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Rho inhibition decreases TNF-induced endothelial MAPK activation and monolayer permeability

Fiemu E. Nwariaku,[1] Patricia Rothenbach,[1] Zijuan Liu,[1] Xudong Zhu,[1] Richard H. Turnage,[2] and Lance S. Terada[1]

[1]*Departments of Surgery and Pulmonary Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9156; and* [2]*Department of Surgery, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71115*

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Nwariaku, Fiemu E., Patricia Rothenbach, Zijuan Liu, Xudong Zhu, Richard H. Turnage, and Lance S. Terada. Rho inhibition decreases TNF-induced endothelial MAPK activation and monolayer permeability. *J Appl Physiol* 95: 1889–1895, 2003. First published July 3, 2003; 10.1152/japplphysiol.00225.2003.—Our laboratory previously demonstrated that MAPK activation is an important signal during cytokine-induced endothelial permeability (Nwariaku FE, Liu Z, Terada L, Duffy S, Sarosi G, and Turnage R. *Shock* 18: 82–85, 2002). Because GTP-binding proteins have been implicated in MAPK activation, we now hypothesize that the GTP-binding protein Rho is a mediator of TNF-induced MAPK activation and increased endothelial permeability. Transmonolayer permeability was assessed in human lung microvascular cells by measuring transmonolayer electrical resistance. MAPK activity was assessed by using a phospho-specific immunoprecipitation kinase assay and by comparing Western blots for phospho-MAPK with total MAPK. MAPK inhibitors used were SB-202190 and PD-098059, whereas *Clostridium botulinum* C3 transferase was used as a Rho inactivator. Rho-associated coiled-coil kinase was inhibited with Y-27632. TNF increased pulmonary endothelial permeability in vitro and caused a rapid, sustained increase in endothelial p38 and extracellular signal-regulated kinase MAPK activity. Inhibition of p38 and extracellular signal-regulated kinase MAPK with SB-202190 and PD-098059, respectively, decreased TNF-induced endothelial permeability. C3 transferase attenuated TNF-induced MAPK activation and blocked TNF-induced endothelial permeability. Finally, inhibition of Rho-associated coiled-coil kinase with Y-27632 prevented both MAPK activation and TNF-induced decreases in transmonolayer resistance. Rho acts upstream of mitogen-activated protein kinases in mediating TNF-induced pulmonary endothelial leak.

endothelium; guanosine 5′-triphosphate-binding proteins; cytokines; tumor necrosis factor; mitogen-activated protein kinase; extracellular signal-regulated kinase; p38; cell signaling

ENDOTHELIAL DYSFUNCTION IS a major component of a variety of inflammatory states such as systemic inflammatory response syndrome and ischemia-reperfusion. Elevated levels of cytokines have been implicated in the endothelial barrier dysfunction associated with these conditions (7, 36, 37). TNF appears to be an important cytokine that mediates endothelial dysfunction, in part, through dramatic cytoskeletal rearrangement, leading to loss of endothelial barrier function (5). In addition to cytoskeletal changes, TNF exposure is associated with intercellular gap formation, endothelial cell (EC) retraction, and redistribution of junctional adhesion molecules (2, 12, 21). However, the intracellular mediators of gap formation and junctional redistribution remain elusive. We previously demonstrated that TNF increases endothelial permeability and causes redistribution of the lateral junctional protein, vascular endothelial cadherin, in confluent human umbilical vein endothelial monolayers. Inhibitors of the MAPKs prevented these changes. Rho family GTPases induce actin microfilament remodeling and potentially act upstream of MAPK pathways (18, 22, 39, 45). In the present study, we demonstrate that TNF-induced endothelial permeability proceeds through Rho-induced activation of MAPK.

MATERIALS AND METHODS

Cell Lines

Human lung microvascular ECs (HLMEC) (Bio-Whitaker, Walkersville, MD) were grown in EGM-2 (Bio-Whitaker) culture medium. Cultures were maintained at 37°C in a humidified 5% CO_2 environment until confluent (~3–5 days). Confluent ECs were serum starved 12–24 h before each experiment by culturing in serum-free media. For experiments involving Western immunoblot, the cells were grown in 60-mm petri dishes (Corning, Acton, MA).

Cells used for permeability studies were cultured to confluence on Transwell clear polyester membranes (Corning CoStar, Cambridge, MA) at a density of 75,000–100,000 cells/ml. All cells were used for experiments between *passages 2* and *5*. During inhibition experiments, HLMEC were pretreated with MAPK or Rho inhibitors 1 h before TNF exposure.

Transendothelial electrical resistance. We assessed permeability by measuring the decrease in electrical resistance across endothelial monolayers exposed to TNF. HLMEC were cultured to confluence on 24-well Transwell clear polyester

Address for reprint requests and other correspondence: F. E. Nwariaku, 5323 Harry Hines Blvd., Dallas, TX 75390-9156 (E-mail: fiemu.nwariaku@UTSouthwestern.edu).

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membrane cell culture chambers (Corning CoStar) at a density of 75,000–100,000 cells/ml. Resistance measurements were obtained with an EVOM-G voltmeter (World Precision Instruments, Sarasota, FL), with 6-mm tissue culture cups placed in an Endohm-6 resistance measurement chamber (World Precision Instruments). Baseline resistance was measured across a blank tissue culture cup with culture medium, and that value was subtracted from subsequent resistance measurements.

Reagents and Antibodies

Inhibitors of p38 [SB-202190; 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole] and extracellular signal-regulated kinase (ERK) (PD-98059; 2′-amino-3′-methoxyflavone) (Calbiochem, San Diego, CA) were added to monolayers at concentrations of 10 and 20 μM, respectively, 1 h before TNF (100 U/ml) exposure. Both inhibitors are widely described and demonstrate specificity for the respective kinases at the concentrations used (25, 26, 41).

Rho inactivation was accomplished by using *Clostridium botulinum* C3 transferase (5 μM) 18–24 h before TNF exposure. The Rho effector Rho-associated coiled-coil kinase (ROCK, ROK) was inactivated by using the specific inhibitor Y-27632 (10 μM) (20, 31). Antibodies against phospho-Elk and ATF were obtained from Cell Signaling Technology (Beverly, MA) as part of a kinase assay kit. Phospho-MAPK antibodies were purchased from Cell Signaling Technology.

Kinase Assays

We examined activation of p38 and ERK MAPKs using a nonradioactive immunoprecipitation kinase assay (Cell Signaling Technology). Endothelial monolayers were exposed to vehicle or TNF (100 U/ml) for 5, 15, or 30 min, or 4 h. Lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 10 mM $MgCl_2$] was added for 5 min, and cells were scraped, sonicated, and centrifuged for 10 min at 14,000 rpm. Phosphorylated MAPK was immunoprecipitated from the resulting supernatant (200 μl) by using immobilized phospho-specific p38 or ERK monoclonal antibodies (Cell Signaling Technology). The resulting immunoprecipitates were resuspended in kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$] supplemented with 200 μM ATP and 2 μg of either ATF-2 or Elk-1 fusion proteins (Cell Signaling Technology). Substrate phosphorylation was then detected by Western blotting for phospho-ATF-2 or phospho-Elk-1.

To provide further confirmation of MAPK activation, we performed immunoblots for phosphorylated MAPK and total MAPK from HLMEC lysates exposed to vehicle or TNF. Equal amounts of protein from cell lysates were loaded onto SDS-polyacrylamide gels and detected with specific antibodies against phospho-ERK, phospho-p38, or total MAPK (Cell Signaling Technology). The amounts of phosphorylated and total MAPK were then compared within each group.

Statistical Analysis

Data are expressed as means ± SE of the mean. Statistical comparisons were performed by using ANOVA with Tukey's post hoc test. Differences between groups were considered statistically significant at a P value of <0.05. Kinase assays were performed in triplicate, while the sample size for permeability experiments was six or more per experimental group.

RESULTS

TNF Activates Endothelial p38 and ERK MAPK

Baseline MAPK activity was minimal in the control groups. Exposure of endothelial monolayers to TNF resulted in early activation of p38 and ERK MAPK. Specifically, ERK activation was increased eightfold within 5 min and 20-fold after 30 min. The p38 activity also increased approximately fivefold after 30 min of TNF exposure. Activation of ERK MAPK persisted through 8 h of TNF exposure (Fig. 1). TNF-induced p38 activation was cyclical, with a transient and secondary peak at 4 h (Fig. 1). Vehicle control did not induce p38 or ERK activation at any time point.

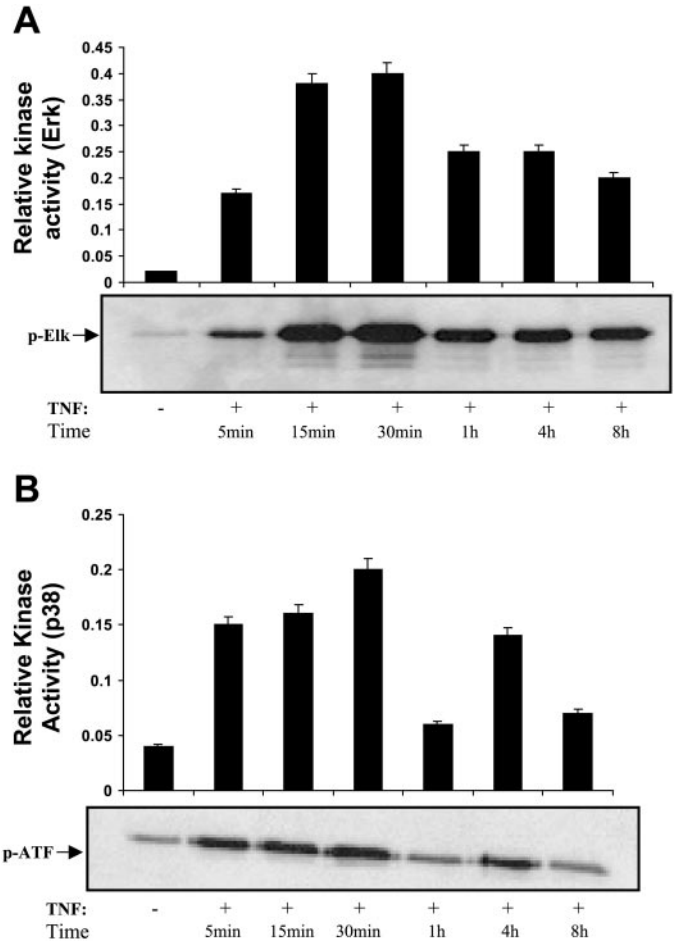

Fig. 1. Effect of TNF on MAPK activation. Kinetics of TNF-induced extracellular signal-regulated kinase (ERK) (*A*) and p38 MAPK activation (*B*). Serum-starved confluent human lung microvascular endothelial cell (HLMEC) monolayers were exposed to vehicle or TNF (100 U/ml) for 5 min through 8 h. Cells were lysed, and phosphorylated MAPKs were immunoprecipitated from the resulting supernatant by using immobilized phospho-specific p38 or ERK monoclonal antibodies. The resulting immunoprecipitates were incubated with 2 μg of either ATF-2 or Elk-1 fusion proteins. Substrate phosphorylation was then detected by Western blotting for phospho-ATF-2 (p-Elk) or phospho-Elk-1 (p-ATF). Histogram shows a mean of three or more experiments. TNF caused early and robust activation of Erk and p38. Activation of both kinases was sustained over 8 h; however, p38 activity diminished at later time points. Values are means ± SE.

Using a different method, we assessed p38 and ERK activation by comparing phospho-p38 and phospho-ERK with total MAPK in cell lysates. Similar to the results using a kinase activity assay, TNF resulted in rapid phosphorylation of both p38 and ERK (Fig. 2).

MAPK Inhibitors Prevent TNF-Induced Endothelial Permeability

Endothelial monolayers exposed to TNF demonstrated a greater decrease in transmonolayer electrical resistance (TER) over 6 h compared with control monolayers or monolayers pretreated with the p38 or ERK inhibitors (Fig. 3). This attenuation of TNF-induced decreased TER appeared within 60 min and persisted through 6 h. Exposure of monolayers to the MAPK inhibitors SB-202190 and PD-098059 alone had no effect on monolayer electrical resistance compared with vehicle controls (Fig. 3).

C3 Transferase Attenuates Endothelial MAPK Activity

Rho inactivation with C3 transferase attenuated both basal and TNF-induced ERK activation at 1 and 4 h. In contrast, whereas p38 activity at 1 h was decreased by C3 transferase, this effect was less marked compared with ERK activity and did not reach control (baseline) levels (Fig. 4). C3 only also decreased

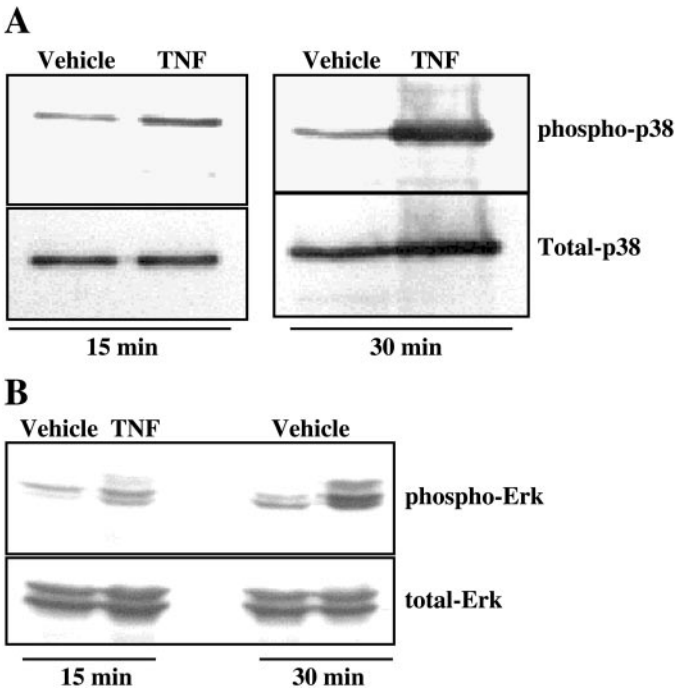

Fig. 2. Effect of TNF on p38 (*A*) and ERK phosphorylation (*B*). To provide further confirmation of MAPK activation, we performed immunoblots for phosphorylated MAPK and total MAPK from HLMEC lysates exposed to vehicle or TNF for 15 and 30 min. Equal amounts of protein were loaded onto SDS-polyacrylamide gels and probed with specific antibodies against phosphorylated and total MAPK. TNF induced phosphorylation of p38 and ERK in the presence of unchanged total MAPK levels. These experiments were performed in duplicate.

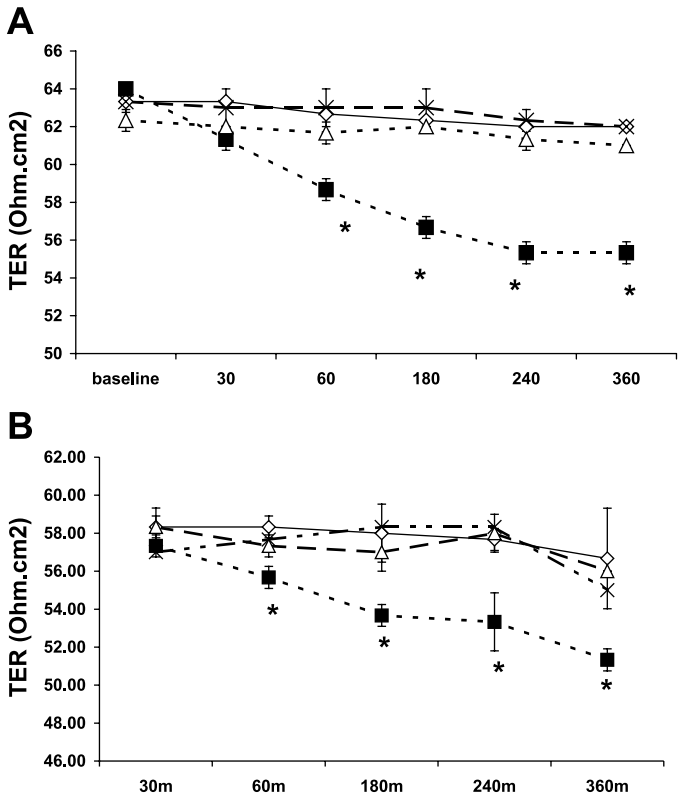

Fig. 3. Effect of MAPK inhibition on TNF-induced transmonolayer electrical resistance (TER). The effect is shown of ERK (*A*) and p38 (*B*) on TNF-induced (■) TER for up to 6 h. *A*: the p38 inhibitor SB-202190 (△) prevented TNF-induced decreased TER, compared with saline controls (◇). SB-202190 alone (×) had no effect on TER. *B*: similarly, the ERK inhibitor PD-098059 (△) prevented TNF-induced decreased TER. PD-098059 alone (×) had no effect on TER. Values are means ± SE; $n > 5$ per group. $^*P < 0.05$.

baseline MAPK activity. C3 transferase also significantly prevented the decreased transendothelial electrical resistance induced by TNF after 30 and 60 min; however, this effect did not persist through 6 h (Fig. 5).

Y-27632 Attenuates TNF-induced MAPK Activation and Endothelial Permeability

An important downstream effector of Rho is the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK). Thus we examined the role of ROCK as an effector of TNF-mediated endothelial permeability and MAPK activation using the specific ROCK inhibitor Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(-4-pyridyl)cyclohexanecarboxamide dihydrochloride] (11, 20, 23, 29, 30). Exposure of endothelial monolayers to Y-27632 caused attenuation of TNF-induced p38 and ERK MAPK activation after 30 min and 4 h of TNF exposure (Fig. 6). ROCK inhibition also prevented TNF-induced decreases in transendothelial electrical resistance (Fig. 7).

DISCUSSION

We report here that TNF-induced microvascular permeability is associated with both p38 and ERK mitogen-activated kinase activation. Inhibition of both ki-

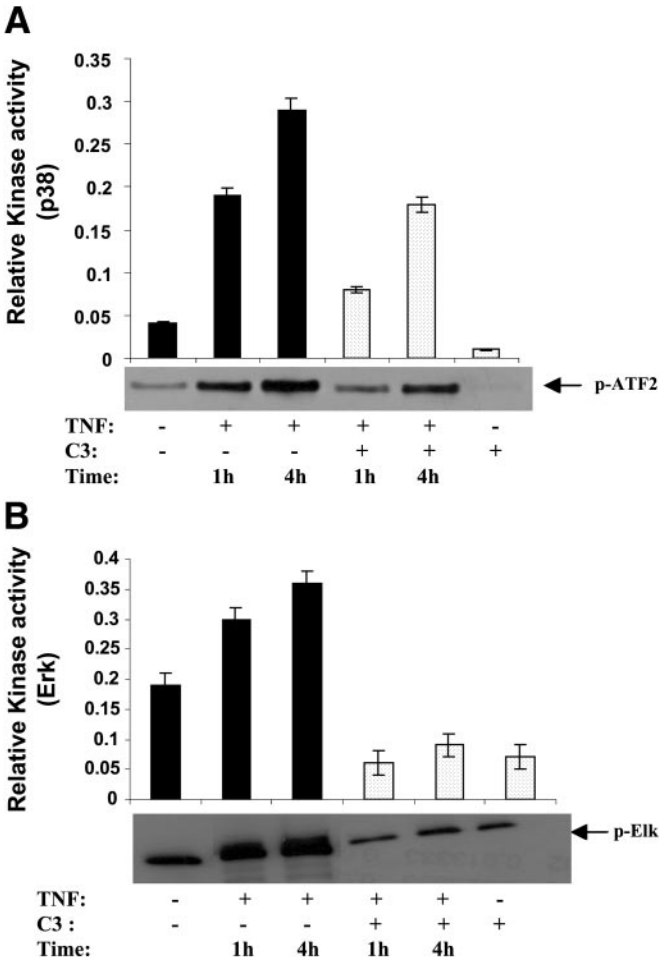

Fig. 4. Effect of C3 transferase on TNF-induced MAPK activation. Confluent HLMEC monolayers were incubated with *Clostridium botulinum* C3 transferase 18 h before vehicle or TNF exposure. MAPK activation was examined at 1 and 4 h. *B*: exposure of monolayers to the Rho inhibitor C3 transferase (5 μM) markedly reduced TNF-induced ERK activation. *A*: in contrast, p38 activity was reduced in the presence of C3, and this difference was less marked at the 4-h time point. Whereas C3 reduced the baseline level of MAPK activation, it did not prevent a response to TNF, suggesting attenuation and not complete abolition of the TNF response. Histograms represent the mean of 3 or more experiments. Values are means ± SE.

nases also reduced TNF-induced endothelial barrier dysfunction, a process that appears to be at least partly dependent on the Rho pathway. Interestingly, ROCK appears to play a role in Rho-mediated MAPK activation and EC dysfunction.

The pattern of MAPK activation in human lung microvascular endothelium appears similar to that observed in other endothelial beds. Using both immunoprecipitation kinase assays and phosphorylation, we observed early and persistent activation of both p38 and ERK. This event peaked ~30 min after TNF exposure for both kinases, in keeping with their roles as proximal signaling molecules. An interesting secondary observation was the biphasic activation of p38 with a transient decrease in activation at 1 h and rebound at 4 h post-TNF exposure. This rebound phenomenon may suggest secondary p38 activation by other cytokines induced by TNF. Indeed, TNF is known to induce interleukin-6 and -8, both of which are involved in MAPK signaling (8, 17). Furthermore, inhibition of either p38 or ERK prevented TNF-induced HLMEC permeability, suggesting a prominent role for both MAPKs in TNF-induced endothelial permeability. This observation is consistent with prior studies examining the role of MAPK in EC permeability, as reported by our laboratory and others (14, 42). The effect of MAPK inhibition in HLMEC contrasts with our findings in human umbilical vein endothelial cells, which suggest that p38, but not ERK, MAPK mediates TNF-induced EC dysfunction (14). Indeed, both p38 and ERK have been shown to play differential roles in cytokine-mediated signaling (26). Taken together, these data suggest that MAPK may have differential signaling roles, depending on cell type, i.e., macrovascular vs. microvascular endothelial beds. This differential role for p38 and ERK was also examined by Niwa and associates (32), who reported that peroxide-induced permeability in bovine pulmonary artery endothelium was inhibited by p38 but not ERK antagonists. Given that TNF signals through oxidant production (1), it is notable that p38 inhibition was shown to have a greater effect than ERK inhibition in decreasing oxidant-induced permeability in bovine pulmonary artery endothelial monolayers (32).

We next explored pathways proximal to the MAPKs, which may mediate TNF-dependent permeability changes. Both TNF and the small GTP-binding protein Rho induce EC shape change, and, in fact, constitutively active RhoA induces cytoskeletal changes similar to those found after TNF exposure (45). Rho family proteins such as Rac1 and Cdc42 are also known to be activated by TNF and cause changes in cell shape, cell-cell adhesion, and MAPK activation (3, 4, 10, 13, 15, 16, 24, 33, 35, 44). Therefore, we examined the

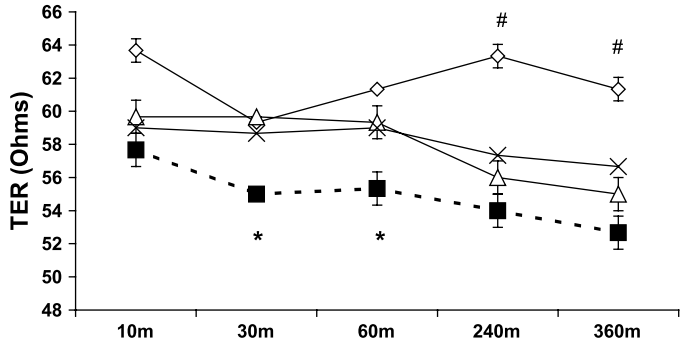

Fig. 5. Effect of C3 transferase on TNF-induced decreased transendothelial electrical resistance (TER). Confluent HLMEC monolayers were cultured in Transwell chambers, and TEER was measured over 6 h. Exposure of HLMEC monolayers to TNF + C3 transferase (△) significantly prevented TNF-induced hyperpermeability (■, dashed line) compared with vehicle controls (◇) at the earlier time points, 30 and 60 min. C3 transferase alone (×) was not significantly different from controls at 30 and 60 min. All treatment groups had significantly lower TER after 2 and 4 h of exposure compared with controls. Values are means ± SE; $n > 6$ per group. $^{*}P < 0.05$, TNF vs. other groups. $^{\#}P < 0.05$, controls vs. other groups.

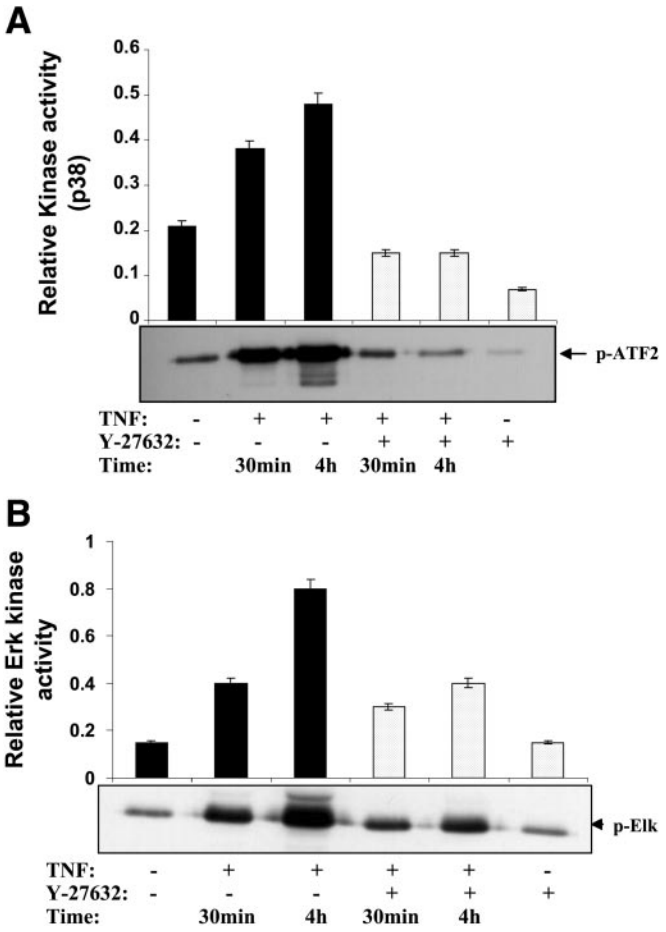

Fig. 6. Effect of Y-27632 inhibition on MAPK activation. Confluent HLMEC were incubated with the Rho-associated coiled-coil kinase-specific inhibitor Y-27632 (10 μM) 1 h before TNF exposure. After 30 min and 4 h of saline or TNF exposure, cell lysates were examined for p38 (*A*) and ERK activity (*B*) as described above. Y-27632 (shaded bars) significantly attenuated TNF-induced p38 and ERK activation (solid bars). These experiments were performed in triplicate. Values are means ± SE.

effect of Rho pathway inhibition on TNF-induced MAPK activity and EC permeability. We found that Rho inactivation using *Clostridium botulinum* C3 transferase markedly reduced TNF-induced HLMEC ERK and p38 activation, as well as permeability, consistent with the involvement of RhoA in TNF signaling upstream of the MAPKs. The inhibitory effect of C3 transferase on ERK activation was more consistent compared with that on p38. Inhibition of p38 activity by C3 transferase appeared to be less effective at the later time point, suggesting that p38 activation may escape TNF-mediated Rho effects. Whereas TNF was found to increase p38 activation, even in the presence of C3 transferase, the overall levels of p38 activity were markedly less compared with that in the TNF group. This suggests a blunting of the TNF response, as opposed to complete abolition of TNF signaling. It could also signify the presence of alternative ERK-independent pathways of TNF-induced MAPK activation independent of Rho (37, 38). Also consistent with an important role for MAPK activation during Rho signaling is the finding by Hippenstiel et al. (18) and Marinissen et al. (28), who demonstrated that Rho-mediated cell signaling and gene expression require activation of p38 and ERK. In this study, Rho inhibition also transiently prevented early TNF-induced EC dysfunction as measured by TER. These effects of C3 appear to disappear at later time points, suggesting either escape from Rho inhibition or recruitment of alternative signaling pathways by TNF or TNF-induced cytokines such as interleukins. Of interest is the observation that monolayers exposed to C3 alone showed lower TER compared with vehicle controls at later time points. This may reflect C3-induced activation of other pathways that alter permeability independent of Rho. Collectively, these data support the hypothesis that Rho, p38, and ERK MAPK are important mediators of EC dysfunction.

Of the studied Rho effectors, ROCK has been shown to be involved in junctional assembly and thrombin-mediated EC permeability (6, 9, 19, 43). ROCK has also been implicated in Rho-induced MAPK activation (20, 27, 30, 38, 46). In this study, ROCK inhibition attenuated TNF-induced MAPK activation and subsequent decreases in transendothelial electrical resistance. These observations provide further support for the involvement of Rho pathways in TNF-mediated EC dysfunction and implicate ROCK as one potential downstream mediator. Inhibition of p38 and ERK by ROCK was consistent and similar to that observed by C3 transferase. One notable difference was that, whereas the C3 transferase blocked baseline p38 activity, ROCK inhibition had no such effect. This may be explained by the long preincubation time (18–24 h) for C3 transferase compared with 1 h for the ROCK inhibitor. Despite this, the ROCK inhibitor appeared to be more effective than C3 transferase at preventing TNF-mediated changes in TER. This observation may be due to a ROCK-dominant pathway during Rho-mediated EC dysfunction, combined with less effective ROCK inhibition by C3 transferase. Our observations differ from those of Petrache and colleagues (34), who

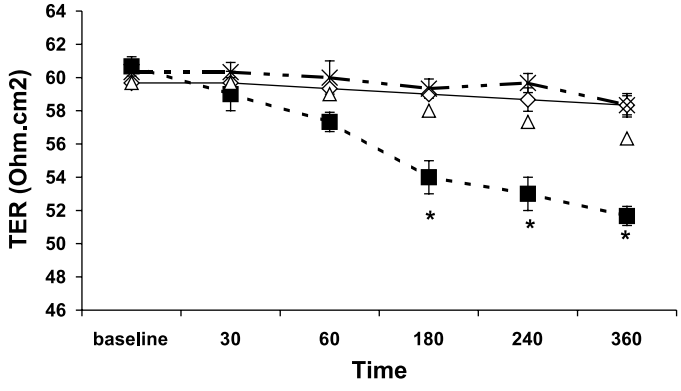

Fig. 7. Effect of Y-27632 on TNF-induced decreased TER. Confluent HLMEC monolayers were cultured in Transwell chambers, and TER was measured as described above. TNF (■) caused an early and sustained decrease in TER compared with controls (◇). Y-27632 (△) prevented TNF-induced decreased TER. Exposure of monolayers to Y-27632 alone (×) had no significant effect on TER compared with controls. Values are means ± SE. $^*P < 0.05$.

noted that ROCK inhibition prevented TNF-induced cytoskeletal changes but had no effect on permeability. This latter study was conducted in pulmonary artery ECs, again supporting the idea of divergent TNF signaling between conduit and microvascular ECs. In other studies, ROCK appears to mediate peroxide-induced pulmonary edema and leukotriene- and thrombin-induced cytoskeletal changes and monolayer permeability (6, 9, 40), suggesting broad relevance for this pathway in cytokine-mediated EC dysfunction.

We conclude that TNF-mediated microvascular endothelial barrier dysfunction involves the activation of Rho and ROCK, acting upstream of p38 and ERK MAPK. These findings differ slightly from findings in human umbilical vein endothelial cells and suggest cell-type differences in dominant signaling pathways during EC dysfunction. These data also imply the possibility that targeting Rho activation pathways may provide targets for modulating inflammatory EC dysfunction.

DISCLOSURES

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