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Involvement of RhoA and Rho kinase in neutrophil-stimulated endothelial hyperpermeability

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Breslin, Jerome W., and Sarah Y. Yuan. Involvement of RhoA and Rho kinase in neutrophil-stimulated endothelial hyperpermeability. Am J Physiol Heart Circ Physiol 286: H1057–H1062, 2004. First published November 20, 2003; 10.1152/ajpheart.00841.2003.—Neutrophil-induced microvascular leakage is an early event in ischemic and inflammatory heart diseases. The specific signaling paradigm by which neutrophils increase microvascular permeability is not yet established. We investigated whether the small GTPase RhoA and its downstream effector Rho kinase mediate neutrophil-stimulated endothelial hyperpermeability. We assessed the effect of neutrophils on Rho activity in bovine coronary venular endothelial cells (CVEC) with a Rho-GTP pull-down assay. Permeability to FITC-albumin was evaluated using CVEC monolayers. We then tested the role of Rho kinase in the permeability response to neutrophils using two structurally distinct pharmacological inhibitors: Y-27632 and HA-1077. Furthermore, neutrophil-stimulated changes in endothelial F-actin organization were examined with fluorescence microscopy. The results show that C5a-activated neutrophils induced an increase in permeability coupled with RhoA activation in CVEC. Inhibition of Rho kinase with either Y-27632 or HA-1077 attenuated the hyperpermeability response. Rho kinase inhibition also attenuated increases in permeability stimulated by the neutrophil supernatant. In addition, activated neutrophils caused actin stress fiber formation in CVEC, which was diminished by either Y-27632 or HA-1077. These findings suggest that RhoA and Rho kinase are involved in the mediation of neutrophil-induced endothelial actin reorganization and barrier dysfunction.

Endothelial cells play an active role in the regulation of microvascular permeability (17). Endothelial barrier function is likely determined by a balance between adhesive forces at intercellular junctions and actomyosin-generated centripetal forces. Loss of adhesive force at the junctions, increased actomyosin-generated force, or a combination of both may increase paracellular flux of fluids and macromolecules (12, 26, 31). Our laboratory and others have previously demonstrated that PMN-induced hyperpermeability is associated with both myosin light chain kinase-mediated cytoskeletal rearrangement and reorganization of the adherens junction proteins VE-cadherin and β-catenin (11, 13, 18, 36–38). However, the precise mechanisms by which PMN cause these changes remain elusive.

The small GTPase RhoA and its downstream effector Rho kinase are known to regulate myosin light chain phosphorylation and the actin cytoskeleton (4, 23). RhoA and Rho kinase also appear to play a role in thrombin-induced endothelial hyperpermeability, which is typically associated with changes in F-actin organization (9, 25, 40, 42). Inhibition of the RhoA/Rho kinase cascade also reportedly enhances endothelial monolayer barrier function (9, 10) and decreases baseline hydraulic permeability (1). These reports and others (43) implicate a role for RhoA/Rho kinase in the regulation of microvascular permeability. However, no previous study has specifically addressed the involvement of RhoA and Rho kinase in neutrophil-induced increases in endothelial permeability.

This study investigated the role of RhoA and Rho kinase in neutrophil-stimulated endothelial hyperpermeability. We report that incubation of bovine coronary venular endothelial cells (CVEC) with C5a-activated PMN causes activation of endothelial RhoA coupled with an increase in the transendothelial flux of albumin, which is attenuated during inhibition of Rho kinase. Furthermore, we report that Rho kinase inhibition diminishes the appearance of centralized stress fibers in CVEC monolayers caused by activated PMN and promotes peripheral localization of F-actin. This work demonstrates for the first time a role for the RhoA/Rho kinase pathway in neutrophil-induced endothelial hyperpermeability.

MATERIALS AND METHODS

Materials. Human C5a, Y-27632, and HA-1077 were obtained from Calbiochem (San Diego, CA). Endothelial basal medium (EBM) and endothelial growth medium (EGM) were obtained from Clonetics (San Diego, CA). FITC-albumin, FITC-phalloidin, and Hoechst 33342 were obtained from Sigma (St. Louis, MO). Vectashield mounting medium was from Vector Laboratories (Burlingame, CA).

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THE ENDOTHELIAL CELLS of capillaries and postcapillary venules form a semipermeable barrier that regulates the exchange of fluids and solutes between blood and tissues. Abnormally excessive leakage across the microvascular barrier can lead to edema and tissue dysfunction. Increased coronary microvascular leakage is an early event in heart disease and is largely attributed to inflammatory mediators and the activation of polymorphonuclear leukocytes (PMN), predominantly neutrophils (17, 19, 20, 24). At a site of inflammation or injury, neutrophils interact with endothelial cells, triggering rapid signaling reactions and reversible conformational changes in the endothelium (16, 32). These coordinated events allow neutrophils to adhere to and migrate across the endothelium and into the surrounding tissues (15, 21). As stated above, the process of PMN adherence and migration is associated with an increase in endothelial permeability (8, 14, 22, 48).

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Isolation and activation of porcine neutrophils. Porcine neutrophils (PMN) were isolated as previously described (36, 37, 45). Briefly, porcine arterial blood was centrifuged for 20 min at 300 g to separate plasma and blood cells. The plasma was centrifuged for 10 min at 2,500 g to obtain platelet-poor plasma (PPP). The buffy coat was removed, and the pellet containing red blood cells and PMN was incubated in a solution containing 2% gelatin and 20% PPP in Hanks’ balanced salt solution at 37°C for 45 min. The supernatant was centrifuged at 300 g for 10 min, and PMN were further purified from the supernatant by hypotonic hemolysis. Viability and normal chemotactic function were verified as previously reported (46, 48).

Endothelial cell culture and monolayer permeability assay. CVEC, harvested from the bovine heart (30), were routinely maintained in EGM supplemented with 10% fetal bovine serum. For permeability assays, cells were grown 4–5 days on gelatin-coated Costar Transwell membranes (VWR; Houston, TX) as previously described (37). For time course experiments, CVEC were treated with C5a-activated PMN (10^6 PMN/ml) in the presence of FITC-albumin for the indicated times. In some experiments, CVEC were exposed to PMN and/or drugs (as indicated) for 30 min, and FITC-albumin was then added for 45 min. Samples were collected from both the upper (luminal) and lower (abluminal) chambers for fluorometry analysis. Albumin concentrations were determined using a standard curve, and the permeability coefficient for albumin (P_a) was calculated as follows: 

\[ P_a = \frac{[A]r \times V}{1/A \times V/[L]} \]

where [A] is the abluminal albumin concentration, \( t \) is time (in s), \( A \) is the area of the membrane (in cm^2), \( V \) is the volume of the abluminal chamber, and \( [L] \) is the luminal albumin concentration.

RhoA activation assay. RhoA activation was assessed using a Rho-GTP pull-down assay kit purchased from Upstate (Lake Placid, NY). CVEC were grown to confluence in 15-cm-diameter culture dishes. After treatment with PMN or vehicle, the cells were washed twice with ice-cold Tris-buffered saline containing 25 mM NaF and 1 mM EDTA, 10% glycerol, 10% buffer (MLB; containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% aprotinin, 10 μg/ml leupeptin, 25 mM NaF, and 1 mM vanadate) and incubated on a rotator at 4°C for 15 min. After clarification by centrifugation at 13,000 g for 10 min, an aliquot of each lysate was diluted in MLB, followed by incubation with Rheotekin-Rho-binding domain-agarose on a rotator at 4°C for 1 h. The beads were washed three times with MLB, followed by incubation in 2× SDS reducing sample buffer at 95°C for 5 min to elute Rho. Active Rho was then detected by immunoblotting.

Fluorescence microscopy. CVEC were grown on gelatin-coated round no. 1 coverslips (VWR) and allowed to reach confluence. After treatment with PMN and/or drugs, the cells were fixed in 4% paraformaldehyde in PBS for 5 min and permeabilized with 0.5% Triton X-100 for 5 min. F-actin was labeled with FITC-phalloidin, and nuclei were labeled with Hoechst 33342. The coverslips were mounted on glass slides with Vectashield. Images were obtained using a Zeiss Axiosvert 300M fluorescence microscope equipped with an AxioCam MRm black and white digital camera (Carl Zeiss; Thornwood, NY). Digital images were collected using Zeiss Axiovision 4.0 software.

Data analysis. For each experiment, the P_a values obtained from different culture plates were normalized to control, averaged, and reported as a percentage of control (means ± SE). ANOVA was used to evaluate the significance of intergroup differences. Significance was accepted at \( P < 0.05 \).

RESULTS

Incubation with C5a-activated PMN caused an increase in CVEC monolayer permeability to FITC-albumin. This increase became significant 30 min after the addition of activated PMN. The maximum response we observed occurred in the time period of 30–75 min after activated PMN were added (Fig. 1A). The response to C5a-activated PMN was also concentration dependent (Fig. 1B). Suspensions of 10^3, 10^5, and 10^7 activated PMN/ml increased \( P_a \) to ~142%, 162%, and 171% of control, respectively. Incubation with C5a alone or inactive PMN (10^6 PMN/ml) had no effect on \( P_a \) (Fig. 2), indicating that the permeability response was caused by factors secreted by or present on activated PMN.

Treatment of CVEC monolayers with activated PMN (10^6 PMN/ml) also caused RhoA activation (Fig. 3). A slight increase in activated RhoA was apparent 5 min after the addition of activated PMN, and the maximum RhoA activation we observed occurred at 30 min (Fig. 3A). Incubation with PMN that were not activated with C5a, or with C5a alone, did not cause RhoA activation in CVEC monolayers. In addition, RhoA activation in the volume of a suspension of C5a-activated PMN alone (not added to CVEC) was insufficient to increase the band intensity of CVEC treated with activated PMN (Fig. 3B).

We next tested the role of Rho kinase in PMN-induced endothelial hyperpermeability. Y-27632, a specific Rho kinase inhibitor, attenuated PMN-induced increases in \( P_a \) in a con-
centration-related manner (Fig. 4A). This range of concentrations (from $10^{-6}$ to $2.5 	imes 10^{-5}$ M) did not affect baseline permeability in the absence of activated PMN. Likewise, a structurally distinct Rho kinase inhibitor, HA-1077, also attenuated PMN-induced hyperpermeability (Fig. 4B).

Our laboratory has previously shown that C5a-activated PMN can increase $P_a$ in an adhesion-independent manner (36, 45). In CVEC, the addition of the supernatant from a suspension of C5a-activated PMN ($10^7$ PMN/ml, supernatant added 1:10 to the CVEC monolayer) caused activation of RhoA (Fig. 5A). The addition of this supernatant also caused a significant increase in $P_a$ (Fig. 5B). Although slightly lower, this increase in $P_a$ was not significantly different from the increase observed when CVEC were incubated with $10^6$ activated PMN/ml. Treatment with either Y-27632 or HA-1077 significantly attenuated the PMN-induced, adhesion-independent hyperpermeability (Fig. 5).
Rho kinase activity has been implicated in actin polymerization (4). Because actin localization and polymerization are thought to have a key role in the regulation of endothelial barrier function (7, 12, 27), we examined whether Rho kinase inhibition affected PMN-induced changes in endothelial actin organization. In control CVEC, actin was typically observed as peripheral bands predominantly at cell-cell contacts and also as stress fibers in some cells (Fig. 6A). When CVEC were treated with C5a-activated PMN, a marked increase in actin stress fibers was observed, and less actin at intercellular junctions was evident (Fig. 6B). Treatment of nonstimulated CVEC with Y-27632 did not cause any apparent change in F-actin organization (Fig. 6C). However, Y-27632 did change the organization of F-actin in CVEC stimulated with activated PMN (Fig. 6D). Although the amount of F-actin appeared to be the same as in PMN-treated CVEC in which Y-27632 was not present, the appearance of long stress fibers was diminished in most cells and was replaced with very short fibers attached at foci in a lattice-like structure. More F-actin at intercellular junctions and fewer stress fibers were observed in CVEC treated with HA-1077 (Fig. 6E). In addition, HA-1077 treatment attenuated actin polymerization in CVEC treated with activated PMN (Fig. 6F). There were some long stress fibers and also lattice structures of F-actin similar to those seen in CVEC treated with Y-27632 and activated PMN. However, the overall degree of actin polymerization appeared to be diminished further with HA-1077 treatment.

DISCUSSION

The data from the present study suggest that RhoA and Rho kinase mediate neutrophil-induced increases in endothelial permeability. We demonstrated that C5a-activated PMN increase RhoA activity in endothelial monolayers and that inhibition of Rho kinase by either of two structurally distinct pharmacological agents attenuates PMN-induced increases in endothelial permeability. We also showed that C5a-activated PMN can increase endothelial permeability in an adhesion-independent manner, to approximately the same level as when PMN were allowed to adhere to endothelial cells, and that this was also attenuated by Rho kinase inhibition. In addition, we showed that Rho kinase plays a role in PMN-induced F-actin polymerization, an event associated with the regulation of endothelial permeability (35, 37, 45).
We demonstrated that C5a-activated PMN increase endothelial monolayer permeability in a concentration-dependent manner. This finding is in agreement with our laboratory’s previous results using an isolated coronary venule model to assess permeability changes (45). In addition, the supernatant from activated PMN is sufficient to stimulate increases in endothelial permeability to almost the same degree as adding activated PMN directly to the endothelial monolayer, consistent with our previous findings (36, 45). This latter observation proved important for distinguishing the effects of pharmacological inhibitors on endothelial cells versus PMN (see below). The fact that there was no significant difference between the increases in permeability caused by direct addition of activated PMN versus the supernatant from activated PMN also suggests that adhesion of PMN may not be a prerequisite for PMN-induced hyperpermeability.

We also showed that C5a-activated PMN cause RhoA activation in endothelial cells. The maximal RhoA activation we observed occurred at the beginning of the time in which we observed the maximal permeability response, supporting the idea that these two events are associated. This is a new finding because to our knowledge no other study has addressed the issue of RhoA activation and hyperpermeability in endothelial cells during neutrophil stimulation. Other investigators have shown that soluble permeability-increasing agents such as thrombin and VEGF can activate endothelial RhoA (39, 40), providing further support for an association between RhoA activation and increased endothelial permeability.

Our data show that inhibition of Rho kinase by two structurally distinct pharmacological agents attenuated PMN-induced hyperpermeability. This is concordant with studies in which RhoA or Rho kinase blockade attenuated increased permeability in response to thrombin, VEGF, and histamine (9, 25, 39, 40, 42). In addition, our data support other reports showing that Rho kinase inhibition diminishes neutrophil migration across endothelial monolayers (28) and neutrophil infiltration after subarachnoid hemorrhage (29) or hepatic ischemia-reperfusion injury (34). Our data support the notion that RhoA and Rho kinase mediate PMN-induced increases in permeability. One study, however, reported that Rho kinase does not mediate increases in permeability caused by platelet-activating factor (PAF) or bradykinin (1). Moreover, PAF- and bradykinin-induced hyperpermeability does not appear to involve actomyosin contraction (2), indicating that RhoA/Rho kinase-mediated actin polymerization may not serve as a general mechanism for all agonists that cause increased permeability.

Rho kinase inhibition also attenuates adhesion-independent PMN-induced hyperpermeability, i.e., increases in permeability stimulated by the supernatant from activated PMN after centrifugation. These studies served two important purposes: 1) to demonstrate that Rho kinase mediates the hyper permeability response stimulated by factors released by activated PMN, and 2) to help distinguish whether the Rho kinase inhibitors act directly on the CVEC monolayer to attenuate PMN-induced increases in permeability. The latter was especially important because RhoA activation in PMN appears to be involved in their chemotactic activity and migration (3, 33, 41). We cannot exclude the possibility that the pharmacological agents used may have acted upon the PMN, when applied directly to CVEC. However, we feel confident that endothelial Rho kinase mediates PMN-induced hyperpermeability, largely because Rho kinase inhibition attenuated this response when it was stimulated by the supernatant from activated PMN.

Actin polymerization plays a major role in the determination of cell shape, which in turn is a vital factor in the organization of tissues. Our laboratory and others have previously reported the importance of F-actin arrangement in the regulation of the endothelial barrier (6, 12, 27, 35, 44, 45, 47). Moreover, RhoA and Rho kinase are known to mediate actin polymerization (5). We report here that C5a-activated PMN stimulate polymerization of actin in venular endothelial cells, with increased appearance of long, centralized stress fibers, as previously shown (37, 45). Pharmacological inhibition of Rho kinase diminished the PMN-stimulated appearance of long stress fibers in CVEC. Interestingly, a lattice formation of F-actin was observed in the place of stress fibers. These data suggest that, whereas PMN-induced actin stress fiber formation may be Rho kinase dependent, a Rho kinase-independent pathway may also be involved in actin polymerization during PMN stimulation. We speculate that the altered F-actin arrangement due to Rho kinase inhibition may be sufficient to attenuate PMN-induced endothelial hyperpermeability.

In summary, our data suggest that endothelial RhoA and Rho kinase mediate PMN-induced increases in endothelial permeability. Rho kinase plays an important role in PMN-induced changes in the endothelial barrier, regardless of whether PMN adhere to the endothelium. These PMN-stimulated increases in permeability are associated with Rho kinase-mediated changes in the actin cytoskeleton, which is likely an important player in the regulation of the endothelial barrier.

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