Myosin Light Chain Phosphorylation in Neutrophil-Stimulated Coronary Microvascular Leakage

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Abstract—Neutrophil-induced coronary microvascular leakage represents an important pathophysiological consequence of ischemic and inflammatory heart diseases. The precise mechanism by which neutrophils regulate endothelial barrier function remains to be established. The aim of this study was to examine the microvascular endothelial response to neutrophil activation with a focus on myosin light chain kinase (MLCK)-mediated myosin light chain (MLC) phosphorylation, a regulatory process that controls cell contraction. The apparent permeability coefficient of albumin (Pa) was measured in intact isolated porcine coronary venules. Incubation of the vessels with C5a-activated neutrophils induced a time- and concentration-dependent increase in Pa. The hyperpermeability response was significantly attenuated during inhibition of endothelial MLC phosphorylation with the selective MLCK inhibitor ML-7 and transfection of a specific MLCK-inhibiting peptide. In contrast, transfection of constitutively active MLCK elevated Pa, which was abolished by ML-7. In addition to the vessel study, albumin transendothelial flux was measured in cultured bovine coronary venular endothelial monolayers, which displayed a hyperpermeability response to neutrophils and MLCK in a pattern similar to that in venules. Importantly, neutrophil stimulation caused MLC phosphorylation in endothelial cells in a time course closely correlated with that of the hyperpermeability response. Consistently, the MLCK inhibitors abolished neutrophil-induced MLC phosphorylation. Furthermore, immunohistochemical observation of neutrophil-stimulated endothelial cells revealed an increased staining for phosphorylated MLC in association with contractile stress fiber formation and intercellular gap development. Taken together, the results suggest that endothelial MLCK activation and MLC phosphorylation play an important role in mediating endothelial barrier dysfunction during neutrophil activation. (Circ Res. 2002;90:1214-1221.)

Key Words: microvascular permeability ■ neutrophil-endothelium interaction ■ signal transduction

Coronary microvascular barrier dysfunction represents an early pathological event in the development of ischemic disease, reperfusion injury, diabetic cardiomyopathy, atherosclerosis, and inflammatory disease of the heart. The disorder is largely attributed to the elaboration of inflammatory mediators as well as the activation of polymorphonuclear leukocytes (PMNs), predominantly neutrophils. In response to injurious or inflammatory stimulation, neutrophils undergo a series of kinetic and metabolic changes, characterized by adherence to the venular endothelium, followed by transendothelial migration and release of vasoactive mediators.1–4 Despite the great efforts that have been dedicated to the identification of neutrophil-derived agonists and their second messengers,5–7 our knowledge is rather limited regarding the precise molecular mechanism by which activated neutrophils cause microvascular barrier dysfunction. In this regard, conventional concepts of neutrophil-induced vascular leakage emphasize mechanical disruption of the endothelial barrier due to neutrophil migration and release of proteases.8–9 Not until recently has the active role of endothelial cells been fully appreciated. It is now recognized that neutrophil-endothelium interaction elicits a dynamic process involving rapid signaling reactions and reversible conformational changes in the endothelium.10–11 Structurally, cytoskeletal rearrangement and junctional reorganization have been reported in endothelial cells on neutrophil stimulation.12–16 Consistently, our previous studies have demonstrated that neutrophil adherence to coronary venular endothelial cell monolayers causes tyrosine phosphorylation of adherens junctional proteins, which is associated with intercellular gap formation and endothelial hyperpermeability.17 The same observation also revealed significant stress fiber formation in endothelial cells exposed to activated neutrophils, indicating a possible involvement of the endothelial contractile process. However, whether this cytoskeletal response contributes to neutrophil-dependent barrier dysfunction remains inconclusive. Therefore, the purposes of the present study were (1) to examine molecular reactions occurring at the endothelial contractile cytoskeleton...
in response to neutrophil stimulation and (2) to specify the contribution of cytoskeletal contraction to neutrophil-dependent increase in venular endothelial permeability.

In endothelial cells, actin-myosin contraction is mainly mediated by myosin light chain kinase (MLCK)-catalyzed myosin light chain (MLC) phosphorylation.\textsuperscript{18–21} We have recently shown that transfection of constitutively active MLCK induces MLC phosphorylation coupled with hyperpermeability in endothelial cells,\textsuperscript{22} whereas inhibition of MLC phosphorylation with an MLCK antagonist greatly attenuates the increase in venular permeability in response to soluble inflammatory mediators.\textsuperscript{23} The present study extends these original investigations to a neutrophil-dependent regulatory pathway. The endothelial permeability was measured in intact isolated coronary venules in combination with cultured endothelial cells derived from the same type of microvasculature, which enabled a close correlation between molecular events and vascular function under the same experimental conditions. The results provide direct evidence of the role of endothelial MLC phosphorylation in the mediation of neutrophil-induced leakage in coronary exchange vessels.

**Materials and Methods**

**Isolation of Coronary Venules**

Pigs weighing 9 to 13 kg were anesthetized with sodium pentobarbital (30 mg/kg IV) and heparinized (250 U/kg IV). A left thoracotomy was performed, and the heart was electrically fibrillated, excised, and placed in 4°C physiological saline. The technique of isolation and cannulation of coronary venules has been described in detail in our previous publications.\textsuperscript{23–25} Briefly, a venule 20 to 50 μm in diameter was dissected and cannulated with a micropipette on each end, with a third smaller pipette inserted into the inflow micropipette. Each micropipette was connected to a reservoir to allow independent control of intraluminal perfusion pressure and flow. The vessel was interchangeably perfused with either physiological salt solution through the outer pipette or the same perfusate allowing for the study of neutrophil-dependent endothelial responses. Compared with C5a, the effects of platelet-activating factor (PAF) on neutrophil dynamics and permeability were examined in the same isolated microvessel model.\textsuperscript{22} The results showed that PAF was able to induce microvascular hyperpermeability by activating both the endothelium and neutrophils. The dual effect of PAF renders it of limited utility in obtaining information exclusive to neutrophil-dependent events.

**Urea Gel and Western Blot Analyses**

Cells were lysed in a Tris-HCl lysis buffer containing 1% Triton X-100 and subjected to urea PAGE, followed by immunoblotting with a monoclonal anti-MLC antibody (clone MY-21), as previously described,\textsuperscript{22,27} to intact microvessels is described in our previous publications.\textsuperscript{23,25} The apparent solute permeability coefficient of albumin (Pa) was calculated by using the following equation: Pa = (1/ΔL) × [(dI/dt) × (r/t)], where ΔL is the initial step increase in fluorescent intensity, (dI/dt) is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel, and r is the venular radius. In each experiment, the venule was perfused under a relatively constant perfusion pressure of 10 cm H\textsubscript{2}O and a flow velocity of 7 mm/s. Samples were discarded if fluorochrome leakage was detected.

**In Vitro Permeability Assay**

Coronary venular endothelial cells were harvested from the bovine heart and routinely maintained in a culture medium containing 2% FBS.\textsuperscript{37,22} For permeability assays, cells were seeded at 10^4 cells/cm\textsuperscript{2} on gelatin-coated Costar Transwell membranes (VWR International) and grown to confluence. Fluorescently labeled BSA was added to the top (luminal) chamber at 10 mg/mL and a flow velocity of 7 mm/s. Samples were collected from both the luminal and abluminal (bottom) chambers and analyzed with a fluorescence microplate reader. Sample readings were converted with a standard curve to albumin concentration. The permeability coefficient of albumin was based on the following equation: Pa = [A] × x × I/A × V/L, where [A] is albumin concentration, t is time in seconds, A is area of the membrane in cm\textsuperscript{2}, V is volume of the abluminal chamber, and L is luminal concentration.\textsuperscript{38} Control experiments were performed to measure tracer flux across the gelatin-coated microporous filter without cells. Monolayers that failed to form an effective barrier, as indicated by a >20-fold decrease in Pa, were discarded.

**Protein Transfection of Venules and Cells**

The technique of transfecting proteins to endothelial cells as well as to intact microvessels is described in our previous publications.\textsuperscript{22,27–29} Briefly, a cannulated venule was perfused at a constant perfusion pressure gradient of 20 cm H\textsubscript{2}O for 1 hour with a transfection mixture containing the polycation reagent TransIT (Pan Vera) at 10 μL/mL and a specific MLCK-inhibiting peptide (Calbiochem) at 10^-4 to 10^-3 mol/L or truncated MLCK protein (TMLCK) at 5 μg/mL. The latter was isolated from chicken gizzards, purified by affinity chromatography, and activated by digesting with trypsin as previously described,\textsuperscript{22,26} which generated an MLCK protein fragment that was active in the absence of calcium/calmodulin.\textsuperscript{31} After transfection, the vessel was washed with the regular perfusate and then subjected to neutrophil or chemical stimulation. The same procedure was used for cell transfection.

**Isolation and Activation of Neutrophils**

Porcine neutrophils were isolated and purified as previously described.\textsuperscript{32,33} For activation, neutrophils were mixed with human recombinant C5a (10^-8 mol/L) and added to the suffusion bath at 10^7 per mL. The stimulus intensity of C5a was derived from previous dose-response studies by us and others in which an optimal effect on neutrophil activation and interaction with microvascular endothelium was observed at 10^-8 mol/L.\textsuperscript{33,34} The selection of C5a as the neutrophil activator was based on the finding that human C5a cross-reacts with porcine neutrophils, leading to respiratory burst and chemotaxis.\textsuperscript{35} Furthermore, the fact that C5a exerts a minimal direct effect on porcine microvascular endothelium\textsuperscript{33,35,36} makes it a unique probe for the study of neutrophil-dependent endothelial responses. Compared with C5a, the effects of platelet-activating factor (PAF) on neutrophil dynamics and permeability were examined in the same isolated microvessel model.\textsuperscript{22} The results showed that PAF was able to induce microvascular hyperpermeability by activating both the endothelium and neutrophils. The dual effect of PAF renders it of limited utility in obtaining information exclusive to neutrophil-dependent events.

**Immunocytochemistry**

Coronary venular endothelial cells were grown to confluence on gelatin-coated coverslips and incubated with C5a-activated neutrophils at 10^6 cells/mL for 10 minutes. Cells were immediately fixed with 2% paraformaldehyde for 15 minutes and permeabilized with 0.2% Triton X-100 for 2 minutes. For double labeling of actin and MLC, cells were incubated for 30 minutes with rhodamine phalloidin (Molecular Probes) at 10 U/mL and rabbit polyclonal anti-phospho-MLC (Santa Cruz) at 1:100 dilution, followed by an FITC-labeled anti-rabbit antibody. Coverslips were then mounted on slides for fluorescence microscopic observation.
Results

Incubation of coronary venules with C5a-activated neutrophils induced a significant increase in permeability in a time- and concentration-dependent pattern (Figure 1). The hyperpermeability response occurred rapidly within minutes, reached a peak at 5 to 10 minutes, and gradually declined to the control level at 30 to 40 minutes after adding neutrophils. Inhibition of MLC phosphorylation by pretreatment with the selective MLCK inhibitor ML-7 greatly attenuated neutrophil-induced hyperpermeability (Figure 2). A similar inhibitory effect was observed in venules that were pretreated with an MLCK-inhibiting peptide (Figure 3). To test whether MLCK activation mimicked the hyperpermeability effect of neutrophils, venules were transfected with MLCK for dominant activation of MLCK. As shown in Figure 4, the transfection increased Pa by 2- to 3-fold, which lasted for at least 2 hours. The effect was specific to the transected tMLCK because treatment with the same transfection mixture in the absence of tMLCK did not significantly alter the basal permeability. Furthermore, the increase in Pa caused by tMLCK transfection was dose-dependently attenuated by ML-7 (Figure 5). The presence of neutrophils potentiated the hyperpermeability effect of MLCK transfection (data not shown), indicating that mechanisms in addition to MLCK activation might be involved in the venular response to neutrophils.

Cultured coronary venular endothelial cell monolayers displayed similar permeability responses to neutrophils and to the MLCK activator or inhibitor (Figure 6). In particular, incubation with C5a-activated neutrophils (10⁶ cells/mL) for 10 minutes elevated Pa by 2-fold. This effect was significantly reduced by either pretreatment with ML-7 or transfection with MLCK-inhibiting peptide. In contrast, transfection of active MLCK produced a hyperpermeability effect similar to that seen with neutrophils (Figure 6).
Urea gel electrophoresis showed that activated neutrophils induced MLC phosphorylation in a concentration-dependent (Figure 7, top panel) and time-dependent (Figure 7, middle panel) manner, correlating with that of the permeability response in intact venules. Western blot analysis of neutrophil-stimulated endothelial cells further indicated that the phosphorylation occurred at Thr18 and Ser19 in MLC (Figure 7, bottom panel). The response was prevented by pretreatment with ML-7 (10^{-7} mol/L) or transfection of MLCK-inhibiting peptide (5 \mu g/mL) and mimicked by transfecting tMLCK (5 \mu g/mL). In some control experiments, incubation with the same concentration of neutrophils without C5a activation caused a slightly increase in MLC phosphorylation that was presumably due to the basal activity of neutrophils or a low level of activation of neutrophils during isolation.

Immunocytochemical analysis of coronary venular endothelial cells exposed to C5a-activated neutrophils revealed an increased formation of actin stress fibers and intercellular gaps (Figure 8). The staining of phosphorylated MLC appeared to be increased and was colocalized with stress fibers. The morphological changes were observed within 5 to 10 minutes after neutrophil stimulation, which was concomitant with the hyperpermeability response to neutrophils.

Discussion
The present study suggests a novel mechanism in the pathophysiological regulation of coronary microvascular exchange function. In particular, our experiments demonstrated the following: (1) C5a-activated neutrophils increased coronary venular permeability in a time- and concentration-dependent manner through the activation of MLCK. (2) The selective MLCK inhibitor ML-7 dose-dependently reduced the hyperpermeability response to tMLCK transfection in coronary venules. In the absence of ML-7, tMLCK transfection produced a Pa value of 6.71 \pm 0.33 \times 10^{-6} cm/s (259.30 \pm 12.33\% of basal permeability, P=0.001 vs basal). ML-7 reduced the Pa value to 5.21 \pm 0.59 \times 10^{-6} cm/s at 10^{-7} mol/L (201.69 \pm 23.05\% of basal value, P=0.09 vs the hyperpermeability response to tMLCK in the absence of ML-7), 3.79 \pm 0.27 \times 10^{-6} cm/s at 10^{-6} mol/L (146.44 \pm 10.26\% of basal value, P=0.001 vs the hyperpermeability response to tMLCK in the absence of ML-7), and 3.08 \pm 0.31 \times 10^{-6} cm/s at 10^{-5} mol/L (119.11 \pm 12.72\% of basal value, P=0.001 vs the hyperpermeability response to tMLCK in the absence of ML-7). Numbers in parentheses represent the numbers of vessels studied.
The paracellular permeability of the endothelial barrier is dynamically controlled by an equilibrium between the contractile force generated at the cytoskeleton and the adhesive force maintained by cell-cell junctions. Alterations in either or both structures can cause an imbalance of the competing forces, resulting in opening of the paracellular pathway for transendothelial flux of fluid and macromolecules. At the cytoskeleton, actin and myosin binding and cross-bridge movement provide a mechanical basis for the development of centripetal tension. In vascular endothelial cells, actin-myosin interaction is mainly regulated by an MLCK-dependent process. Many agonists can activate the process by increasing MLCK activity through the calcium/calmodulin signaling or by directly phosphorylating MLCK, which in turn phosphorylates the regulatory light chain of myosin at Thr18 and Ser19, resulting in increased myosin activity. Activated myosin then interacts with actin, leading to cell contraction and shape change.

Although research involving actomyosin regulation of cell morphology has increased over the past few years, the functional importance of the contractile elements in controlling microvascular permeability remains to be established. Some in vitro experiments have suggested that MLCK phosphorylation is involved in endothelial barrier modulation by inflammatory agonists, including thrombin, histamine, cyto-

Figure 6. Transfection of constitutively active MLCK or stimulation with C5a-activated neutrophils induced an increase of albumin permeability in cultured coronary venular endothelial cell monolayers. In particular, tMLCK transfection increased Pa to 259.73±11.66% of its basal value (P=0.00005 vs basal), and neutrophil (10^6 cells/mL) stimulation elevated Pa to 207.67±19.36% of the basal value (P=0.0001 vs basal). The MLCK inhibitor ML-7 (10^{-7} mol/L) reduced the PMN-induced increase in Pa to 142.40±2.71% (P=0.00005 vs basal, P=0.04 vs PMN response without inhibitors). In cells transfected with MLCK-inhibiting peptide (10^{-4} mol/L), the permeability after PMN stimulation was 121.85±5.03% of basal value (P=0.00008 vs basal, P=0.01 vs PMN response without inhibitors). Numbers in parentheses represent the numbers of vessels studied.

Figure 7. MLC phosphorylation in coronary venular endothelial cells treated with C5a-activated neutrophils. Top, Urea gel electrophoresis showing a shift of MLC from unphosphorylated form in control (column 1) to monophosphorylated and diphosphorylated forms on stimulation by C5a-activated neutrophils (columns 2 through 4). The hyperphosphorylation was prevented by either ML-7 (column 5) or MLCK-inhibiting peptide (column 6) and mimicked by transfecting constitutively active tMLCK (column 7). Middle, Urea gel analysis of the time course of MLC phosphorylation in response to activated neutrophils (10^6 cells/mL). MLC phosphorylation is indicated by the increase in the content of monophosphorylated or diphosphorylated MLC. Bottom, SDS-PAGE followed by blotting with a polyclonal antibody directed against phospho-Thr18/Ser19 of MLC. Activated neutrophils induced MLC phosphorylation at Thr18/Ser19 (column 2). The effect was diminished in endothelial cells transfected with MLCK-inhibiting peptide (column 4) or pretreatment with ML-7 (column 8). Transfection with active tMLCK upregulated MLC phosphorylation (column 5), which was not augmented or attenuated by the addition of activated neutrophils (column 6).
activation of endothelial morphology and biomechanical properties, characterized by cytoskeletal reorganization, stress fiber formation, cell contraction, and the opening of intercellular gaps.13,14,16,17,44,46 The present study has demonstrated that this cellular process occurs in coronary venules via a signaling cascade involving MLCK-catalyzed MLC phosphorylation. Activated neutrophils produce a rapid and concentration-dependent MLC phosphorylation in a time course strongly correlated with that of venular hyperpermeability, whereas blockage of MLCK abolishes the hyperphosphorylation and greatly attenuates the hyperpermeability effect of neutrophils. Although the result indicates a causal relationship between endothelial MLCK phosphorylation and barrier dysfunction, it was noted that the MLCK inhibitors failed to completely abolish the hyperpermeability response to neutrophils. Therefore, it is unlikely that MLCK phosphorylation accounts for the entire mechanism of endothelial hyperpermeability. Discerning that the addition of neutrophils potentiates an MLCK-induced increase in venular permeability further supports the involvement of other mechanisms, of which endothelial junctional disorganization remains as a potential pathway for neutrophil-induced paracellular permeability.12,15,47 Within this context, our previous experiments have shown that neutrophil adherence to venular endothelial monolayers results in a tyrosine phosphorylation–associated sequestration of VE-cadherin and β-catenin.17 Taking the data from the present study into consideration, we postulate that the junctional constituents may interact with the contractile elements to synergistically cause the barrier opening. The relative contribution of the cytoskeletal and junctional mechanisms to neutrophil-regulated endothelial permeability requires further studies.

Our investigation of neutrophil-regulated barrier function focused on coronary venules. We used an experimental approach that correlates in situ functional measurements with in vitro molecular analyses. First, the apparent permeability coefficient was measured in isolated and perfused coronary venules. This model enables a quantitative analysis of endothelial barrier function in intact physiologically functional microvessels under tightly controlled experimental conditions.23,24,29 Second, in addition to the pharmacological inhibition, protein transfection of coronary venules was applied to specify the effect of MLCK activation on endothelial permeability. The microvessel transfection technique, which was recently developed in our laboratory,28 provides a specific analysis for particular endothelial molecules in the controlling of microvascular function. Finally, the neutrophil response was compared between venules and cultured endothelial cells derived from the same type of microvessels. The present study provides a close correlation between molecular reactions at the cytoskeleton and functional changes at the microvascular level.

Neutrophil-endothelium interactions trigger a series of intracellular signaling events followed by dynamic modifica-

Figure 8. Immunocytochemistry of coronary venular endothelial cells exposed to C5a-activated neutrophils. Phalloidin-labeled actin changed from a meshwork staining pattern with significant peripheral bands in control cells (A) to centralized stress fibers in neutrophil-stimulated cells (B). There was an overall increase in the staining of phosphorylated MLC after neutrophil stimulation (D) compared with control nonstimulated cells (C), and the phosphorylated MLC appeared to be colocalized with stress fibers. Intercellular gaps were frequently observed in neutrophil-stimulated cells.
hyperpermeability response was observed during neutrophil activation at the abluminal (basement membrane) side of the venule, where no significant adhesion or migration was observed. Therefore, it is unlikely that neutrophil adhesion and migration are prerequisites for microvascular leakage in inflammation.

In summary, the present study provides experimental evidence supporting the contribution of the endothelial cytoskeleton to the pathological regulation of coronary venular barrier function. We suggest that MLCK-mediated MLC phosphorylation and actomyosin reorganization play an important role in the development of microvascular leakage during neutrophil stimulation.

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