux out of the vessels, while the tissue remains connected to a live anaesthetized animal. The hamster cheek pouch is another commonly used model, where the time-dependent tracer distribution in the intravascular vs. extravascular space is monitored and tracer flux measured as an indicator of permeability.[102] In hamster cheek pouch vessels, agonists that elevate endothelial intracellular NO/cGMP and increase paracellular permeability include platelet activating factor (PAF),[103] ADP,[104] and bradykinin. By inhibiting eNOS or its downstream signalling, microvascular permeability to water and macromolecules is significantly reduced. Therefore, NO production and cGMP are important for inducing hyperpermeability *in vivo*, as we have shown in isolated microvessels and in cultured cells.

Our *in vivo* studies have focused on rodent models of full-thickness burns covering 25–40% total body surface area. Severe burns are a common form of trauma that often induces a systemic inflammatory response affecting multiple organs.[23,105–107] The reaction is initiated by overproduction of inflammatory mediators, many of which target the microvasculature leading to impaired blood-tissue perfusion and exchange. As a cardinal component of systemic inflammation, microvascular leak occurs not only at the local wound, but also in distal tissues, especially in the splanchnic microvessels.[108,109] Plasma fluid loss and accumulation in tissues result in hypovolemic shock, pulmonary oedema, abdominal compartment syndrome, and generalized tissue malperfusion that ultimately lead to multiple organ failure.[109,110] Our previous *in vivo* studies show that plasma extravasation in the splanchnic microvasculature is significantly increased following burns.[111] Consistent with the *in vivo* observation, our experiments with endothelial monolayers and isolated microvessels show that endothelial permeability is increased by circulating factors released during burn injury.[18,112] However, clinical studies have shown that targeting specific inflammatory pathways has limited efficacy in treating burn oedema. Likewise, we have seen that pharmacological inhibition of signalling molecules generally considered to lie upstream of the hyperpermeability response, such as Src and PKC, has negligible inhibitory effects on burn-induced microvascular leakage.[111] This is not surprising considering the wide spectrum of extracellular inflammatory mediators and intracellular signalling intermediaries that cause endothelial hyperpermeability,[113–118] notwithstanding that there is crosstalk between parallel signalling pathways. These events can compensate for each other such that selective inhibition of individual pathways may not be sufficient to block the massive, collective detrimental response. We believe that a better therapeutic strategy for treatment of burn oedema is one that specifically targets common terminal effectors of these signalling pathways. Our studies show that treatment with ML-7 blocks MLC phosphorylation and significantly attenuates burn-induced venular hyperpermeability in a dose-dependent manner.[111] This finding supports our hypothesis that MLCK is a common endpoint effector for multiple signalling pathways triggered by circulating inflammatory factors that induce endothelial hyperpermeability in trauma.

The construction of MLCK-210 knock-out mice[119] has enabled *in vivo* testing of the hypothesis that nmMLCK kinase activity is necessary for microvascular barrier response to stress or injury. The lungs are frequently involved in trauma- or sepsis-induced inflammation,[18,120] and we have observed that pulmonary microvessels are particularly susceptible to hyperpermeability in response to inflammatory mediators. Studies of human populations show altered susceptibility to acute lung injury or acute respiratory distress syndrome associated with single nucleotide polymorphisms in the mylk1 gene, in the regions specifically coding nmMLCK.[121,122] This suggests that lung hyperpermeability during trauma or sepsis is specifically mediated by nmMLCK isoforms. In addition, MLCK210 knockout animals have lowered susceptibility to septic injury, especially in the lung tissue.[119] Therefore, we investigated the specific role of nmMLCK in microvascular hyperpermeability during severe burns using MLCK210 knockout mice.[123] When compared with wild-type mice that show substantially increased albumin transflux and hydraulic conductivity (Lp) after severe burns, microvascular hyperpermeability was significantly attenuated in MLCK210 knockouts and accompanied by improved survival.[123] This study provides strong *in vivo* evidence that nmMLCK mediates microvascular barrier dysfunction in response to systemic inflammation during severe traumatic injury. Further studies of endothelial-specific overexpression of MLCK2 in transgenic mice shows enhanced serum protein leakage into lung tissue during sepsis or injury, suggesting that individual nmMLCK isoforms mediate endothelial hyperpermeability *in vivo*.[124]

# 8. Summary

Microvascular endothelial barriers face diverse challenges in the form of physical forces, chemical factors, and circulating cells, and are critical for maintaining fluid/electrolyte homeostasis and physiological organ function. As the initiator and consequence of many diseases and disorders associated with microvascular inflammation, disruption of endothelial integrity is a critical problem that is difficult to correct clinically. The research efforts of several laboratories spanning decades has revealed that endothelial barrier dysfunction is generated by an imbalance between interendothelial adhesive forces and actomyosin-based centripetal tension. MLCK is a central regulator of actomyosin-based contractile cytoskeleton for a multitude of inflammatory cell signalling pathways. In this review, we provided experimental evidence ranging from *in vitro* to *ex vivo* and *in vivo* that supports the concept that MLCK is a common mediator for microvascular endothelial barrier dysfunction induced by several signalling molecules (e.g. NO/GMP and PKC), activated neutrophils, and severe thermal injury. Notably, MLCK-mediated MLC phosphorylation does not account for the action of all inflammatory agonists. There is evidence that MLCK-dependent actomyosin contractile mechanisms do not contribute significantly to PAF- or bradykinin-induced hyperpermeability in rat venular microvessels.[49] Additionally, evidence suggests that the role of actomyosin cytoskeletal contraction in controlling endothelial permeability *in vivo* is different from endothelial cell monolayers *in vitro*. For example, in cultured endothelial cells, inhibition of the ROCK/MLC cascade exhibits a strong protective effect on the endothelial barrier during lethal toxin-induced inhibition of Rac1 activity, an effect believed to stabilize the endothelial barrier at the level of VE-cadherin.[125] In the same study, antagonism of actomyosin contractility did not prevent toxin-induced hyperpermeability. Hence the mechanisms that mediate endothelial hyperpermeability may be more complex for intact vessels *in vivo* than for cell monolayers *in vitro*. Given the central role of nmMLCK in mediating microvascular leakage associated with the many inflammatory conditions described above, targeting nmMLCK as a therapeutic intervention may prove an effective alternative for treating these problems. Thus far, clinical use of non-specific MLCK inhibitors such as ML-7 and

ML-9 has not been possible, due to systemic complications and non-specific effects on other kinases. Optimally, more specific small molecule or peptide inhibitors of nmMLCK isoforms can be designed that selectively target endothelial tissues and prevent oedema. More recent evidence regarding the protective effects of sphingosine-1-phosphate (S1P) indicate that activation of the receptor SIPR1 or of downstream tyrosine kinases may offer other approaches to therapeutic inhibition of endothelial nmMLCK activity, and suppression of vascular hyperpermeability.[126,127]

**Conflict of interest:** none declared.

## Funding

This work is supported by the National Institutes of Health grants HL61507, HL70752, HL73324, HL84542, and HL96640.

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