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Myosin Light Chain Kinase Signaling in Endothelial Barrier Dysfunction

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Abstract

Microvascular barrier dysfunction is a serious problem that occurs in many inflammatory conditions, including sepsis, trauma, ischemia–reperfusion injury, cardiovascular disease, and diabetes. Barrier dysfunction permits extravasation of serum components into the surrounding tissue, leading to edema formation and organ failure. The basis for microvascular barrier dysfunction is hyperpermeability at endothelial cell–cell junctions. Endothelial hyperpermeability is increased by actomyosin contractile activity in response to phosphorylation of myosin light chain by myosin light chain kinase (MLCK). MLCK-dependent endothelial hyperpermeability occurs in response to inflammatory mediators (e.g., activated neutrophils, thrombin, histamine, tumor necrosis factor alpha, etc.), through multiple cell signaling pathways and signaling molecules (e.g., Ca^{++}, protein kinase C, Src kinase, nitric oxide synthase, etc.). Other signaling molecules protect against MLCK-dependent hyperpermeability (e.g., sphingosine-1-phosphate or cAMP). In addition, individual MLCK isoforms play specific roles in endothelial barrier dysfunction, suggesting that isoform-specific inhibitors could be useful for treating inflammatory disorders and preventing multiple organ failure. Because endothelial barrier dysfunction depends upon signaling through MLCK in many instances, MLCK-dependent signaling comprises multiple potential therapeutic targets for preventing edema formation and multiple organ failure. The following review is a discussion of MLCK-dependent mechanisms and cell signaling events that mediate endothelial hyperpermeability.

Keywords

myosin light chain kinase (MLCK); protein kinases; edema; endothelial barrier dysfunction; microvascular permeability

1. INTRODUCTION

The microvascular endothelium is a protective barrier that controls the exchange of fluid and plasma components between blood and biological tissues (for review see[1, 2]). Under inflammatory conditions, or upon exposure to inflammatory agents (e.g., histamine, thrombin, etc.), microvascular endothelial (micro-VE) barriers are compromised, leading to tissue edema, decreased perfusion, and multiple organ failure. Micro-VE barrier dysfunction

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permits plasma components and blood cells (e.g., neutrophils) to infiltrate surrounding tissues. This occurs in many adverse conditions, including acute lung injury (ALI), cardiovascular disease/atherosclerosis, dermal burn injury, ischemia–reperfusion (I/R), and traumatic brain injury (TBI), affecting the lungs, heart, gut, brain, or other tissues.

Endothelial barrier dysfunction is triggered by misregulation of intracellular signaling events that normally maintain barrier integrity, or by inappropriate activation of cell signaling pathways that induce endothelial hyperpermeability.[1, 3–5] In many cases, cell signaling events that induce barrier dysfunction act by increasing the activity of myosin light chain kinase (MLCK). A normal amount of basal MLCK activity is required to maintain physiological microvascular permeability. In contrast, pathologically elevated MLCK activity induces microvascular barrier dysfunction (reviewed in[3]). Appropriate control of MLCK activity in endothelium is necessary for preventing barrier dysfunction in inflammatory conditions.

The main biological function of MLCK is to phosphorylate the regulatory myosin light chain (MLC-2).[3, 6] Phosphorylation of MLC-2 at Ser-19, and subsequently at Thr-18, induces ATP-dependent actomyosin contraction (Fig. 1)[3, 7, 8]; MLCK-dependent actomyosin contractility regulates arteriolar vasoconstriction, and capillary/venule permeability. MLCK-dependent actin-myosin mediated cytoskeletal contraction followed by endothelial cell membrane retraction and intercellular gap formation is the classical mechanism of endothelial barrier dysfunction. In some instances, MLCK activity causes barrier dysfunction through phosphorylation and decreased expression of endothelial cell–cell junction proteins, via mechanisms that are poorly understood. The following review is an examination of cell signaling events related to MLCK-dependent endothelial barrier dysfunction, and is directed at understanding the role of MLCK in microvasculature under inflammatory conditions.

2. PHYSIOLOGY, STRUCTURE, AND REGULATION OF MLCK AT ENDOTHELIAL BARRIERS

A. Endothelial Cell–Cell Junctions and Hyperpermeability

The bases for microvascular barrier function are endothelial intercellular junctions that restrict the passage of plasma components into the tissue interstitial space.[1, 9, 10] While plasma components can be transported across endothelial barriers in a transcellular fashion, facilitated by vesicles or transporter proteins at the endothelial cell membrane, most plasma extravasation is paracellular, through cell–cell junctions. Endothelial cell–cell junctions consist of integral membrane proteins expressed on the lateral surfaces of adjacent endothelial cells that interact via extracellular domains to form a barrier to the passage of solutes between cells. On the intracellular side, junction proteins are linked to adaptor proteins that are in turn connected to the actin cytoskeleton. These linkages are a physical basis for control of junction integrity, translating cytoskeletal tension into retraction at cell–cell interfaces.

Microvascular cell–cell junctions consist of adherens junctions (AJs) and tight junctions (TJs). AJs are present in the vascular endothelium of most organs and tissues,[10–12] whereas TJs are found in the microvasculature of specialized tissues (e.g., the blood–brain barrier, or blood–retinal barrier).[13–15] AJs are composed of VE-cadherin and junction adhesion molecules (JAMs), connected intracellularly to the actin cytoskeleton via catenins (α, β, γ, and p120).[12, 16–18] TJs have additional specialized junction proteins: occludin and claudins,[13, 19, 20] anchored to the actin cytoskeleton via zona occludens (ZO-1 or ZO-2), and α-catenin.[12, 16–18]

Control of cell–cell junction permeability is dependent upon stability and organization of the actin cytoskeleton. Cytoskeletal actin filaments are stabilized by cross-linking proteins (e.g., spectrin or filamen), and are regulated by molecules that control actin polymerization (e.g., cofilin, gelsolin, or heat shock protein (HSP27)). In addition to their connections at cell–cell junctions, the opposing ends of actin filaments are connected via actin-linking proteins (e.g., talin, paxillin, and vinculin) to transmembrane integrins at focal adhesions.[16, 18, 21] Focal adhesions anchor endothelium to the basement membrane extracellular matrix, and provide a fulcrum for actin cytoskeletal tension to control cell–cell junction permeability.[17, 22] Therefore, endothelial hyperpermeability can be mediated in three ways, through (i) loss of cell–cell or cell–matrix adhesion due to decreased expression or impaired function of junction proteins, cross-linker proteins, or focal adhesion proteins, (ii) cytoskeletal instability and reorganization, or (iii) actomyosin contractile forces and tension propagated by the actin cytoskeleton.

B. MLCK-Dependent Hyperpermeability

Cytoskeletal tension is controlled dynamically by the phosphorylation status of MLC, which is the result of a balance between phosphorylation by MLCK and dephosphorylation by MLC phosphatase (MLCP) (Fig. 1).[3, 18] MLC phosphorylation permits ATP-dependent mechanochemical interaction between actin and myosin, which increases cytoskeletal tension and causes endothelial hyperpermeability.[23–25] Conversely, MLC dephosphorylation by MLCP decreases actomyosin contractility, which relaxes the actin cytoskeleton and decreases paracellular permeability.[26] In addition, MLCK and MLCP are controlled reciprocally by phosphorylation: MLCK activity is increased while MLCP activity is decreased. MLCP is phosphorylated and inactivated by Rho kinase, downstream of the Rho GTPase, RhoA (Fig. 1).[12, 27, 28] MLCP inhibition increases MLC phosphorylation, actomyosin contractility, and endothelial hyperpermeability in a manner that is MLCK-dependent, but does not require increased MLCK activity.

C. MLCK Isoforms in Endothelium

MLCK is a member of the immunoglobin superfamily of proteins (see[3, 8]). All MLCKs are derived from the genes *mylk1–3*, on human chromosomes 3 (3q21), 20 (20q13.31), or 16 (16q11.2), respectively,[29–31] including muscle MLCK variants: cardiac (cMLCK), skeletal (skMLCK), and smooth muscle (smMLCK), products of genes *mylk*3, *mylk*2, and *mylk*1, respectively,[6, 29, 32, 33] and nonmuscle (nmMLCK) isoforms, products of *mylk1*.[8] The structural components required for activity (catalytic kinase domain) and binding to MLC (C2-type immunoglobin-like domain) are highly conserved in all MLCK variants across species. Expression of cMLCK and skMLCK is restricted to cardiac and skeletal muscle, respectively. In contrast, smMLCK and nmMLCK are highly expressed in endothelium, and several other tissues.

smMLCK and nmMLCK, splice variants derived from *mylk*1, differ in that nmMLCK isoforms have a 922-amino acid insertion near the N-terminus.[34] Hence, these variants have markedly different molecular weights: 108 kD for smMLCK and approximately 210 kD for nmMLCK, referred to as MLCK108 and MLCK210, respectively.[35, 36] The nmMLCK isoforms (MLCK1–4), originally known as endothelial MLCK (eMLCK),[34] are variations based on the 1914 amino acid sequence of the prototypic isoform, MLCK1.[8] Compared to MLCK1, MLCK2 lacks a 69-amino acid regulatory region containing two sites for tyrosine phosphorylation by p60Src.[8,37] MLCK 3a and 3b lack 51 amino acids corresponding to exon 30, and are otherwise identical to MLCK1 and MLCK2, respectively. Only partial sequence information is available for MLCK4, and little is known about the function of this isoform. MLCK1–4 have broad tissue distribution, however, MLCK1 and MLCK2 are the most abundant nmMLCK isoforms in endothelial tissue.

D. MLCK Activity and Regulation

The principle function of MLCK activity is to phosphorylate MLC. Control of MLCK activity is a basis for cellular regulation of actomyosin contractile activity and permeability at endothelial intercellular junctions. Ca^{2+}/calmodulin are central to control of MLCK activity[6,38] in that calmodulin binding to MLCK causes release of MLCK autoinhibition.[32,36,39] In smooth muscle, Ca^{2+} binding to calmodulin activates smMLCK and induces actin-myosin contraction. However, in endothelium and other nonmuscle cell types, Ca^{2+}/calmodulin binding alone is not sufficient to elicit actomyosin contraction, and requires additional signaling.[34] For example, MLCK1 can be activated by tyrosine kinase-mediated phosphorylation at Tyr-464 and Tyr-471.[7,40,41] The 922 amino acid insertion to the N-terminal region of nmMLCK contains multiple consensus phosphorylation recognition sequences for protein kinases including protein kinase A (PKA), protein kinase C (PKC), and calmodulin kinase II (CaMKII).[7,10,37,42] Signaling to MLCK through these pathways is crucial for barrier function in vascular endothelium (summarized in Fig. 1).

E. MLCK-Dependent Maintenance of Basal Microvascular Permeability

Elevated MLCK activity causes endothelial hyperpermeability and endothelial barrier dysfunction, however, tonic MLCK activity is important for maintenance of basal microvascular permeability. In isolated porcine coronary venules as well as microvessels from rodent skeletal muscle and mesentery, basal microvessel permeability to fluorescein isothiocyanate (FITC)-albumin is significantly attenuated by the MLCK inhibitor ML-7.[43,44] In addition, treatment with the protein phosphatase (PP1/PP2A) inhibitor calyculin-A increases both MLC phosphorylation and vascular permeability, supporting the notion that residual MLCK activity is present and maintains normal endothelial permeability in intact microvasculature.

In cultured endothelial cell monolayers, MLCK inhibition often has no effect on basal transendothelial albumin flux as seen in rat heart or lung micro-VE cells, or on transendothelial electrical resistance (TER) as seen in bovine brain micro-VE cells.[45–47] This lack of responsiveness may be due to inherent differences between intact microvessels and cultured endothelial monolayers, or to phenotypic heterogeneity between endothelial cells.[9] For example, Curry and Adamson have noted that FITC-albumin leakage occurs adjacent to a very small percentage of endothelial cells in microvessels in situ.[9,48] Hence homogeneous populations of cultured endothelial cells may display barrier properties that differ from those of heterogeneous populations of endothelial cells in intact microvessels. Barrier properties may also vary between endothelial cells originated from different tissues. In particular, endothelial cells derived from nonexchange vessels, for example, human umbilical vein endothelial cells (HUVEC) or aortic endothelial cells, may display permeability responses that differ from that of exchange microvessels. Endothelial cells in vivo also receive signals from cells in the surrounding tissue. This is well-established for brain endothelial cells where the presence of astrocytes enhances endothelial barrier function, in part by increasing endothelial expression of junction proteins,[49,50] or for skin endothelium, where contraction of surrounding fibroblasts modulates microvascular permeability by regulating compaction of the extracellular matrix.[51] These dynamic interactions with surrounding nonendothelial cells in situ further explain why some treatments can increase or decrease endothelial permeability differently in cultured micro-VE cells versus microvessels in vivo.

3. MLCK-DEPENDENT SIGNALING AND ENDOTHELIAL HYPERPERMEABILITY

A. Ca^{++} Channels

In many cases, the molecular basis of MLCK-induced hyperpermeability is not well-understood, and involves mechanisms other than actomyosin contractility, including activation of Ca^{++} channels.[11] For example, in pulmonary artery or brain capillary endothelial cells, hyperpermeability is mediated by activation of store-operated membrane transient receptor potential (TRPC) Ca^{++} channels (Fig. 2).[52,53] Activation of TRPC is prevented by treatment with the MLCK inhibitor ML-9, or by agents that either disrupt or stabilize actin filaments. Therefore, activation of TRPC requires both MLCK activity and the intact actin cytoskeleton. MLCK-dependent endothelial hyperpermeability may also depend upon activation of store-operated Ca^{++} (SOC) channels, and subsequent decreased expression, or modification, of junction proteins or cytoskeletal linker proteins (Fig. 2). Activation of SOC channels is triggered by depletion of Ca^{++} from intracellular endoplasmic reticulum (ER) stores, and MLCK activity prior to SOC channel activation depends on Ca^{++} released from the ER in response to inositol 1,4,5-trisphosphate receptor (IP_3R) activation. IP_3R activation leads to phosphorylation of MLC, and MLC phosphorylation-dependent phosphorylation of TJ proteins occludin and claudin-5.[54,55] MLCK-dependent phosphorylation of occludin and claudin-5 occurs at the blood-brain barrier (BBB) during the hyperpermeability responses to controlled cortical impact TBI, or exposure to C-reactive protein (CRP) or methamphetamine, suggesting a general role for this mechanism in BBB endothelial hyperpermeability.[56–58] However, a causal relationship between MLCK activation and SOC channel activation is challenged by the recent observation in immortalized mouse brain endothelial (bend.3) cells that treatment with the TRPC channel inhibitor SKF 96365 prevents both MLC phosphorylation and junction hyperpermeability following hypoxia.[53] Ca^{++}-dependent events independent of MLCK activation also contribute to endothelial hyperpermeability. For example, in lung endothelium, TRPC Ca^{++} channel activation by thrombin induces actin stress fiber formation and hyperpermeability, possibly through activation of RhoA kinase.[10,59]

B. Protein Kinase C

Signaling for endothelial hyperpermeability in response to a variety of inflammatory agents is dependent upon PKC (Fig. 3).[1,5,10–12] However, signaling through PKC is complex involving effects on cytoskeletal organization and contractility, Ca^{++} channels, and multiple cell signaling pathways, including opposing roles for specific PKC isoforms in controlling endothelial hyperpermeability. Classical PKC isoforms α and β are well-known inducers of hyperpermeability. For example, PKC-induced endothelial dysfunction in the progression of diabetes requires $PKC\beta_{II}$.[60–62] In isolated coronary venules, KDR receptor-mediated vascular endothelial growth factor (VEGF)-induced hyperpermeability is prevented by the phospholipase C (PLC) inhibitor U-73122, or by the classical PKC isoform-selective inhibitor bis-indolylmaleimide (BIM). These treatments also prevent VEGF-induced tyrosine phosphorylation of endothelial nitric oxide synthase (eNOS), suggesting that PKC-mediated hyperpermeability is signaled through increased nitric oxide (NO) synthesis (Fig. 3).[63] VEGF binding to KDR also initiated PLC-mediated cytosolic Ca^{2+} elevation, which may be necessary for activation of classical (Ca^{++}-dependent) PKC isoforms ($\alpha/\beta_{I/II}/\gamma$) and subsequent hyperpermeability. In coronary venules, PKC-induced hyperpermeability is also significantly attenuated by ML-7.[43] Therefore, PKC-induced microvascular permeability in isolated coronary venules may occur partially via activation of MLCK. In support of these observations, we observe synergy between elevated intracellular Ca^{2+} and PMA treatment to increase MLC phosphorylation and hyperpermeability in cultured endothelial (HUVEC) cell

monolayers.[40] Therefore, activation of PKC isoforms and elevated cytosolic Ca^{++} may act in concert to signal MLCK-dependent microvascular hyperpermeability.

Because of the existence of at least 11 PKC isoforms and heterogeneous responses of endothelial cells from different vascular segments, PKC signaling for hyperpermeability is complex. Studies of hypoxia in rat brain micro-VE cells show that increased PKC activity and expression of multiple PKC isoforms (β_{II}/γ /η/μ/θ/λ) may contribute to endothelial hyperpermeability during hypoxia/I/R injury.[64,65] However, in mouse brain endothelium, TNF-α induced hyperpermeability is mediated through PKC-α.[66] Similarly, blood–retinal barrier dysfunction may be mediated through PKC-β_{II},[67,68] PKC-δ,[69] or PKC-ζ.[70] In contrast, coronary endothelial hyperpermeability is achieved either by overexpression of PKCβ_{II} or by inhibition of PKCδ,[61] and inflammatory cytokine-dependent hyperpermeability requires both classical PKC and MLCK activity.[47] Other investigators have shown in bovine aortic endothelial cells that VEGF-induced hyperpermeability is prevented by PKCζ-dependent phosphorylation of eNOS (at Thr497) and inhibition of eNOS activity,[71] or that thrombin-induced eNOS phosphorylation (at Ser1179) and activation is inhibited by expression of dominant negative PKCα or PKCδ.[72] Therefore, individual PKC isoforms may induce or prevent endothelial hyperpermeability by phosphorylation of eNOS at sites that either stimulate (Ser1179) or inhibit (Thr497) eNOS activity (Fig. 3).

Clearly, the notion that the effects of Ca^{++} and PKC are mediated through the effects of eNOS on MLCK is an oversimplification. VEGF induced PLC-mediated hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) leads to diacylglygerol (DAG)-mediated effects on classical and novel PKC isoforms, and IP_3-induced release of Ca^{++} from intracellular stores. Release of ER Ca^{++} activates MLCK (through calmodulin), eNOS, classical PKCs, and many other signaling molecules that affect MLCK activity or cytoskeletal organization, leading to endothelial hyperpermeability (reviewed extensively elsewhere).[10,11]

C. NO and Cyclic GMP

There is significant debate about the effects of NO and guanosine 3′, 5′-cyclic monophosphate (cGMP) which induce hyperpermeability in intact microvessels, yet are protective against hyperpermeability in cultured endothelial cells.[73–75] In porcine coronary venules, NO production and cGMP mediate endothelial shear stress- and agonist-induced hyperpermeability responses.[76′78] For example, histamine increases permeability through a PLC′NOS′cGMP signaling cascade (Fig. 3), where permeability is inhibited by the PLC inhibitor 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC), the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA), or the guanylate cyclase inhibitor LY83583, respectively.[76] The NO donor sodium nitroprusside (SNP) or cGMP analog 8Br-cGMP also induce hyperpermeability to albumin, indicating dependence on NO and cGMP-dependent protein kinase (PKG).[78,79] Hyperpermeability in response to these agonists is significantly attenuated by treatment with 10 μM ML-7. Although nmMLCK has consensus sites for phosphorylation by PKG,[7] there is no conclusive evidence that MLCK is directly phosphorylated by PKG. Rather, evidence suggests that PKG-dependent activation of MLCK is mediated by mitogen-activated protein kinases (MAPK), and extracellular-regulated kinase (ERK1/2) (Fig. 3). For example, in HUVEC cells, the Raf-1/MAPK/ERK1/2 signaling cascade is activated by PKG downstream of NO, in response to VEGF treatment.[80,81] Furthermore, in isolated coronary venules, VEGF-, histamine-, SNP- or 8Br-cGMP-induced hyperpermeability responses are decreased by inhibition of MEK1 and ERK1/2 with U0126 or PD98059.[82]

In endothelial cell culture systems, NO and cGMP decrease MLC phosphorylation, and decrease endothelial permeability.[83,84] Several laboratories have suggested that control of

endothelial permeability by cGMP is due to cross-talk between cGMP and cAMP.[75,85] The cAMP-dependent protein kinase, PKA suppresses nmMLCK activity and hyperpermeability in most endothelial systems.[34,86] In HUVEC cells, differential effects of PKG on phosphodiesterase isoforms can increase or decrease the amount of cellular cAMP in a biphasic fashion dependent upon the concentration of cGMP.[87] Thus, the hyperpermeability-inducing versus protective effects of NO-cGMP could be explained by tissue/cell-specific differences in expression or regulation of phosphodiesterase isoforms, resulting in increases or decreases in cellular cAMP in response to cGMP (Fig. 3). This is supported by studies of ventilator-induced lung injury in mice showing that pathological expression of the phosphodiesterase isoform PDE2A in the pulmonary endothelium, coupled with cGMP production, leads to increased hydrolysis of cAMP.[88] Therefore, PKG-dependent activation of MLCK and hyperpermeability in some endothelial systems may be signaled through decreased cAMP and decreased PKA activity. A recent study in HUVEC cells, using a fluorescence resonance energy transfer (FRET)-based indicator of cAMP, demonstrated that increased cytosolic Ca^{++} in response to thrombin treatment also decreases cAMP levels through modulation of adenylate cyclase isoforms (AC5/6).[89] Therefore, decreased cAMP may contribute to endothelial hyperpermeability in trauma or prothrombotic conditions.

Direct protective effects of cGMP on endothelial junction integrity are also reported. In rat coronary endothelial cells, treatment with the NO donor DEA-nonoate or the soluble guanylate cyclase activator HMR1766 causes Ca^{++} sequestration into the ER through phosphorylation of phospholamban and subsequent activation of the ER Ca^{++} ATPase, leading to decreased intercellular gap formation and increased MLC phosphorylation during I/R.[83] Conversely, inhibition of PKG with KT5823 prevents Ca^{++} sequestration and increases gap formation during I/R injury in these cells. Therefore, PKG activity may affect MLC phosphorylation (and intercellular gap formation) by limiting cytosolic Ca^{++} available for MLCK activation.

NO affects multiple signaling pathways, and therefore the effects of NO are not confined to signaling through cGMP. For example, NO modifies the activity of many proteins by S-nitrosylation at cysteine residues.[90] NO combines with reactive oxygen species (ROS) to form the highly reactive species, peroxynitrate ($ONOO^-$) (Fig. 3).[91] NO also impairs mitochondrial function by competing with O_2 for binding to complex IV of the electron transport chain, and can thereby interfere with mitochondrial Ca^{++} sequestration, contributing to a rise in cytosolic Ca^{++}.[92] NO affects many other aspects of cell Ca^{++} homeostasis, including ER Ca^{++} loading or membrane Ca^{++} channel activity, and NOS activity is Ca^{++}-dependent.[93] Because of the brief and unstable existence of NO, the effects of NO are often localized to specific subcellular or membrane regions where NOS isoforms are expressed.[90] Therefore, while NO-induced cGMP production is central to control of endothelial hyperpermeability, the effects of NO signaling on MLCK-dependent actomyosin contraction and endothelial hyperpermeability may be exceptionally complex.

4. PATHOPHYSIOLOGICAL RELEVANCE OF MLCK-DEPENDENT ENDOTHELIAL HYPERPERMEABILITY

A. Leukocytes

In several inflammatory conditions, activated neutrophils induce endothelial hyperpermeability.[22,94,95] Some investigators have argued that neutrophil-induced endothelial hyperpermeability is a prerequisite for neutrophil extravasation.[95,96] Yet, in some instances there is a lack of temporal correlation between leukocyte adhesion and vascular leakage.[97] In general, leukocyte transendothelial migration occurs via two routes: transcellular or paracellular.[98] Fluorescence microscopy images of leukocytes migrating

across endothelium in a transcellular fashion show podosomes in close apposition with the endothelial vesicular membrane, suggesting that transcellular migration is not accompanied by gap formation or leakage.[99,100] Classic histological evidence by Lewis and Granger has shown that neutrophil transmigration across microvessel endothelial wall in a paracellular fashion occurs in the absence of serum protein extravasation.[101] In addition, in a rat aseptic dermal injury model with fluorescent-labeled neutrophils and albumin, endothelial hyperpermeability and neutrophil transendothelial migration were temporally separated events, where neutrophil transmigration did not cause nor was accompanied by hyperpermeability.[102] It has been suggested that cell junction leakage of chemoattractants is necessary to attract leukocytes to sites of inflammation.[103] However, this is refuted by the observation that the chemokine interleukin-8 (IL-8) must be presented at the endothelial luminal surface, by vesicle-mediated transcytosis from the basolateral to the apical cell membrane, for IL-8 to attract leukocytes. Together, these lines of evidence indicate that neutrophil transmigration and neutrophil-induced endothelial hyperpermeability are separate and not necessarily related events.

In isolated coronary venules, activated neutrophil-induced hyperpermeability to albumin is significantly attenuated by treatment with ML-7, indicating the involvement of MLCK.[104] The requirement for MLCK in this process was demonstrated in isolated, perfused coronary venules transfected with either an MLCK-inhibiting peptide or dominant negative, inactivated MLCK.[104,105] Following transfection with either agent, activated neutrophils were incapable of inducing microvessel hyperpermeability. Although these transfections were directed at the microvessel endothelium, it is possible that decreased neutrophil-induced hyperpermeability is due to inhibition of MLCK in neutrophils. This is supported by the observation that nmMLCK$^{-/-}$ neutrophils fail to attach to wild-type lung endothelium during lipopolysaccharide (LPS)-induced lung injury in mice.[106] Related to these phenomena, we have observed that endothelial hyperpermeability is attenuated in nmMLCK −/− aortic endothelial cell monolayers in response to thrombin, but that monocyte transendothelial migration across these monolayers is unaffected by endothelial MLCK gene knockout.[107] Thus, monocyte transmigration across aortic endothelium is not dependent upon endothelial MLCK or hyperpermeability. The relative importance of MLCK-dependent signaling in endothelium versus neutrophils with regard to neutrophil-induced endothelial hyperpermeability and neutrophil transmigration remains to be determined.

Neutrophil-induced endothelial hyperpermeability is dependent upon neutrophil granule secretion, including the release of many hyperpermeability-inducing compounds, for example, proteases, elastase, arachadonic acid, serum proteins, and ROS.[95] ROS are additionally produced within endothelial cells by NAPH oxidase or xanthine oxidoreductase, therefore, the combination of ROS from intracellular and extracellular sources can be potent mediators of neutrophil-induced endothelial hyperpermeability.[91] In brain endothelial cells, ROS generated in response to interleukin-17, CRP, or hypoxia activate MLCK.[45,57,108] ROS generation is dependent upon p38MAPK activity in response to CRP, and upon cytosolic Ca^{++} in response to hypoxia. In porcine brain capillary endothelial cells, hydrogen peroxide (H_2O_2) increases junctional permeability via a pathway dependent upon ERK1/2 activity downstream of intracellular Ca^{++} release.[109] In pulmonary endothelial cell monolayers, neutrophil-induced endothelial hyperpermeability is dependent upon ROS signaling through PKCα-dependent activation of the transient receptor potential melastatin cation channel (TRPM2).[110,111] Therefore, neutrophil-induced ROS generation may cause endothelial hyperpermeability through PKCα-dependent activation of Ca^{++} channels, and activation of MAP/ERK kinases. ROS activates many other signaling molecules that could play a role in endothelial hyperpermeability. For example, CaMKII is rapidly autophosphorylated and activated by exposure to H_2O_2,[112,113] and is capable of phosphorylating MLCK. Oxidative stress also affects RhoA (known to inhibit MLCP and to induce actin stress fiber formation),

though the specific mechanism for RhoA activation by ROS is not known.[91] In brain micro-VE cells, H_2O_2 decreases actin stress fiber formation and decreases expression of TJ proteins, occludin, and claudin-5.[114] These effects are prevented by inhibition of RhoA, PI3 kinase, or Protein Kinase B (PKB) (Akt), suggesting that multiple control mechanisms mediate ROS-induced endothelial hyperpermeability.

B. Severe Burn Injury

Severe burns cause systemic inflammation, leading to systemic inflammatory response syndrome (SIRS) characterized by microvascular leak in tissues far removed from the site of injury.[115–118] Leakage of fluid across microvascular barriers decreases blood volume, leading to hypovolemic shock and poor tissue perfusion, and increases fluid accumulation in tissues, leading to pulmonary edema, abdominal compartment syndrome, and multiple organ failure.[119–122] In clinical patients, these pathologies can be further exacerbated by accompanying smoke inhalation, or infection and sepsis, precipitating further ALI, inflammation, and edema. In addition, the splanchnic microvessels are highly susceptible to vascular leakage during severe dermal burn injury, and are an excellent model for examining microvascular permeability to albumin in rodents.[123] Microvascular permeability in the gut microvasculature can be examined either in isolated perfused venules, or in vivo in the intact splanchnic mesentery using fluorescence intravital microscopy.[124,125] The time-dependent distribution of fluorescent FITC-albumin in the intravascular versus extravascular space is monitored and used as an indicator of permeability.[126] In rat splanchnic mesenteric microvasculature, FITC-albumin leakage is significantly increased over 6 hr following a 25% total body surface area (TBSA) full-thickness scald burn.[127] In these experiments, the splanchnic microvascular is far-removed from the injury site, suggesting that hyperpermeability-inducing factors produced by the dermal burn injury travel in the blood circulation and affect microvascular permeability remotely in the gut. To test this hypothesis, isolated, perfused mesenteric venules were treated with plasma collected from rats following burn injury. In this study, burn plasma significantly increased venule permeability to albumin, compared to venules treated with plasma obtained from unburned animals. Therefore, endothelial hyperpermeability is induced by circulating factors present in the plasma during burn injury. In humans and rodents, circulating factors released into the plasma during burn injury include multiple inflammatory cytokines that are known to induce endothelial hyperpermeability.[128] However, blocking specific individual inflammatory cytokine pathways has not yielded effective clinical treatments for burn-induced microvascular dysfunction.[129] Consistent with this observation, treatment of isolated perfused venules with inhibitors of intermediate inflammatory signaling molecules, for example, Src or PKC, fails to prevent burn plasma-induced microvascular leakage.[127] Based on this evidence, we suspect that multiple inflammatory signaling pathways operate in parallel during burn injury to induce endothelial hyperpermeability. A better therapeutic strategy would be to identify and target common terminal effectors of multiple inflammatory pathways that mediate the hyperpermeability response. Because multiple extracellular inflammatory mediators and intracellular signaling intermediaries can cause endothelial hyperpermeability by increasing MLCK-dependent MLC phosphorylation, MLCK is a strong candidate target for preventing burn-induced microvascular dysfunction.[130–135] This notion is supported by the observation that treatment of isolated venules with ML-7 blocks MLC phosphorylation and significantly attenuates burn-induced venular hyperpermeability.[127] In addition, albumin leakage is fully attenuated in the splanchnic mesentary of MLCK210 knockout mice following severe dermal burn injury, compared to that of wild-type mice,[136] further indicating that endothelial MLCK is required for burn-induced endothelial hyperpermeability.

C. Trauma and Sepsis

The lungs are especially vulnerable to trauma- or sepsis-induced inflammation, and are frequently involved in multiple-organ failure.[18,137] Increased susceptibility to ALI or acute respiratory distress syndrome is associated with single nucleotide polymorphisms (SNPs) in the human *mylk1* gene.[138,139] Moreover, SNPs associated with ALI in this study, occurred at loci corresponding to regions that are unique to nmMLCK, whereas SNPs in regions also encoding smMLCK were unrelated to ALI. This suggests that lung microvascular hyperpermeability during ALI is mediated by nmMLCK isoforms. In support of this suggestion, MLCK210 knockout mice have lowered susceptibility to sepsis-induced lung injury.[140] In a subsequent study, endothelial-specific transgenic overexpression of MLCK2 in mice greatly enhanced serum protein extravasation into lung tissue during LPS-induced sepsis, or mechanical ventilator injury.[141] Although artifacts are associated with protein overexpression models, this suggests that MLCK2 contributes to lung vascular hyperpermeability in response to sepsis or trauma. Thus, a likely future direction for studies of microvascular dysfunction is to distinguish the respective contributions of MLCK isoforms to endothelial hyperpermeability.

D. Chronic Inflammatory Diseases

Endothelial barrier dysfunction also occurs in chronic inflammatory conditions including atherosclerosis, BBB dysfunction in multiple sclerosis, and diabetic microvascular complications in several organs including the peripheral circulation and the blood–retinal barrier. Interestingly, in an apolipoprotein E deficient mouse model of atherosclerosis, macrophage deposition and albumin (Evans Blue) leakage across the aortic wall were attenuated by nmMLCK (−/−) gene knockout.[107] Further mechanistic studies revealed that MLCK-dependent barrier dysfunction in aortic endothelium is only partially dependent upon MLCK kinase activity, and that MLCK participates in endothelial barrier dysfunction through both kinase activity-dependent and kinase activity-independent molecular mechanisms.

5. PROTECTIVE STRATEGIES AND THERAPEUTIC TARGETS IN MLCK-DEPENDENT ENDOTHELIAL HYPERPERMEABILITY

A. Sphingosine-1-Phosphate (S1P)

S1P, a derivative of sphingomyelin, enhances the barrier properties of lung endothelium, and protects against hyperpermeability.[142,143] S1P in the circulation is supplied by blood cells, including erythrocytes and activated platelets, helping to maintain VE integrity. In mice, selective knockout of erythrocyte SIP eliminates S1P from the blood circulation, causing increased basal endothelial leakage and decreased resistance to hyperpermeablility in response to PAF or histamine.[144] S1P binds to G-protein coupled S1P receptor isoforms (S1PR1–5).[143] In a study of LPS-induced toxicity with an S1P analog FTY720, and SIPR knock-out mice, it was determined that the protective effects of SIP against vascular leakage in the lungs are mediated by G_i-coupled SIPR1.[145] In human pulmonary artery endothelial cells, SIP binding to S1PR1 recruits nmMLCK and multiple tyrosine phosphorylated proteins to caveolin-1 containing lipid rafts in the plasma membrane, and increases TER.[146] Recruitment to lipid rafts brings nmMLCK in close proximity with the actin binding proteins cortactin and β-catenin. S1P treatment also triggers tyrosine phosphorylation of both nmMLCK (Y^{464}) and cortactin (Y^{486}) by the actin-binding tyrosine kinase c-Abl, and promotes nmMLCK binding to cortactin coinciding with localization to the peripheral actin ring.[147,148] Because MLCK1 and MLCK2 are the most abundant nmMLCK isoforms in endothelium, phosphorylation at Y^{464} indicates that the protective effects of S1P are mediated through MLCK1. However, in human pulmonary endothelial cells, it was shown

that S1P treatment induces tyrosine kinase-dependent mobilization of cortactin and MLCK2 to the cell periphery.[149] In this study, it is hypothesized that the tyrosine kinase-dependent association of MLCK with cortactin strengthens the cortical actin band, and improves endothelial barrier function. However, recruitment to the cell membrane also removes MLCK from its usual location within the cell, thereby decreasing the amount of nmMLCK available to induce actomyosin contraction in response to inflammatory agents.

B. MLCK as a Therapeutic Drug Target

Microvascular barrier dysfunction is a consequence of highly complex molecular events and signaling cascades affecting intercellular junctions and cell-matrix adhesion. Many of these events are signaled through MLCK. Therefore, the ability to selectively and appropriately inhibit MLCK activity in vascular endothelium could be useful for treatment of inflammatory conditions. The pharmacological inhibitors, ML-7 and ML-9, target the ATP binding site of MLCK, which is highly homologous with other kinases, including PKA and PKC.[150] In addition, these compounds broadly affect all MLCK isoforms in many tissue types, and cause unintended physiological effects in vivo. Hence, these compounds are not suitable for clinical use. Because of this, Watterson and co-workers synthesized a 6-phenyl-3-aminopyridazine compound that inhibits MLCK more specifically at low micromolar concentrations, and prevents LPS-induced Evans blue extravasation and edema in the lungs of mice.[151,152] This compound is an improvement over previous MLCK inhibitors because of increased specificity for MLCK.

It is also possible to generate highly specific peptide inhibitors of MLCK. For example, the peptide “membrane permeant inhibitor of MLCK” (PIK) is a fragment of the MLCK inhibitory domain that specifically inhibits MLCK without affecting other kinases such as PKA.[150] Inhibition of MLCK with PIK prevents albumin flux through epithelial TJs in the gut during T-cell activation-induced diarrhea in mice.[153] Therefore, with improved methods of delivery, inhibition of MLCK with engineered peptides could be a useful therapeutic approach. Optimally, engineered peptides would be delivered specifically to endothelial tissue, and designed to target specific MLCK isoforms. In support of this claim, MLCK210 knock-out mice are resistant to endothelial hyperpermeability, and show no significant decline in cardiovascular function.[154] This suggests that targeting specific nmMLCK isoforms would have fewer adverse physiological consequences than does nonselective inhibition of MLCK activity.

C. Targeting Signaling Upstream of MLCK

An alternative strategy for therapeutic intervention in vascular barrier dysfunction is to target cell signaling events that modulate MLCK-dependent endothelial hyperpermeability.[155] Many therapeutic compounds currently in clinical use are kinase inhibitors.[112] Therefore, selective kinase inhibition may be a useful approach for improving vascular barrier function. For example, p60Src phosphorylates and activates nmMLCK isoforms 1 and 3a in response to thrombin exposure. Src inhibition or src gene knockout also protect mice from vascular leakage at the BBB in experimental cerebral ischemia.[156] This suggests that Src kinase is a worthwhile target for inhibition of MLCK activity and attenuation of vascular hyperpermeability in some inflammatory conditions. Other cell signaling pathways decrease MLCK activity and preserve endothelial barrier function. For example, treatments that increase cellular cAMP, such as inhibition of PDE2,[157] may improve vascular function, as it has long been known that cAMP decreases MLCK activity and attenuates endothelial hyperpermeability. Further evidence indicates that S1P signaling through the S1P receptor (SIPR1) improves endothelial barrier function by recruiting MLCK to the cortical actin cytoskeleton.[146,147,149] Thus, S1P signaling may contain additional molecular targets for inhibition of MLCK-dependent vascular barrier dysfunction.

6. CONCLUSION

Endothelial barrier dysfunction is an integral component of many inflammatory conditions and disease states, including severe burn injury, trauma, and sepsis. Microvascular leakage and tissue edema, due to compromised barrier integrity, are serious pathophysiologies associated with inflammation that are difficult to correct clinically. This is in part due to the lack of effective molecular targets for therapeutic treatment. The data presented here support the conclusion that MLCK activity by way of MLC phosphorylation is a central mediator of endothelial barrier dysfunction. MLCK is an endpoint mediator of microvascular barrier dysfunction in response to many cell signaling pathways triggered by inflammatory mediators, activated neutrophils, and severe trauma or sepsis. Because MLCK is a downstream effector of multiple signaling pathways leading to endothelial hyperpermeability, selective inhibition of MLCK is a reasonable strategy for correcting microvascular barrier dysfunction. More specifically, nmMLCK isoforms mediate endothelial hyperpermeability in response to many inflammatory stimuli, and should be further investigated as drug targets for preventing or treating vascular dysfunction and edema. In addition, signaling pathways that protect endothelial barriers by suppressing MLCK activity, such as the S1P pathway, should be investigated to reveal the downstream molecular mechanisms that prevent MLCK activation. Finally, because endothelial hyperpermeability can be induced by MLCK-independent mechanisms, hyperpermeability-associated events such as actin stress fiber formation, and phosphorylation or decreased expression of cell junction or cytoskeletal adapter proteins need to be further investigated to determine if these events are physiologically important for microvascular barrier dysfunction independent of MLCK activity.

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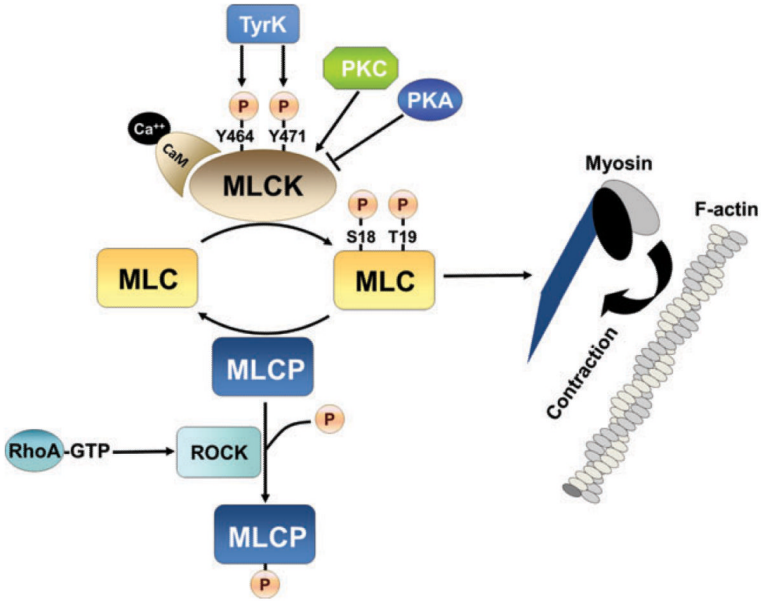

Figure 1.

Myosin light chain kinase (MLCK)-dependent control of actin-myosin contraction in endothelium. Increased myosin light chain (MLC) phosphorylation at Ser-18 and Tyr-19 in response to MLCK activation or myosin light chain phosphatase (MLCP) inhibition increases MLC ATPase-driven force generation relative to actin. MLCK activity is increased by Ca^{2+}-calmodulin binding, and/or by kinase-dependent phosphorylation including phosphorylation by protein kinase C (PKC) or tyrosine kinase phosphorylation at Tyr-464 and Tyr-471. MLCK activity is decreased in response to protein kinase A (PKA) activity. Inhibition of MLCP is achieved by ROCK activation downstream of RhoA activation, resulting in phosphorylation-dependent inhibition of MLCP.

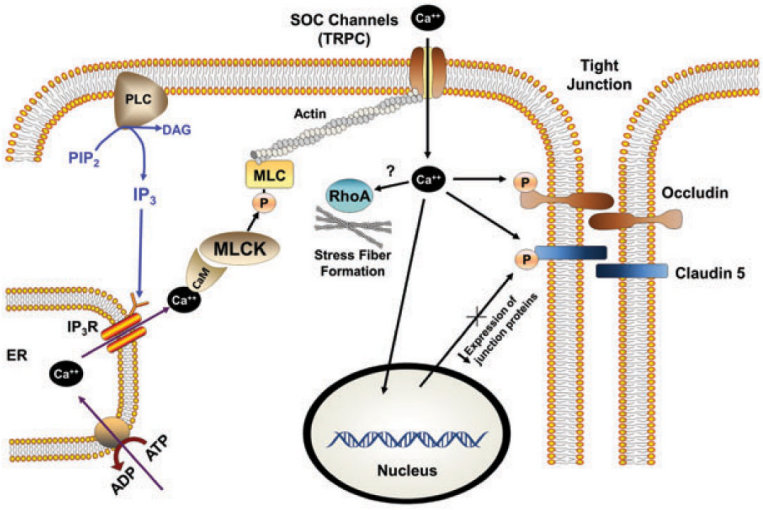

Figure 2.

Myosin light chain kinase (MLCK)-dependent endothelial tight junction (TJ) hyperpermeability signaled through store-operated Ca^{++} (SOC) channel activation. Intracellular stores (endoplasmic reticulum (ER)) Ca^{++} released in response to inositol triphosphate (IP_3) receptor (IP_3R) activation by inositol triphosphate (IP_3), downstream of phospholipase C (PLC)-mediated hydrolysis of phosphotidylinositol bisphosphate (PIP_2), increases MLCK activity through Ca^{++} binding to calmodulin (CaM). MLCK activity in turn activates SOC channels, further increasing cytosolic Ca^{++} concentration, resulting in phosphorylation of occludin and claudin-5, transcriptional downregulation of junction proteins and increased actin stress fiber formation.

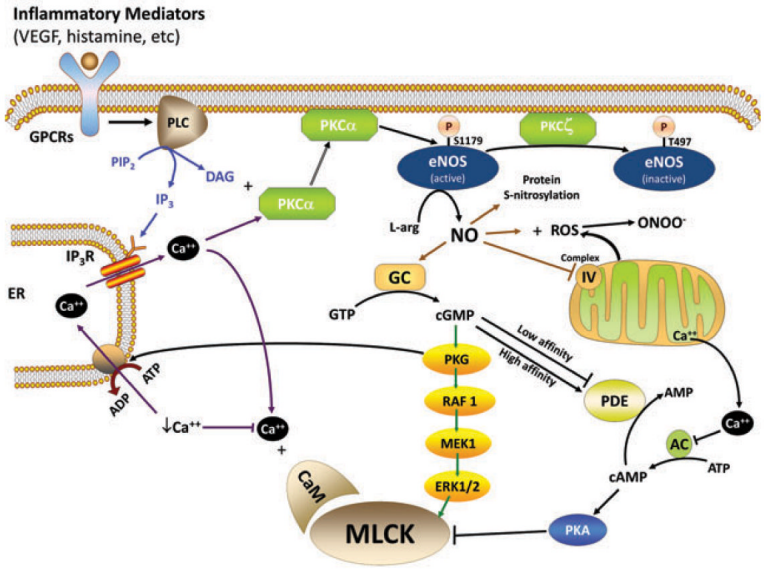

Figure 3.

MLCK signaling in endothelial hyperpermeability. MLCK activity is increased by multiple signaling pathways in response to inflammatory mediators, downstream of G-protein coupled receptor (GPCR) activation. Activation of phospholipase C (PLC) leads to hydrolysis of phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 triggers intracellular Ca^{++} release from intracellular stores (endoplasmic reticulum (ER)) via activation of the IP_3 receptor. Stores Ca^{++} release activates MLCK directly through Ca^{++} binding to calmodulin (CaM). DAG and Ca^{++} together activate PKC-α and other classical PKC isoforms. PKC-α activates endothelial nitric oxide synthase (eNOS) (inhibited by PKC-ζ mediated phosphorylation at T497) by phosphorylation at S1179, to increase nitric oxide (NO) production. NO activates many signaling processes, including NO combines with reactive oxygen species (ROS) to form peroxynitrite ($ONOO^-$); NO causes S-nitrosylation of numerous proteins; NO impairs function of complex IV of the mitochondrial oxidative phosphorylation system causing release of sequestered Ca^{++}; and NO activates guanylate cyclase (GC) to increase conversion of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). cGMP can increase or decrease endothelial permeability through various mechanisms depending upon the cell type examined. cGMP activates MLCK through activation of PKG, which may be mediated through activation of the Raf-MEK-ERK pathway. cGMP may also modulate phosphodiesterase activity to increase or decrease abundance of cAMP and correspondingly affect PKA activity.