Adherent neutrophils activate endothelial myosin light chain kinase: role in transendothelial migration

JOE G. N. GARCIA, ALEXANDER D. VERIN, MARIA HERENYIOVA, AND DENIS ENGLISH

Departments of Medicine, Physiology, and Biophysics, Indiana University School of Medicine, Richard Roudebush Veterans Affairs Center, and the Methodist Research Institute, Indianapolis, Indiana 46202

Garcia, Joe G. N., Alexander D. Verin, Maria Herenyiova, and Denis English. Adherent neutrophils activate endothelial myosin light chain kinase: role in transendothelial migration. J. Appl. Physiol. 84(5): 1817–1821, 1998.—Increased vascular endothelial cell (EC) permeability and neutrophilic leukocyte (PMN) diapedesis through paracellular gaps are cardinal features of acute inflammation. Activation of the EC contractile apparatus is necessary and sufficient to increase vascular permeability in specific models of EC barrier dysfunction. However, it is unknown whether EC contraction with subsequent paracellular gap formation is required for PMN transendothelial migration in response to chemotactic factors. To test this possibility, we assessed migration of human PMNs across confluent bovine pulmonary arterial EC monolayers. Transendothelial PMN migration in the absence of a chemotactic gradient was minimal, whereas abluminal addition of leukotriene B4 (LTB4; 5 µM) resulted in significantly increased PMN migration. Reductions in EC myosin light chain kinase (MLCK) activity by EC monolayer pretreatment with specific MLCK inhibitors (KT-5926 or ML-7) or by increases in cAMP-protein kinase A (PKA) activity (cholera toxin) significantly reduced PMN transmigration. In contrast, pretreatment with the myosin-associated phosphatase inhibitor calyculin resulted in the accumulation of phosphorylated myosin light chains, EC contraction, and significantly enhanced PMN migration. Finally, the interaction of PMNs with 32P-labeled EC monolayers was shown to directly increase EC myosin phosphorylation in a time-dependent fashion. Taken together, these results are consistent with the hypothesis that the phosphorylation status of EC myosin regulates PMN migration and further indicate that EC MLCK is activated by chemotractant-stimulated PMNs. Neutrophil-dependent activation of the EC contractile apparatus with subsequent paracellular gap formation may be a key determinant of transendothelial PMN migration responses to chemotactic agents.

PMN migration responses to chemotactic agents.

Increased vascular endothelial cell (EC) permeability and neutrophil diapedesis

THE MARGINATION AND MIGRATION of circulating neutrophilic leukocytes (PMNs) are critical components of the inflammatory process. Emigration of PMNs from the blood into the extravascular tissue is a multistep process, with leukocytes rolling along the postcapillary vascular wall with subsequent tethering to the endothelial cell (EC) surface. PMN migration through the vessel wall is regulated in a complex fashion by specific recognition molecules on PMN and EC plasmalemmal membranes (1, 10, 13). The consequences of the intimate cell-cell association between EC and adhering PMNs are poorly understood. However, this interaction likely exerts potentially important cross-cellular regulatory influences on signaling pathways on both the endothelium and responding PMNs. For example, Cui et al. (2) showed that PMN chemotactic migration across confluent endothelium, in the presence of a chemotactic gradient produced by leukotriene B4 (LTB4), resulted in activation of EC phospholipase D. Neither direct EC challenge with LTB4, nor simple physical contact of EC with PMNs in the absence of the chemotactic gradient was sufficient to trigger phospholipidase D activation (2).

Increasing information regarding juxtacrine interactions between adherent or migrating PMNs and EC has raised the possibility that similar cross-cellular signaling pathways may be involved in the process of PMN migration into inflamed tissues. For example, EC responses to adherent PMNs may lead to a loss of vessel wall integrity and the formation of paracellular gaps, thereby facilitating PMN migration. Paracellular gap formation is now recognized as critical to enhanced vascular permeability to solutes and fluids (4, 6, 12, 18), which, like PMN diapedesis, is a key feature of the inflammatory process. The premise that PMNs may regulate EC paracellular gaps was first postulated by Huang et al. (9), who described increases in EC cytosolic Ca2+ in response to adherent PMNs. Similar increases in EC cytosolic Ca2+ are evoked by adherent tumor cells (16). The recognized role of Ca2+ in regulating the integrity of the EC monolayer (6, 7, 12, 18) opens the possibility that adherent, migrating PMNs may also regulate EC paracellular gap formation.

Although the biochemical and molecular processes that regulate the vascular paracellular space are incompletely understood, it has been proposed that EC monolayer integrity is maintained by a balance of barrier-disrupting contractile forces existing in equilibrium with tethering forces comprised of adherens junction and focal adhesion proteins (6, 12, 14). In previous work, our laboratory has shown that the phosphorylation status of EC myosin light chains (MLCs) is a key determinant of EC gap formation (4, 21). Our laboratory has cloned and characterized the critical involve-
ment of a novel high-molecular-weight EC MLC kinase (MLCK) isoform in EC contraction, paracellular gap formation, and barrier dysfunction (3, 5). The activity of this contractile effector appears to be regulated by cytosolic calcium as well as by the phosphorylation status of the kinase (4, 5). For example, cAMP-dependent protein kinase A (PKA)-mediated increases in EC MLCK phosphorylation reduce MLCK activity (5), thereby abolishing EC contraction and gap formation evoked by bioactive agents (4, 15). Furthermore, inhibition of myosin-associated type 1 serine-threonine (Ser/Thr) phosphatase activity produces significant EC contraction and paracellular gap formation because of the accumulation of phosphorylated myosin (21).

In this study, we hypothesized that the MLCK-dependent activation of the EC contractile apparatus regulates PMN diapedesis by allowing PMN to emigrate between EC into inflamed tissues. Our data indicate that PMN-EC interaction in response to chemoattractant stimuli produces EC MLCK activation, thereby facilitating EC gap formation and transendothelial migration. These results suggest EC contractile proteins play an essential role in regulating the migration of leukocytes into sites of infection and inflammation.

MATERIALS AND METHODS

Reagents. Chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO), Transwell chambers (24 mm diameter, 3 µm pore size) for EC culture and PMN chemotaxis were obtained from Costar (Cambridge, MA). Bovine pulmonary arterial EC were from American Type Culture Collection (Rockville, MD). Bovine pulmonary artery EC culture. EC were cultured as described previously (4, 15) under a humidified atmosphere of 95% air-5% CO2. After 16–20 passages in gelatin-coated T-75 cm2 flasks, the cells were trypsinized and seeded onto the 3-µm polycarbonate filters at the base of inserts of the added, and the Transwell plates were placed in a CO2 incubator at 37°C for 30 min before addition of PMNs. EC monolayers grown in Transwell chambers were rinsed extensively and placed in a CO2 incubator at 37°C for 30 min before addition of PMNs. Neutrophil migration assay. Human PMNs were freshly purified from the peripheral blood of healthy subjects by Ficoll-Paque density-gradient centrifugation as previously described (2). Final cell preparations were resuspended in DMEM including 0.1% BSA (107 cells/ml). EC monolayers grown in Transwell chambers were rinsed extensively and gently with fresh DMEM (without BSA), fresh media were added, and the Transwell plates were placed in a CO2 incubator at 37°C for 30 min before either pretreatment with specific agents or the addition of PMNs. Neutrophils (0.5 ml, 107 cells/ml) were loaded into the upper compartment, and migration was initiated by adding LTB4 (5 µM) to the lower compartment to establish a chemotactic gradient. The actual PMN-to-EC ratio was therefore ~2:1. The number of PMNs in the abluminal chamber was determined at specified times over a 1- to 2-h incubation as previously described (2). To verify that EC-pretreatment conditions with MLCK inhibitors did not alter PMN migration capacity in a nonspecific manner, EC monolayers were pretreated with either KT-5926 (60 min) or vehicle, followed by washing and PMN addition. After 30 min of coincubation, PMNs were retrieved and utilized for direct assessment of PMN chemotactic activity in response to LTB4. PMNs recovered from either vehicle- or KT-5926-pretreated EC monolayers demonstrated comparable chemotactic activity (data not shown).

Determination of MLCK activity in EC monolayers. Confluent EC monolayers grown in either Transwell chambers or 60-mm dishes were prelabeled with 32P (0.2 mCi/ml) in phosphate-free DMEM media including 1% fetal calf serum and incubated for 3 h at 37°C in a humidified atmosphere of 5% CO2-95% air. At specific times after PMN contact, proteins were harvested by precipitation into ice-cold 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol. The cellular proteins present in TCA-treated pellets were washed three times with diethyl ether and resuspended in sample buffer containing 6.7 M urea and (in mM) 10 dithiothreitol, 20 Tris, 2 glycine, and 270 sucrose, pH 9.0. MLC phosphorylation was analyzed as we have described by a modification of the urea PAGE separation (20 µg/lane) of the mono- and diphosphorylated forms of MLC, which migrate more rapidly than do unphosphorylated forms (4). Radioactive protein bands were detected by autoradiography at ~70°C. Positions of both phosphorylated and unphosphorylated MLC were detected by immunoblotting with 1:1,000 MLC-specific antibody followed by staining with goat anti-rabbit-horseradish peroxidase system (4). EC MLCK phosphorylation was measured by densitometry of autoradiographs of nitrocellulose blots containing phosphorylated labeled MLC and normalized by protein loading.

RESULTS

Effect of EC MLCK inhibition on PMN migration. Transendothelial migration of human PMNs across confluent EC monolayers over a 2-h period in the absence of LTB4 was significantly less than migration after the addition of LTB4 (5 µM) to the abluminal media (Fig. 1). The increase in PMN transendothelial migration began as early as 5 min and plateaued between 60 and 120 min. In prior work, our laboratory has shown that the addition of equimolar amounts of LTB4 to both the upper and lower chambers fails to significantly augment PMN migration into the ablumi-

Fig. 1. Effect of leukotriene B4 (LTB4) on transendothelial neutrophilic leukocyte (PMN) migration. Endothelial cell (EC) monolayers grown in Transwell chambers were rinsed extensively and placed in a CO2 incubator at 37°C for 30 min before addition of PMNs. Neutrophils (0.5 ml, 107 cells/ml) suspended in DMEM containing 0.1% BSA were loaded into upper compartment, and migration was assessed in presence (+) or absence of (-) LTB4 (5 µM) in lower compartment. No. of PMNs in abluminal chamber was determined at specified times over a 2-h incubation as previously described (2). Data represent 8–10 independent experiments.
nal compartment (2). To examine the hypothesis that EC MLCK activation is required for subsequent PMN paracellular migration, EC monolayers were pretreated with two enzymatic inhibitors of MLCK activity, KT-5926 and ML-7, at concentrations we have shown to effectively reduce MLC phosphorylation (4). A prior study has shown that basal EC MLC phosphorylation exhibits a stoichiometry of 0.3–0.4 mol/mol catalyzed by constitutively active EC MLCK, with further increases to ~0.8–0.9 mol/mol after agonists such as thrombin (4). Inhibition of MLC phosphorylation with KT-5926 significantly reduces basal and thrombin-induced MLC phosphorylation to ~0.2–0.3 mol/mol, as well as attenuates EC gap formation and albumin permeability (4). EC monolayers in Transwell chambers were pretreated with either 4 µM KT-5926 or 10 µM ML-7 for 60 min and carefully washed twice to remove the inhibitor before the addition of human PMNs. As shown in Fig. 2, despite the presence of LTB₄, both MLCK inhibitors produced a dramatic and significant reduction in PMN transendothelial migration. Thus EC MLCK activity appears to be a critical participant in the process of PMN transmigration after the establishment of an LTB₄ gradient across EC monolayers.

We have previously shown that increases in both intracellular cAMP concentrations and PKA activities by choleratoxin reduces both MLCK enzymatic activity and thrombin-induced gap formation and permeability via, at least in part, direct MLCK phosphorylation (4, 5, 15). To extend the observations noted above with the selective MLCK inhibitors, we pretreated EC monolayers with choleratoxin (1 h), washed the EC monolayers carefully, and assessed PMN migration. As noted in Fig. 2, cholera toxin significantly reduced transendothelial PMN migration in response to LTB₄. These results are consistent with a central role of EC MLCK in the regulation of PMN movement across the vessel wall in response to chemotactic signals.

Role of myosin-associated phosphatases in PMN transmigration. We have previously shown the involvement of a type 1 Ser/Thr phosphatase in the regulation of EC MLC phosphorylation (21). Inhibition of this phosphatase with calyculin results in dose-dependent increases in MLC phosphorylation, EC paracellular gap formation, and EC permeability to albumin (21). To examine whether increases in EC paracellular gaps produced by myosin-associated phosphatase inhibition would facilitate PMN migration, we pretreated EC monolayers with 10 nM calyculin for 15 min, carefully washed the EC monolayers, and added PMNs to the upper compartment. Although calyculin treatment did not affect PMN migration in the absence of LTB₄ (data not shown), as noted in Fig. 2, calyculin significantly increased PMN transmigration in response to LTB₄ compared with vehicle-pretreated controls. These results were further confirmed in studies wherein PMNs were added to EC monolayers briefly stimulated with thrombin (2 min). Thrombin pretreatment, known to produce an increase in MLC phosphorylation (4), also significantly enhanced PMN migration only in the presence of the LTB₄ chemotactic gradient (Fig. 2). These results are consistent with the hypothesis that EC MLCK phosphorylation, a process that is tightly linked to paracellular gap formation, is critical to PMN diapedesis.

Phosphorylation of EC MLC by transmigrating PMNs. To further extend these observations, EC monolayers were incubated with ³²P to label intracellular ATP. After careful washing to remove ³²P from the medium, labeled EC were incubated at 37°C with PMNs, either in the presence or absence of LTB₄. EC MLC phosphorylation profiles of TCA-precipitated cellular proteins (containing both PMN and EC MLCKs) were obtained by urea gel electrophoresis and autoradiography. Compared with phosphorylation profiles of ³²P-labeled EC unexposed to PMNs, adherent PMNs stimulated an ~2.3-fold increase in EC MLC phosphorylation in the absence of LTB₄ and a 3.5-fold increase in the presence of LTB₄ (Fig. 3). The increase in EC MLC phosphorylation elicited by LTB₄-challenged PMNs was maximal at 15 min, returning to baseline by 60 min. Direct challenge with LTB₄ alone (in the absence of PMNs) did not alter basal MLC phosphorylation (data not shown). These results indicate that PMN interactions with the EC monolayer, particularly in the presence of chemotactic agents, are sufficient for activation of EC MLCK in this system.

**DISCUSSION**

Given its role as a semiselective diffusion barrier between the plasma and interstitial fluid, the vascular endothelium is a key participant in multiple components of inflammatory processes. For example, tissue edema, a cardinal feature of the acute inflammatory
response, is the result of a complex but well-orchestrated series of biochemical events that are linked to paracellular gap formation evoked by permeability-inducing bioactive agents (6, 12). Evidence now exists that EC gap formation is controlled, at least in part, by actin and/or myosin interactions that are regulated via the phosphorylation of MLCs, an event catalyzed by Ca²⁺/calmodulin-dependent MLCK (4, 8, 11, 21). In recent reports we have characterized the involvement of this kinase in EC gap formation and permeability responses (4), with molecular cloning revealing the presence of a novel MLCK isoform in vascular endothelium (5).

In addition to increases in vascular permeability, the margination and subsequent migration of circulating PMNs through the endothelium into inflamed tissues are other critical components of the inflammatory process (1, 10, 13). After metabolic activation, PMNs mediate EC damage associated with the inflammatory response (17). However, in addition to causing direct vascular injury, PMNs may also disrupt vascular EC monolayer integrity by inducing responses in EC during the process of chemotactic migration (1, 2, 19, 22). In prior studies, our laboratory has demonstrated that PMN-EC interaction and subsequent transendothelial migration result in activation of EC phospholipase D (2), thereby generating important inflammatory mediators such as phosphatidic acid and diacylglycerol. The data reported in the present study indicate that the activity of EC MLCK, via its capacity to increase EC contraction and paracellular gap formation, is critical to the migration of chemotactic-stimulated PMNs. Subsequent experiments performed with radiolabeled EC demonstrated that adherent PMNs can directly increase EC MLC phosphorylation, with maximal effect elicited in the presence of a chemotactant gradient. Because increases in EC MLC phosphorylation were not observed in EC exposed to LTβ in the absence of PMNs, our results are consistent with our hypothesis that PMN-EC interaction, particularly in the presence of a chemotactic stimulus, is essential and sufficient to activate EC MLCK.

Our experiments demonstrate that direct EC MLCK inhibition via pharmacological inhibitors (KT-5926 and ML-7) or via posttranslational modification (cholera toxin) decreased PMN transmigration in response to a chemotactic stimulus. Increasing cAMP-dependent PKA activity with cholera toxin (7, 15) attenuates MLCK activation (5); abolishes thrombin-induced MLC phosphorylation, EC gap formation, and permeability (4); and reverses established barrier dysfunction subsequent to thrombin activation (15). Although the exact PKA substrates involved in regulating PMN diapedesis are unknown, cholera toxin does not prevent adhesion of PMNs to EC monolayers (20). Thus it is unlikely that the protective effects of cAMP on PMN-mediated EC barrier dysfunction (20) or on PMN migration (Fig. 2) can be ascribed to a reduction in PMN adhesion. We speculate that the effect of cholera toxin on PMN migration and macromolecule permeability occurs, at least in part, via its capacity to reduce MLC phosphorylation (4), and subsequent paracellular gap formation (15).

In prior work our laboratory has shown that, in addition to MLCK activity, the phosphorylation status of EC is determined by specific type 1 Ser/Thr myosin-associated phosphatase activities (21). Inhibition of this phosphatase activity with the protein phosphatase inhibitor calyculin A produced dose-dependent enhancement of both EC MLC phosphorylation and EC permeability (21). In the present study, compared with vehicle-pretreated controls, PMN transmigration through EC monolayers pretreated with calyculin was significantly increased in the presence of the chemotactic agent LTβ. Although we have not excluded a potential effect of calyculin on target proteins other than MLC, these data are consistent with our hypothesis that PMN migration into tissues occurs through EC intercellular gaps linked to the phosphory-

Fig. 3. Effect of PMNs on MLC phosphorylation in EC monolayers. EC monolayers were preloaded with 32P-labeled orthophosphate for 3 h and washed and further incubated in medium containing 5 × 10⁶ PMNs in presence or absence of 5 µM LTβ at 37°C for 30 min. MLC were separated by urea gel electrophoresis as described in MATERIALS AND METHODS. Amount of 32P-labeled MLC contained on nitrocellulose membranes was detected by autoradiography followed by immunoblotting with MLC-specific antibodies. A: MLC immunoblot under control conditions. Un-P, Mono-P, and Di-P: positions of un-, mono-, and di-phosphorylated MLC bands, respectively. B: autoradiogram of 32P-labeled EC MLC in control conditions and after PMN addition. Acutal extent of phosphorylation is expressed as %control MLC phosphorylation, normalized by protein loading and in table below autoradiogram. Interaction of PMNs with 32P-labeled EC monolayers increased MLC phosphorylation in a time-dependent fashion, with greatest extent of PMN-mediated MLC phosphorylation observed in presence of LTβ.
Huang et al. (9) noted that the rise in EC Ca\textsuperscript{2+} and/or receptor complexes that remain undefined (22) with a role for PMN-stimulated EC intracellular Ca\textsuperscript{2+} adhesion but was essential for EC gap formation and cytosolic Ca\textsuperscript{2+} increases in EC monolayer permeability (4), a rise in bin-mediated EC gap formation, MLC phosphorylation, though a prompt rise in Ca\textsuperscript{2+} mediated increases in EC Ca\textsuperscript{2+} chemotaxant-stimulated PMNs produce EC MLCK activity and the EC contractile apparatus is an active participant in tissue inflammation. The exact mechanism by which chemotaxant-stimulated PMNs produce EC MLCK activation is not known but likely involves PMN-mediated increases in EC Ca\textsuperscript{2+} (9) through ligand and/or receptor complexes that remain undefined (22). Huang et al. (9) noted that the rise in EC Ca\textsuperscript{2+} evoked by PMN-EC interaction was not required for PMN adhesion but was essential for EC gap formation and PMN migration through EC monolayers, consistent with a role for PMN-stimulated EC intracellular Ca\textsuperscript{2+} mobilization in subsequent EC MLCK activation. Although a prompt rise in Ca\textsuperscript{2+} precedes maximal thrombin-mediated EC gap formation, MLC phosphorylation, decreases in EC monolayer electrical resistance, and increases in EC monolayer permeability (4), a rise in cytosolic Ca\textsuperscript{2+} alone is insufficient to activate EC MLCK (4, 7). Furthermore, characterization of additional PMN-elicted EC signaling pathways that modulate EC MLCK activity and the EC contractile apparatus awaits further study.

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REFERENCES