Myosin light chain phosphorylation and pulmonary endothelial cell hyperpermeability in burns

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Tinsley, John H., Nicole R. Teasdale, and Sarah Y. Yuan. Myosin light chain phosphorylation and pulmonary endothelial cell hyperpermeability in burns. Am J Physiol Lung Cell Mol Physiol 286: L841–L847, 2004.—Major cutaneous burns result in not only localized tissue damage but broad systemic inflammation causing organ system damage distal to the burn site. It is well recognized that many problems result from the release of inflammatory mediators that target vascular endothelial cells, causing organ dysfunction. The pulmonary microvessels are particularly susceptible to functional abnormalities as a direct consequence of exposure to burn-induced inflammatory mediators. Traditional therapeutic intervention is quite often ineffective in treating burn patients suffering from systemic problems. A possible explanation for this ineffectiveness may be that because so many mediators are released, supposedly activating numerous signaling cascades that interact with each other, targeting of upstream factors in these cascades on an individual basis becomes futile. Therefore, if an end-point effector responsible for endothelial dysfunction following burn injury could be identified, it may present a target for intervention. In this study, we identified phosphorylation of myosin light chain (MLC) as a required element of burn plasma-induced hyperpermeability across rat lung microvascular endothelial cell monolayers. In addition, pharmacological inhibition of myosin light chain kinase (MLCK) and Rho kinase as well as transfection of MLCK-inhibiting peptide blocked actin stress fiber formation and MLC phosphorylation in response to burn plasma. The results suggest that blocking MLC phosphorylation may provide therapeutic intervention in burn patients with the goal of alleviating systemic inflammation-induced endothelial dysfunction.

signal transduction; permeability; pulmonary endothelium; microvasculature

BURN INJURIES provoke traumatic responses not only at the site of the wound but throughout distal organ systems. The development of systemic inflammatory response syndrome (SIRS) represents a major cause of death in patients with full-thickness burns that exceed 25% of the total body surface area (TBSA) (3). SIRS is characterized by plasma leakage at both the burn site and in distant organ systems, a reaction that peaks in the first 3 h and diminishes over the next 6–12 h (3, 6, 12). The extreme loss of blood fluid and proteins results in shock, respiratory distress syndrome, and inadequate perfusion incidents leading to multiple organ failure (1, 6). Although fluid resuscitation following a major thermal injury is a necessary component of treatment, this same intervention often exacerbates systemic edema. Understanding the molecular mechanisms governing this acute, traumatic condition may lead to therapeutic intervention during this initial stage of burn injury that would increase the chances of survival. However, pharmacological intervention targeting specific mediators involved in postburn inflammation have not proven very effective.

Our recent in vivo study reveals that perturbations in the microvascular endothelium play a role in the pathogenesis of thermal injury (8). Changes in endothelial cell function and morphology in response to external stimuli are usually propagated by signaling cascades. Endothelial barrier function is affected by many agonists, such as histamine and prostaglandins, as well as the inflammatory cells known as polymorphonuclear leukocytes (2, 24, 25, 31). In fact, SIRS is associated with an overproduction of many inflammatory mediators capable of activating intracellular signaling pathways in response to thermal injury (1, 6, 12). However, these pathways often have redundancies in function, and this may play a big role in the relative ineffectiveness of specific pharmacological treatments directed against burn-induced injury. Perhaps targeting an end-point component of these signaling mechanisms would provide a better chance of lessening the impact of postburn abnormalities.

To identify a potential therapeutic target, one must first understand how the microvascular endothelium maintains its barrier function. Transmembrane adherens junctions are the main structures preventing macromolecular leakage through the interendothelial pathway. Contractile forces generated by actomyosin interaction tend to pull the tightly connected cells apart. A delicate balance between these adhesive and contractile forces maintains the endothelial monolayer in a semipermeable state. A disruption of the equilibrium results in endothelial barrier dysfunction and subsequent microvascular leakage. We and others (9, 17, 22) have shown that phosphorylation of myosin light chain (MLC) at Ser19/Thr18 by activated myosin light chain kinase (MLCK) plays a critical role in the development and regulation of contractile forces within cells. In addition, activated Rho kinase is known to indirectly result in MLC phosphorylation at these same two residues by inactivating myosin phosphatase (19, 26). The phosphorylation condition of MLC affects the permeability of cultured endothelial cells and intact venular endothelium (5, 17, 32). Phosphorylated MLC leads to myosin association with actin stress fibers to produce cell contraction (7).

The physical process of “pulling” endothelial cells apart is a downstream event in multiple signaling pathways activated by inflammatory mediators, such as those generated by thermal injury. For this reason, as opposed to the redundancy found in upstream events, we sought to determine whether MLC phosphorylation and the cytoskeleton play roles in endothelial...
These concentrations were then used in the following equation to convert with the use of a standard curve to albumin concentration.

\[ P_a = \frac{[A]}{t} \times \frac{1}{P_v} \times \frac{V}{[L]} \]  

where [A] is abluminal concentration; t is time in seconds; A is area of membrane in cm²; V is volume of abluminal chamber; and [L] is luminal concentration.

**Transfection of inhibitor peptide.** MLCKi peptide was delivered to the RLMEC as previously described (23). Briefly, MLCKi (10 μg/ml) was mixed with TransIT-LT1 (10 μl/ml) in a small volume of OptiMEM-reduced serum medium and incubated at room temperature for 30 min. This mixture was then added to the cells, which had been switched from complete medium to OptiMEM, for a period of 4 h. The cells were then washed with PBS, and complete medium was restored before continuation of treatments.

**Thermal injury and collection of plasma.** The protocols using Sprague-Dawley rats (250–350 g) were approved by the Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guidelines of the Animal Welfare Act. Rats were anesthetized with an intramuscular injection of urethane (1.75 mg/kg).

Thermal injury was inflicted on the basis of a modified Walker and Mason burn model (10, 13, 27). A dorsal area that equaled 25% of the TBSA was shaved. The rat was placed in a mold with an adjustable opening to expose the shaved area to 100°C for 30 s, which produced a clearly defined full-thickness burn without detectable visceral injury, as confirmed by pathological studies (10). Ringer lactate solution (4 ml·kg⁻¹·%TBSA⁻¹) was constantly infused through a jugular vein cannula to mimic clinical condition of fluid resuscitation. In the control (nonburn) group, animals were shaved and subjected to the same burn procedure except the water temperature was 37°C. At 3 h postburn or nonburn, blood was collected into heparinized tubes and plasma was obtained by centrifugation at 3,000 g for 20 min. The 3-h postburn time point was selected for plasma collection on the basis of our own in vivo observations and numerous clinical studies showing a maximal plasma leakage at this point (1, 3, 6, 12). The RLMEC were exposed to the plasma at 1:3 dilution. This dilution was chosen due to the fact that a higher concentration of plasma interfered with albumin flux across the Transwell inserts.

**Western analysis.** RLMEC were treated and total cellular protein was obtained by lysis with modified RIPA buffer for 30 min on ice followed by centrifugation at 10,000 g for 10 min at 4°C. Protein concentrations were determined by the Bradford assay, and 15 μg treatment were subjected to SDS-PAGE (12%) gel followed by blotting to nitrocellulose. A polyclonal phosphospecific MLCK (Ser19/Thr18) primary antibody (Cell Signaling) followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling) and chemiluminescent detection were employed for band detection.

**Actin and phosho-MLC localization.** RLMEC were grown to confluence and treated as appropriate. Cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. Rhodamine phalloidin (Molecular Probes) at 5 U/ml and primary antibody to phosho-MLC (Cell Signaling) at 1:100 dilution were applied for 2 h. After PBS washes, secondary antibody conjugated to FITC was applied to visualize the phosho-MLC. Observations were made using a Zeiss inverted microscope at ×630 magnification with appropriate rhodamine and phalloidin filters.

**Quantification of F-actin.** F-actin was quantified using a modified fluorimetric microplate assay (11). RLMEC were grown to confluence and treated as appropriate on clear-bottom, black 96-well plates. After treatment, cells were fixed with 18.5% formaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 15 min, and 500 nM FITC-phalloidin was added for 30 min. Several washes with PBS were followed by measurement of stained F-actin in a fluorescence microplate reader. Background levels of fluorescence obtained from cells that had not been permeabilized as well as background from empty wells were subtracted from all readings.

**RESULTS**

**Burn plasma-induced hyperpermeability dependent on MLCK activity.** With the knowledge that endothelial barrier function is maintained by a delicate balance between tethering and contractile forces that involve the cytoskeleton, we sought to determine whether MLCK activity is involved in burn-induced barrier dysfunction. As Fig. 1 shows, exposure of RLMEC to burn plasma, even at 1:3 dilution, elicits an approximate twofold increase in albumin flux across the monolayer. The widely used pharmacological MLCK inhibitor ML-7 attenuated the hyperpermeability response in a dose-dependent manner (Fig. 1). Doses restoring barrier function to near basal levels had no significant effect on permeability, suggesting that MLCK activity is not involved in basal permeability (Fig. 1, ML-7 only).

With the knowledge that the specificity of pharmacological inhibitors is often called into question, particularly when they are used at high concentrations, we sought a more specific method to block MLCK activity. With the use of a protein transfection technique we had previously developed, an 11-amino acid, MLCKi, corresponding to part of the calmodulin-binding sequence of MLCK was introduced to the cells. As Fig. 2 shows, the presence of this peptide attenuated burn plasma-induced hyperpermeability responses to near basal levels. In agreement with the ML-7 data, the MLCKi had no
significant effect on basal permeability (Fig. 2). Furthermore, exposure of the RLMEC to plasma from control or nonburned rats did not lead to barrier dysfunction as measured by albumin flux (Fig. 2).

**MLCK-dependent, burn plasma-induced MLC phosphorylation.** The activation of MLCK is marked by increases in serine/threonine phosphorylation (9). Having shown that MLCK activity is necessary for thermal injury-induced hyperpermeability responses, we sought to examine the phosphorylation status of MLC, the phosphorylation target of MLCK. Western blotting showed that phosphorylation of MLC increased dramatically after 5-min exposure to burn plasma, and the elevated level was maintained for 30 min (Fig. 3A). In agreement with the permeability studies, ML-7 attenuated burn plasma-induced MLC phosphorylation in a dosage-dependent manner (Fig. 3B). Furthermore, we found that transfection of MLCKi had the same inhibitory consequence as ML-7 when examining MLC phosphorylation (Fig. 3C). These findings suggest that MLCK is required for most, if not all, MLC phosphorylation under conditions of thermal injury.
Fig. 5. Actin and phosphorylated MLC localization in RLMEC. Cells were grown to confluence on coverslips and treated with ML-7 (10^{-5} M) for 15 min; MLCKi transfection for 4 h; Y-27632 (5 \mu M) for 30 min; and BP at 1:3 for 30 min as shown. Cells were fixed, permeabilized, and stained for actin using rhodamine phalloidin and/or phosphorylated MLC using phosphospecific antibody followed by secondary antibody conjugated to FITC as shown. Images were obtained with a Zeiss inverted microscope at \times630 magnification.
Rho kinase-dependent, burn-induced hyperpermeability and MLC phosphorylation. To this point, our study has shown that phosphorylation of MLC is essential for hyperpermeability in response to burn plasma. However, MLCK is not the only enzyme that has been shown to activate MLC via Ser19/Thr18 phosphorylation. Rho kinase is also responsible for this activation in an indirect, but nevertheless efficient, manner. For this reason, and the fact that burn plasma contains numerous inflammatory mediators, we sought to determine whether or not Rho kinase plays a role in thermal injury-induced hyperpermeability. As shown in Fig. 4, when cells were exposed to the Rho kinase inhibitor Y-27632 before exposure to burn plasma, the permeability response was greatly attenuated. In addition, we found that Y-27632 blocked burn plasma-induced MLC phosphorylation (Fig. 4). Together with the permeability experiments using MLCK inhibitors, the data strongly suggest that both MLCK and Rho kinase are essential for burn plasma-induced hyperpermeability and MLC phosphorylation.

Actin and phosho-MLC localization. As stated earlier, cytoskeletal elements play a major role in endothelial barrier function. With the observation that permeability increases in response to burn plasma were both MLCK- and Rho kinase-dependent and MLC phosphorylation occurred concomitantly with this phenomenon, we hypothesized that changes in actin distribution may also occur. Indeed, increases in actin stress fiber formation were quite evident when RLMEC were exposed to burn plasma (Fig. 5C). When the cells were preexposed to ML-7, Y-27632, or transfected with MLCKi before application of burn plasma, the actin cytoskeleton maintained a basal appearance (Fig. 5, E, G, and I). This is in agreement with our previous study that showed that inhibition of MLCK blocked actin stress fiber formation (22). Immunostaining revealed increases in phosho-MLC in response to burn plasma as well (Fig. 5D), confirming the increases we observed with Western analysis. Not surprisingly, phosho-MLC staining in burn plasma-exposed cells that were pretreated with ML-7, Y-27632, or MLCKi resembled basal levels (Fig. 5, F, H, and J). It should be pointed out that when these experiments were performed using FITC-conjugated secondary antibody in the absence of phosho-specific MLC primary antibody, no signal was seen.

F-actin quantification. Having observed qualitatively the burn plasma-induced increase in actin stress fiber formation, we sought a way to express the increase in relation to basal levels. To this end, we employed a quantitative fluorometric microplate assay. With the use of this approach, we found that F-actin levels increased by ~50% in RLMEC exposed to burn plasma (Fig. 6). This increase was attenuated by pretreatment with either ML-7 or MLCKi (Fig. 6). Additionally, ML-7 and MLCKi as well as nonburn plasma had no significant effects on basal F-actin levels (Fig. 6).

DISCUSSION

One of the most traumatic and damaging effects of thermal injury is the systemic inflammation that occurs in the first 24–48 h after a major cutaneous burn. Recent studies have focused on damage to the microvascular endothelium as a prominent event leading to leakage of fluid and solutes from the circulation into the interstitial tissues. In particular, pulmonary damage is disproportionately great, and treatment of lung ischemia and edema is challenging to say the least. For this reason, we chose pulmonary microvascular endothelial cells as our model for examining the effects of burn-induced plasma on the structure and function of these barrier-forming cells in the circulation of the lung.

The release of mediators such as histamine as well as leukocyte adhesion and release of toxic metabolites (14, 16, 18) occur in the circulation of burn patients and animals. Our previous studies on histamine signaling and VEGF-induced hyperpermeability via phospholipase C, PKC, nitric oxide, and Ca2+ pathways show that these phenomena are complicated in their scope (30, 31). Inhibition of histamine and VEGF receptors may not prove very effective in reducing the deleterious effects that burns initiate on the microvascular endothelium. On the other hand, a hallmark of burn-induced pulmonary injury is accumulation of neutrophils within the lung (20). Exposure of endothelial cells to neutrophils from burned patients results in rounding and shrinking of the cells and subsequent hyperpermeability across the monolayer (29). The neutrophils themselves undergo dramatic changes after thermal injury. They are known to release numerous inflammatory mediators that induce hyperpermeability, i.e., platelet-activating factor, glutamate, elastase, and oxygen radicals (4, 15, 21, 28). Again, these mediators initiate complicated, multiple pathways, so anti-neutrophil adhesion therapy would not prove effective in treating microvascular dysfunction.

As stated in the introduction, endothelial barrier function is maintained in a semipermeable state by the balance between adhesive forces at intercellular junctions and contractile forces, which are produced by the actomyosin complex, within the cells. In previous studies, we have observed that activated neutrophils induce both alterations in adherens junction proteins, which are connected to the actin cytoskeleton, and hyperpermeability across endothelial cell monolayers (24, 25). Additionally, we showed that neutrophil adhesion is not required for endothelial dysfunction; supernatant derived from activated neutrophils had the same effect as actual physical contact between the endothelial cells and neutrophils (24). In other words, the neutrophil-released inflammatory mediators play a more important role in hyperpermeability responses than...
neutrophil adhesion. Furthermore, introduction of activated MLCK resulted in F-actin stress fiber formation and cellular retraction with subsequent gap formation (22). With the knowledge that neutrophil activation leads to endothelial barrier dysfunction and that burn injuries elicit systemic inflammation characterized by neutrophil accumulation in the lungs, we sought a link between burns and the pulmonary actomyosin complex in the hopes of revealing a possible target for therapeutic strategies.

This study, for the first time, linked burn plasma to development of pulmonary microvascular endothelial cell hyperpermeability in an MLC phosphorylation-dependent manner. In addition, we found that burn plasma-induced F-actin increases and actin/phospho-MLC colocalization are both MLCK- and Rho kinase-dependent. These results suggest that the phosphorylating effect of MLCK and the Rho kinase inhibitory effect on myosin phosphatase work in tandem to effectively phosphorylate MLC under conditions of thermal injury in the pulmonary endothelium.

Previously, our studies had determined by pharmacological inhibition that PKC, Src family tyrosine kinase family, and mast cell activation are not required for burn-induced hyperpermeability of rat mesenteric vascular beds (8). It is precisely these findings that may explain why therapeutic intervention after thermal injury is often ineffective. The PKC and Src pathways, as well as histamine production, are early-stage effectors. These signaling pathways are often interactive and carry redundancies in their means to an end. In other words, inhibition of one may not interfere with the culmination of etiological responses in a situation where multiple inflammatory mediators are present, i.e., following burn injury. Endothelial barrier dysfunction based on cellular retraction and dissolution of adherens junction proteins is propagated by the actomyosin complex, and more specifically, myosin activation as a result of MLCK-dependent MLC phosphorylation (22). Successful targeting and inhibition of a downstream event such as MLC phosphorylation would make the issue of the numerous upstream signaling events triggered by multiple mediators released after thermal injury a moot point. On the basis of our results and those of others, we suggest that specific MLC phosphorylation inhibition presents a valid objective for development of therapeutic strategies directed at minimizing the effects of major burns on pulmonary endothelial dysfunction as a consequence of systemic inflammation.

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