PKC-dependent, burn-induced adherens junction reorganization and barrier dysfunction in pulmonary microvascular endothelial cells

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The wall of microvascular exchange vessels is composed of endothelial cells that connect to each other via closely opposed intercellular junctions. These junctions maintain the semipermeable property of the endothelial barrier and control the transvascular passage of solutes, fluid, and blood cells. The adherens junctions (AJ; formed by transmembrane adhesive proteins called cadherins) are important complexes involved in the regulation of paracellular permeability in microvascular endothelium. Vascular endothelial (VE)-cadherin is associated with the actin cytoskeleton through a family of proteins called catenins, including α-catenin, β-catenin, and plakoglobin (11, 13). Whereas the AJ guard against macromolecular leakage by holding the cells together, the contractile forces generated by actomyosin interaction tend to pull the tightly connected endothelial cells apart. The balance between these two forces maintains the endothelial monolayer in a semipermeable status, and disruption of this equilibrium can lead to barrier dysfunction.

Endothelial permeability is affected by many agonists including thrombin, histamine, phorbol esters, growth factors, and polymorphonuclear leukocytes (1, 3–5, 24, 25). Characteristic phenomena resulting from endothelial cell exposure to various agonists are phosphorylation of myosin light chain (MLC), actomyosin contractile forces, and modification/reorganization of the AJ (23–25, 27). We have shown that activated neutrophils induce tyrosine phosphorylation of VE-cadherin and β-catenin followed by gap formation and hyperpermeability (25). Furthermore, we found that application of plasma from burned rats to cultured microvascular endothelial cells induced MLC phosphorylation-dependent permeability increases and actin stress fiber formation (23).

The traumatic response to burn injuries is seen not only at the site of the wound but also throughout distal organ systems. This is referred to as systemic inflammatory response syndrome (SIRS), a malady that contributes to high mortality rates in patients with full-thickness burns that exceed 25% of the total body surface area (TBSA) (6). SIRS is characterized by plasma leakage at both the burn site and in distant organ systems, peaking in the first 3 h and diminishing over the next 6–12 h (6, 9, 14). SIRS is associated with an overproduction of many inflammatory mediators capable of activating intracellular signaling events such as the arachidonic acid and bradykinin cascades in the endothelium in response to thermal injury (2, 9, 14). A previous study of ours has also shown that MLC kinase (MLCK) and Rho kinase activity is upregulated in response to burns (23). MLCK and Rho kinase activity were both shown to be necessary for MLC phosphorylation. Furthermore, others have shown a role for PKC in the mechanisms governing MLC activity (26). We wondered then whether PKC played a role in the pathogenesis of burn-induced microvascular barrier dysfunction.

Pharmacological intervention targeting specific mediators involved in postburn inflammation has not proven very effective. This may be due to the fact that these pathways often have redundancies in function. We feel that understanding the molecular changes that occur at the end point of these signaling mechanisms would elucidate possible therapeutic targets, with the goal being to lessen the impact of postburn abnormalities.

Along these lines, we have begun to examine the events that lead to AJ disruption and gap formation in the microvascular endothelium, and our model for this study is rat lung microvascular endothelial cells (RLMEC). As stated above, our previous study showed that burn-induced actin stress fiber formation and barrier dysfunction is dependent on MLC phosphorylation (23). We found that inhibition of either MLCK, which directly phosphorylates MLC, or Rho kinase, which is regulated by the PKC inhibitor bisindolylmaleimide, suggesting that PKC is required for burn-induced pulmonary endothelial dysfunction.

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incubated at 37°C in 5% CO2. RLMEC exhibited properties characteristic of the endothelial cell, such as typical cobblestone morphology and incorporation of acetylated low-density lipoprotein.

**Measurement of endothelial permeability.** RLMEC permeability was determined by measuring FITC-albumin flux across the monolayer as previously described (23-25). Cells were grown on gelatin-coated Costar Transwell membranes (VWR, Houston, TX). BIM and plasma were added to the luminal chamber. FITC-albumin (3 μg/ml) was added to the luminal chamber for 60 min, and samples were removed from both luminal and abluminal chambers for fluorometry analysis. The readings were converted with the use of a standard curve to albumin concentration. These concentrations were then used in the following equation to determine the permeability coefficient of albumin (P)

\[ P = (\frac{A}{I}) \times (\frac{A}{I}) \times (V/[L]) \]

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.

**Assessment of barrier function by TER.** TER was measured using an Electrical Cell Motility System (CET, Iowa City, IA) as previously reported (8, 10, 18, 19). Briefly, RLMEC were cultured (10^4 cells/cm²) on a small gold electrode array (Applied Biophysics, Troy, NY). MCDB-131 served as the electrolyte, and barrier function was dynamically measured by determining the impedance of a cell-covered electrode. A 1-V, 4,000-Hz alternate current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. The in-phase voltage (proportional to resistance) and the out-of-phase voltage (proportional to capacitive resistance) were measured. Endothelial barrier function is expressed as the fractional change in TER.

**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 (Texas A & M University and Scott & White Hospital) and were in accordance with the 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 based on a modified Walker and Mason burn model (12, 15, 29). 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 water for 30 s, which produced a clearly defined full-thickness burn without detectable visceral injury as confirmed by pathological studies (12). 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 based on our own in vivo observations and numerous clinical studies showing a maximal plasma leakage at this point (2, 6, 9, 15). 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.

**Immunoprecipitation and Western blot analysis.** RLMEC were treated as appropriate, 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. For immunoprecipitation, 100 μg of total protein were incubated with 2 μg of either anti-VE-cadherin or anti-β-catenin for 4 h. This was followed by addition of 25 μl of protein A/G agarose and further incubation overnight at 4°C. After five washes with RIPA, the remaining pellet was subjected to SDS-PAGE (6% gel) at a constant 125 V for ~1.5 h followed by blotting to nitrocellulose and band detection using phosphoserine antibody. In the case of total cell lysate, 15 μg of protein were run per lane.

**VE-cadherin and β-catenin localization.** RLMEC were grown to confluence on gelatin-coated coverslips and treated as shown in results and in the figures. Cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. Primary antibody to VE-cadherin or β-catenin at 1:100 dilution was applied 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.

Analysis of variance was used to evaluate the significance of intergroup differences. A value of P < 0.05 was considered significant for the comparison. In addition, Tukey’s honestly significant difference test was performed to examine differences among specific groups, when appropriate.

**RESULTS**

**PKC-dependent burn plasma-induced endothelial barrier dysfunction.** With the assumption that numerous inflammatory mediators are released following a burn injury, it stands to reason that various pathways and signaling cascades will be activated in response to these cytokines. The PKC pathway is known to be involved in maintaining and altering endothelial barrier status (22); therefore, we sought to determine whether PKC plays a role in burn-induced dysfunction. Two methods were used to assess the status of RLMEC monolayers. The first involved measuring transendothelial flux of FITC-albumin, the Fₐ. As Fig. 1 shows, burn plasma induced an ~85% increase in Fₐ compared with basal levels after 1 h, whereas nonburn plasma showed a slight, but not significant, increase. The PKC inhibitor BIM evoked a dosage-dependent attenuation of burn plasma-induced hyperpermeability and at 10 μM reduced Fₐ to...
Burn plasma-induced AJ phosphorylation. Our previous studies had shown that tyrosine phosphorylation of the AJ proteins VE-cadherin and β-catenin in endothelial cells was elevated in response to activated neutrophils (25). Now knowing that PKC activity is required for burn-induced barrier dysfunction, we sought to determine whether PKC-elicited serine phosphorylation of the AJ proteins occurs in response to burns. An initial study showed that burn plasma induced increases in overall serine phosphorylation in RLMEC as shown by Western blotting (Fig. 3, b vs. a), a phenomenon not seen with nonburn plasma (Fig. 3, c vs. a). Furthermore, the PKC inhibitor BIM attenuated the burn plasma-induced serine phosphorylation (Fig. 3, e vs. a). To determine the possible serine phosphorylation status of the AJ proteins, immunoprecipitation of VE-cadherin and β-catenin was followed by immunoblotting for phosphoserine. As shown in Fig. 4, burn plasma induced serine phosphorylation of both of these AJ proteins (b vs. a). Again, the nonburn plasma failed to induce serine phosphorylation (Fig. 4, c vs. a). Addition of BIM attenuated the burn plasma-induced serine phosphorylation of the AJ proteins to near basal levels (Fig. 4, d-f). The bands in Fig. 4 were quantified by scanning densitometry. The average ratio of phosphorylated to total protein from three separate experiments is given above the phosphospecific bands. For both β-catenin and VE-cadherin, the burn plasma-induced phosphorylation increases were significant (P < 0.05; Fig. 4, b

evaluation of plasma-only values and all three of the BIM treatments (Fig. 2A). To determine the possible role of PKC on burn-induced decreases in TER, BIM was added to the cells at t = 60 min followed by burn plasma at t = 90 min. As seen in Fig. 2B, all three concentrations of BIM decreased the effect of burn plasma on TER decrease. Statistical analyses on the data from Fig. 2B have been performed. The mean values for resistance readings at 10-min intervals are shown in Table 1. A significant difference (P < 0.05) was found between the burn plasma-only values and all three of the BIM treatments (−5, −6, and −7)/burn plasma at the 110-min time point and all time points thereafter. Because of the sensitive nature of these experiments, the experiments were repeated on three occasions with n = 4 for each treatment.

Fig. 2. Transendothelial electrical resistance (TER) is measured across RLMEC monolayers. Cells were grown to confluence on gold electrode arrays, and TER was measured as described in MATERIALS AND METHODS. Fresh media were applied, and the resistance was allowed to stabilize for 60 min. A and B: burn or nonburn plasma was added at 1:3 dilution at t (time) = 90 min. In B, BIM was added at the molar concentrations shown at t = 60 min. The experiments were allowed to proceed for ~4 h. For each treatment, n = 4. The experiments were repeated 3 times, and representative graphs are shown.
The nonburn plasma and BIM-treated cells showed no significant phosphorylation increases (Fig. 4, c–f vs. a). There were no significant changes in the overall levels of either VE-cadherin or β-catenin in response to burn plasma (Fig. 4). These data suggest that PKC either directly or indirectly phosphorylates VE-cadherin and β-catenin in the endothelium in response to burn injury.

**VE-cadherin and β-catenin localization.** Our previous work addressing the effects of neutrophils on endothelial cells showed a disruption in AJ organization concomitant with hyperpermeability (25). We have now shown serine phosphorylation increases in VE-cadherin and β-catenin in response to burn plasma, but does this phenomenon result in junctional changes? To address this question, we stained RLMEC for VE-cadherin and β-catenin following treatment with plasma. Figure 5a shows that in control cells, these two junctional proteins are seen in a smooth, continuous layer around the periphery. When exposed to nonburn plasma or burn plasma following pretreatment with BIM, cellular morphology is relatively unchanged (Fig. 5a). However, upon exposure to burn plasma, gap formation occurs and VE-cadherin and β-catenin disappear from areas of the cells that no longer contact adjoining cells (Fig. 5b). They now appear as “finger-like projections” in areas where cells maintain contact with each other (Fig. 5b). In as little as 15 min, loss of VE-cadherin and β-catenin from the cell periphery is evident followed by gap formation (Fig. 5b).

**DISCUSSION**

Pulmonary microvascular endothelial damage is a hallmark consequence of thermal injury. This is due to systemic inflammation occurring in the first 24–48 h after a major cutaneous burn. Treatment of lung ischemia and edema is difficult, and, in

**Table 1. RLMEC mean resistance readings in ohms**

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RLMEC, rat lung microvascular endothelial cells; BIM, bisindolylmaleimide I.
fact, many of the currently applied therapeutic interventions themselves have detrimental effects in addition to their limited beneficial outcomes. The inflammatory mediators released in the circulation of burn patients are well known and numerous. In addition, neutrophil accumulation in the lungs occurs following burn injury (20). Along with an array of proinflammatory cytokines that are released, activated neutrophils are known to release hyperpermeability-inducing factors such as platelet-activating factor, glutamate, elastase, and oxygen radicals (7, 17, 21, 30). These inflammatory mediators activate numerous, complicated signaling pathways that often are redundant in function and scope. Targeting of one particular component or pathway may have little, if any, effect on alleviating the overall deleterious effects of burn-induced pulmonary inflammation.

We feel that focusing on an end point consequence of systemic inflammation for possible therapeutic intervention could lead to more effective treatment of burn patients. In particular, what structural modifications does the microvascular endothelium exhibit that lead to barrier dysfunction and subsequent pulmonary edema? As stated in the introduction, the endothelial monolayer is maintained in a semipermeable state by the delicate balance between adhesive forces at intercellular junctions and contractile forces produced by the actomyosin complex. Our studies and those of others have shown that endothelial barrier dysfunction is accompanied by alterations in AJ proteins that interact with the actin cytoskeleton as well as span the cellular membrane to connect neighboring cells. In fact, our recent work demonstrated the requirement for both MLCK and Rho kinase activity in burn-induced endothelial hyperpermeability and actin stress fiber formation (23). These two kinases are both responsible for MLC serine phosphorylation, MLCK directly and Rho kinase indirectly. Because phosphorylation of MLC activates the actomyosin complex and this complex interacts with VE-cadherin through β-catenin, we wanted to determine whether burns result in alterations/modifications of the AJ proteins. Previous studies had shown increased tyrosine phosphorylation of these two AJ proteins as well as their disappearance from the cell periphery concomitant with endothelial gap formation in response to activated neutrophils (25).

This study shows that burn-induced barrier dysfunction in the pulmonary microvasculature is marked by serine phosphorylation of the AJ proteins VE-cadherin and β-catenin, move-
ment from these proteins from the cell periphery in areas of gap formation, and the requirement for PKC activity in the propagation of these phenomena. Barrier dysfunction, as measured by both FITC-albumin flux and TER, occurred rapidly in response to burn plasma, and, in the case of TER, was evident for at least 2 h after RMLCE exposure to the plasma. As shown by the TER results, it appears as though the response to burn plasma is biphasic. Addition of burn plasma caused an immediate drop in resistance followed by a return to basal or near basal levels. Approximately 30 min after treatment, the resistance again decreased below basal levels and remained lower for ~2 more hours. The initial drop in resistance, or barrier perturbation, may be due to inflammatory mediators present in the burn plasma such as nitric oxide. The secondary, longer-lasting resistance decrease may reflect the effects of various cytokines on cellular signaling pathways that lead to more long-lived consequences, such as the phosphorylation/activation of MLC. Increased albumin flux across the monolayer was attenuated in a dosage-dependent manner by the PKC inhibitor BIM. BIM also lessened the effects of burn plasma on TER. All three concentrations of BIM blocked resistance decreases with similar magnitude. This may be due to the fact that TER only measures cell-cell and cell-matrix adhesion (8), and these are only two of several factors that determine diffusive flux of albumin and other macromolecules across the endothelium. Other factors include charge selectivity of the endothelial glyocalyx, the integrity of the glyocalyx, and the possibility of changes in transcellular movement of albumin (through vesicular transport) across the endothelium (16). Alternatively, the differences seen in the PKC inhibitor data may be a reflection of the fact that the albumin flux measurements are done at one time point, 90 min following exposure to plasma. The permeability of albumin is a cumulative measurement. Varying degrees of PKC inhibition through the three dosages of BIM could result in differences in albumin flux following plasma exposure due to the cumulative nature of these data. TER measurements are real-time and do not reflect cumulative changes in barrier integrity. Furthermore, we show that not only does burn plasma-induced serine phosphorylation increase in numerous proteins but, in particular, these increases in VE-cadherin and β-catenin are blocked by PKC inhibition. This suggests that PKC either directly or indirectly phosphorylates these two AJ proteins. Finally, we observed that burn plasma induces cellular gap formation marked by a disappearance of VE-cadherin and β-catenin from areas of the cell periphery where contact with adjoining cells has been lost; this is also dependent on PKC activity.

Signaling pathways activated by numerous inflammatory mediators, such as those released in response to burns, are often interactive and redundant. Therefore, inhibition of one pathway may not alleviate the deleterious effects of thermal injury, especially in the case of pulmonary microvascular dysfunction. Targeting of potential downstream or end point events that result from burns may prove more successful in the treatment of patients in the first 1–2 days following injury. Dissolution of the AJ appears to be a hallmark occurrence in endothelial monolayer gap formation, and we know that the AJ is closely connected to the cytoskeleton via the catenins. Our previous study showed that serine phosphorylation of MLC is essential for burn-induced barrier dysfunction (23), and this current study shows that PKC phosphorylation of AJ proteins is required as well. Although the precise order of these events is unknown, these results indicate that a concerted activation of the actomyosin complex together with breakdown of the AJ are both required for endothelial barrier breakdown in burns. Although not entirely conclusive, based on the time course study reflected in Fig. 5B, we believe that breakdown of the AJ occurs just before gap formation. As early as 5 min after exposure to burn plasma, the morphology of the AJ proteins seems to be taking on a “spiked” or finger-like projection appearance; this phenomenon is more pronounced after 15 min. This phenomenon has been observed by others examining endothelial cell morphology following exposure to growth factor (1). An increase in punctate staining of the AJ proteins in the cytoplasm suggests that they are internalized rather than degraded. This is substantiated by the Western blot data showing no significant changes in total cellular β-catenin or VE-cadherin following treatment with burn plasma (Fig. 4). Gap formation begins to become evident at the 15-min time point and is quite obvious after 30 min (arrows in Fig. 5B). Together, this study and our previous data (23) suggest that specific inhibition of serine phosphorylation of VE-cadherin or β-catenin, as well as MLC, may provide an effective means of combating the detrimental effects of burn injury on pulmonary edema.

GRANTS
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