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Ezrin/Radixin/Moesin Proteins Are Phosphorylated by TNF-α and Modulate Permeability Increases in Human Pulmonary Microvascular Endothelial Cells

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Endothelial cells (ECs) respond to TNF-α by altering their F-actin cytoskeleton and junctional permeability through mechanisms that include protein kinase C (PKC) and p38 MAPK. Ezrin, radixin, and moesin (ERM) regulate many cell processes that often require a conformational change of these proteins as a result of phosphorylation on a conserved threonine residue near the C terminus. This study tested the hypothesis that ERM proteins are phosphorylated on this critical threonine residue through TNF-α-induced activation of PKC and p38 and modulate permeability increases in pulmonary microvascular ECs. TNF-α-induced ERM phosphorylation on the threonine residue that required activation of p38, PKC isoforms, and phosphatidylinositol-4-phosphate 5-kinase Iα, a major enzyme generating phosphatidylinositol 4,5-bisphosphate, and phosphorylated ERM were prominently localized at the EC periphery. TNF-α-induced ERM phosphorylation was accompanied by cytoskeletal changes, paracellular gap formation, and increased permeability to fluxes of dextran and albumin. These changes required activation of p38 and PKC and were completely prevented by inhibition of ERM protein expression using small interfering RNA. Thus, ERM proteins are phosphorylated through p38 and PKC-dependent mechanisms and modulate TNF-α-induced increases in endothelial permeability. Phosphorylation of ERM likely plays important roles in EC responses to TNF-α by modulating the F-actin cytoskeleton, adhesion molecules, and signaling events. The Journal of Immunology, 2006, 176: 1218–1227.

The pulmonary microvascular endothelium is a primary target of inflammatory cytokines during acute inflammation. Responses of endothelial cells (ECs) to TNF-α and IL-1β include up-regulation of adhesion molecules such as ICAM-1, cytoskeletal changes, and permeability increases (1). Mice genetically deficient in receptors for TNF-α and IL-1β have reduced neutrophil emigration and edema formation in Escherichia coli pneumonia, establishing an indispensable role for these two cytokines in response to this stimulus (2).

TNF-α induces signaling events in lung ECs, resulting in cytoskeletal changes and increases in EC permeability. TNF-α induces an increase in permeability in vivo, in the isolated lungs, and in pulmonary microvascular ECs in vitro (1). TNF-α also induces cytoskeletal changes within ECs, and the stabilization of F-actin using phalloidin prevents TNF-α-induced decreases in endothelial barrier functions (3). The actin cytoskeletal changes likely occur through the modulation of actin-binding proteins by TNF-α-induced signaling mechanisms that include activation of p38 MAPK, protein kinase C (PKC) isoforms, and Rho family GTPases, although the downstream targets of these signaling pathways leading to cytoskeletal changes remain to be clearly defined (4–7).

This study focuses on a family of three proteins called ezrin, radixin, and moesin (ERM). These proteins are highly homologous in their primary structures and functions. They contain binding sites for membrane adhesion molecules on the N terminus and actin binding sites on the C terminus and, thus, can function as membrane-cytoskeletal linkers (8, 9). In addition, these proteins regulate PI3K and Rho GTPase pathways by binding to a regulatory subunit of PI3K (p85) and a Rho GDP dissociation inhibitor, respectively (10–12). ERM proteins also promote de novo F-actin polymerization on phagosomes (13). The importance of ERM in many processes has been demonstrated in vitro and in vivo (9, 14, 15). Radixin deficiency in mice results in deafness associated with degeneration of cochlear stereocilia (14), and ezrin is essential for epithelial organization and villus morphogenesis in the developing intestine in mice (15).

One important mechanism for regulating the functions of ERM is through phosphorylation on a conserved threonine residue in the C terminus. Each of the three human ERM proteins has a threonine residue in this region: T567 in ezrin, T564 in radixin, and T558 in moesin. Nonphosphorylated ERM proteins exist in a folded conformation through intramolecular interactions, masking binding sites for other molecules. Phosphorylation on this conserved threonine residue causes conformational changes in ERM, unmasking binding sites. Two kinases postulated to phosphorylate this residue are PKC and Rho kinase, although the identity of these kinases that directly phosphorylate ERM in many other cells remains to be clearly defined (8, 9). In addition, ERM proteins have binding sites.
for phosphatidylinositol 4,5-bisphosphate (PIP$_2$), and binding to PIP$_2$ may be required for ERM phosphorylation (8, 9).

This study tested the hypothesis that ERM proteins are phosphorylated on this critical threonine residue by TNF-$\alpha$-induced signaling events and play important roles in modulating the cytoskeletal changes and permeability increases in human pulmonary microvascular ECs.

**Materials and Methods**

**Treatment of ECs with TNF-$\alpha$ and inhibitors or antisense oligonucleotides**

Human pulmonary microvascular ECs (Camberly) were grown on dishes precoated with 12.5 $\mu$m fibronectin (16, 17). Confluent ECs were treated with either vehicle (PBS containing 0.1% BSA) or recombinant human TNF-$\alpha$ for 0.25–24 h as indicated.

To evaluate the role of p38 MAPK or PKC, ECs were pretreated with either vehicle (0.2% DMSO) or 20 $\mu$m SB203580, 1 $\mu$m bisindolylmaleimide I, or 10 $\mu$m Ro-31-7549 for 30 min (Calbiochem). To evaluate the role of PKC isozymes, ECs were pretreated with 50 $\mu$m rottlerin or a 25 $\mu$m inhibitory peptide specifically targeting PKC$\beta$ (BIOMOL), PKC$\beta$, PKC$\gamma$, or PKC$\delta$ (Calbiochem) or vehicle (PBS) for 30 min. The PKC$\beta$ peptide contains the binding site of PKC$\beta$II to a PKC anchoring protein named RACK1. This binding site is conserved within the conventional PKC subfamily, including PKC$\alpha$, PKC$\beta$, PKC$\delta$, and PKC$\gamma$. This peptide inhibits the translocation and function of PKC$\alpha$ and PKC$\beta$ in cultured cells (18, 19). The inhibitory peptides for PKC$\beta$, PKC$\gamma$, and PKC$\delta$ are specific substrates, and they specifically inhibit the isoform activity in various cells including ECs (20, 21). All of the peptides are myristoylated, which enables them to cross the cell membrane.

To evaluate the role of phosphatidylinositol-4-phosphate 5-kinase Ia (PIP5Kia), an antisense oligonucleotide, was used to inhibit its protein expression. The antisense oligonucleotide targeting human PIP5Kia (5'-AACATATGGCTCTGGCTGG-3') and a control oligonucleotide (5'-CGTTATTAACCTCCGTTGAA-3') were synthesized as uniform phosphorothioate chimeric oligonucleotides with 2'-O-methoxylated groups on bases 1–5 and 16–20 (17).

ECs were treated for 4 h with 100 nM oligonucleotides premixed with 10 $\mu$g/ml lipofectin (Invitrogen Life Technologies) (17). The cells were then incubated with normal culture medium for 72 h before being used in experiments.

**Evaluation of ERM phosphorylation using immunoblotting and immunocytochemistry**

The proteins were solubilized using SDS buffer containing 1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2% PMSF, 5 mg/ml leupeptin, and 1 mM pepstatin in 10 mM Tris (pH 8.0) (16, 17). The level of phosphorylated ERM was examined by immunoblot using a single Ab that recognizes any of the three ERM proteins regardless of their phosphorylation state (Cell Signaling) (16, 17). The distribution of threonine-phosphorylated ERM was evaluated by immunoblot using an Ab that recognizes total ERM, ezrin (Upstate Biotechnology), moesin (BD Biosciences), or actin (Santa Cruz Biotechnology).

**Measurement of EC permeability to fluxes of dextran or albumin**

The fluxes of albumin or dextran across ECs were evaluated using Transwell chambers (5, 24). Both molecules were measured for the following reasons: 1) they have different sizes, with albumin having a molecular mass of $\sim$65 kDa and dextran $\sim$10 kDa; and 2) they may use different transport mechanisms across ECs. In addition to pinocytotic and intercellular transport, albumin transport can be modulated by albumin-binding proteins present on the EC surface. ECs were seeded onto Transwell inserts (6.5-mm diameter and 0.4-$\mu$m pore size) coated with 12.5 $\mu$g/ml fibronectin and stimulated with 20 ng/ml TNF-$\alpha$ or vehicle. One hour later, 0.05 $\mu$Ci of $^{125}$I-labeled human albumin (Iso-Tex Diagnostics) and 250 $\mu$g of FITC-conjugated dextran (10 kDa) were added to the top well, and 100-$\mu$l aliquots of samples were taken from the bottom chamber every 15 min for 2 h. The amounts of albumin and dextran in the samples were measured using a gamma counter and a fluorescence plate reader. Each time after taking a sample, 100 $\mu$l of medium was added back to the bottom chamber. For each time point, the amounts of albumin and dextran present in the bottom wells as well as the amounts in all the samples removed for measurement were calculated, added up, and plotted. In every experiment, fibronectin-coated filter inserts without ECs were included, and the amounts of dextran and albumin in the bottom wells in each sample were expressed relative to the amounts of these two molecules in the filter-alone samples at 2 h.

**Statistical analysis**

Data were analyzed using the Student $t$ test or one-way ANOVA followed by post hoc comparisons (least significant difference test). A $p$ value of $<0.05$ is considered significant. The data are expressed as the mean value $\pm$ SEM.

**Results**

**Threonine-phosphorylated ERM decreased as ECs approached confluence**

Because this study examines the threonine phosphorylation of ERM in cultured ECs, the effect of culture confluence was first evaluated. ECs were plated at different densities to achieve cultures that were sparse, preconfluence, or 3–4 days postconfluence, respectively, 6 days after plating. Threonine-phosphorylated ERM proteins were detected by immunoblotting using an Ab that recognizes phosphorylated ezrin (T567)/radixin (T564)/moesin (T558) (Cell Signaling) (16, 17).

The level of total ERM was similarly examined using a single Ab that recognizes any of the three ERM proteins regardless of their phosphorylation state (Cell Signaling) (16, 17).

**TNF-$\alpha$-induced threonine phosphorylation of ERM and the underlying signaling mechanisms**

The effect of TNF-$\alpha$ on the threonine phosphorylation of ERM in confluent ECs was then examined. Treatment with 20 ng/ml TNF-$\alpha$ induced a significant increase in ERM phosphorylation in a time-dependent manner (Fig. 2A). This phosphorylation of ERM occurred without detectable changes in the protein expression of ERM and preceded ICAM-1 up-regulation (Fig. 2A). A dose-response study indicated that the most consistent increase induced by TNF-$\alpha$ was detected at 20 ng/ml (Fig. 2B).

The following experiments were performed to determine the signaling mechanisms leading to ERM phosphorylation upon TNF-$\alpha$ treatment. In particular, the role of p38 MAPK and PKC was examined. TNF-$\alpha$ treatment induced rapid and sustainable activation of p38 (Fig. 3A). The role of p38 was examined using 20 $\mu$m SB203580, which effectively prevented the activation of p38.
in these ECs following ICAM-1 ligation (16). Pretreatment with 20 μM SB203580 completely prevented ERM phosphorylation at 1 h (Fig. 3B).

The role of PKC was first examined using two PKC-specific pharmacological inhibitors that have different IC50 values for different PKC isoforms, bisindolylmaleimide I and Ro-31-7549. Pretreatment with Ro-31-7549 completely prevented ERM phosphorylation induced by TNF-α (Fig. 3C). By contrast, bisindolylmaleimide I did not prevent ERM phosphorylation (Fig. 3C). These data indicate that PKC is indeed required for ERM phosphorylation. The fact that these two PKC inhibitors have different effects suggests that particular PKC isoforms may be important.

Because the identity of the PKC isoforms targeted by bisindolylmaleimide I or Ro-31-7549 within ECs is unclear, the role of PKC isoforms was examined using a pharmacological inhibitor or isoform-specific inhibitory peptides. Pretreatment with an inhibitory peptide targeting PKCe prevented ERM phosphorylation induced by TNF-α (Fig. 3D). In addition, pretreatment with a PKCβ or PKCδ inhibitory peptide caused a small but statistically significant reduction in ERM phosphorylation (Fig. 3D). These data suggest that multiple PKC isoforms may be required. By contrast, pretreatment with an inhibitory peptide targeting PKCζ did not prevent ERM phosphorylation (Fig. 3D). Rottlerin, a pharmacological inhibitor relatively specific for PKCδ, also did not prevent ERM phosphorylation (Fig. 3D).

Because PIP2 also modulates ERM phosphorylation, the role of PIP2 was examined by inhibiting PIP5K1α, one of the major enzymes generating PIP2. The protein expression of PIP5K1α was inhibited using an antisense oligonucleotide (Fig. 3E). TNF-α treatment induced a significant increase in ERM phosphorylation in ECs treated with a control oligonucleotide, and this increase was inhibited in ECs treated with PIP5K1α antisense oligonucleotide (Fig. 3E). These data indicate that PIP5K1α is required for TNF-α-induced ERM phosphorylation.

These data indicate that TNF-α-induced ERM phosphorylation requires activation of p38, PKC isoforms, and PIP5K1α. To determine whether the PKC inhibitory peptides prevent ERM phosphorylation by inhibiting the activation of p38 MAPK, the effect of these peptides on TNF-α-induced activation of p38 was examined. Pretreatment with any of the PKC inhibitory peptides had no effect.
FIGURE 3. TNF-α-induced ERM phosphorylation required activation of p38 MAPK and PKC isoforms. A, TNF-α induced activation of p38 MAPK in ECs. Phosphorylated p38 MAPK before or after TNF-α treatment for the indicated times was examined by immunoblot using an Ab that recognizes phosphorylated p38. B, Pretreatment with a specific p38 inhibitor prevented TNF-α-induced ERM phosphorylation. ECs were pretreated with either vehicle or 20 μM SB203580 for 30 min before they were treated with buffer or 20 ng/ml TNF-α for 1 h. C, The effect of two pan-PKC inhibitors on TNF-α-induced ERM phosphorylation. ECs were pretreated with either vehicle or 1 μM bisindolylmaleimide I or 10 μM Ro-31-7549, followed by treatment with buffer or TNF-α for 1 h. D, TNF-α-induced ERM phosphorylation required activation of PKC isoforms. ECs were pretreated with control vehicle, an isoform-specific PKC inhibitory peptide, or rottlerin before they were treated with TNF-α for 1 h. E, TNF-α-induced threonine phosphorylation of ERM required PIP5KIα. ECs treated with either control oligonucleotide or PIP5KIα antisense were stimulated with either buffer or TNF-α for 1 h, and threonine-phosphorylated ERM, total ERM, or PIP5KIα were examined. Data are presented as fold changes in phosphorylated ERM over vehicle-treated controls and expressed as means ± SEM. (n = 4 for each sample). *, p < 0.05 when compared with no TNF-α-treated controls; #, p < 0.05 when compared with the corresponding samples treated with control oligonucleotide or vehicle.
on p38 phosphorylation (Fig. 4A). The PIP5K1α antisense also had no effect on p38 phosphorylation (Fig. 4B). Together, these data suggest that p38 acts either upstream or independently of the activation of PKC isoforms or PIP5K1α in the TNF-α-initiated signaling events that lead to ERM phosphorylation.

The role of ERM in modulating endothelial responses to TNF-α

The distribution of phosphorylated ERM in ECs before or after TNF-α treatment was examined. Before TNF-α treatment, phosphorylated ERM proteins were present in the cytoplasm as well as at the cell borders (Fig. 5). Treatment with TNF-α induced an increase in the amount of phosphorylated ERM proteins, consistent with the immunoblotting studies. The phosphorylated ERM proteins were localized primarily at the cell periphery at 1 and 3 h (Fig. 5). The distribution of ezrin within these ECs was not altered by TNF-α (Fig. 5).

The observations that phosphorylated ERM proteins were concentrated along EC edges upon TNF-α treatment led us to examine the role of these proteins in modulating endothelial permeability increases. The role of ERM was evaluated using siRNA targeting ezrin, radixin, or moesin. ECs were treated with control siRNA or the siRNA for ezrin, radixin, or moesin, either singly or in combination, and the protein expression of ezrin, moesin, or actin was examined (Fig. 6A). Treatment with these siRNAs efficiently and specifically inhibited their respective target (Fig. 6A). Treatment with three siRNAs combined prevented the expression of all ERM proteins (Fig. 6A). Treatment with ERM siRNA, either singly or in combination, had no effect on the expression of actin (Fig. 6B).

Interestingly, by comparing ECs treated with these ERM siRNAs, either singly or in combination, these studies indicate that moesin is the major ERM protein expressed by these ECs, because treatment with moesin siRNA caused the greatest decrease in the total ERM, whereas treatment with ezrin or radixin siRNA had little effect on total ERM (Fig. 6C).

Similarly, the effect of ERM siRNA treatment, either singly or in combination, on the phosphorylated ERM before or after TNF-α treatment was examined. Before TNF-α treatment, moesin was the major ERM protein being phosphorylated, because treatment with moesin siRNA caused a complete loss in phosphorylated ERM, whereas ezrin or radixin siRNA had no effect (Fig. 6D). By contrast, after TNF-α treatment, all three ERM proteins were phosphorylated, because inhibiting these proteins singly caused a similar decrease in the phosphorylated ERM (Fig. 6D). Thus, although moesin is the major ERM protein expressed and phosphorylated before TNF-α treatment, all three proteins are similarly phosphorylated by TNF-α.

The role of ERM in modulating the increases in endothelial permeability induced by TNF-α was then examined by comparing the distribution of VE-cadherin and F-actin, as well as the fluxes of albumin and dextran across cells in ECs treated with control siRNAs, with those treated with ERM siRNA. The distribution of F-actin and VE-cadherin was examined using confocal microscopy. Before TNF-α treatment, the distribution of VE-cadherin and F-actin was similar in ECs treated with control or ERM siRNA (Fig. 7A). TNF-α treatment for 3 h in control siRNA-treated ECs caused paracellular gap formation and F-actin reorganization that included thickening and bundling of the F-actin cytoskeleton along the EC periphery (Fig. 7A). In addition, VE-cadherin appeared disjoined and less continuous at the EC borders (Fig. 7A). However, these changes were prevented in ECs treated with ERM siRNA, indicating that ERM proteins are required (Fig. 7A).

To further examine the role of ERM proteins in modulating endothelial permeability increases induced by TNF-α, a Transwell system was used to examine the fluxes of dextran and albumin across ECs. The fluxes of both dextran and albumin across EC-seeded filters were much less when compared with filters alone, indicating that a confluent layer of ECs on the filters indeed provided a barrier to the fluxes of these two molecules (Fig. 7B).

The role of ERM in regulating the permeability increase induced by TNF-α was then evaluated by comparing ECs treated with control siRNA with those treated with ERM siRNA. In each group, ECs were treated with either a control vehicle or 20 ng/ml TNF-α for 1 h before 125I-labeled albumin and dextran were added to the top well. In the absence of TNF-α, ECs treated with control or ERM siRNA had similar permeability to either dextran or albumin (Fig. 7C). In response to TNF-α, ECs treated with control siRNA showed a significant increase in the fluxes of both dextran and albumin that was apparent 1.5 h after TNF-α treatment (Fig. 7C). However, this increase was completely inhibited by ERM siRNA treatment, indicating that ERM proteins are essential for TNF-α-induced changes in permeability (Fig. 7C).

Because ERM phosphorylation was completely prevented by the p38 inhibitor, SB 203580, or the PKC inhibitor, RO-31-7549, the effect of these two inhibitors on TNF-α-induced cytoskeletal changes and permeability increases was also examined. Both inhibitors completely blocked TNF-α-induced cytoskeletal changes and permeability increases (Fig. 8, A–C). Thus, these data together demonstrate that ERM proteins are downstream targets of TNF-α-induced signaling mechanisms essential for cytoskeletal changes and permeability increases.

Discussion

This study examined the ERM proteins in primary pulmonary microvascular ECs and the role of these proteins in modulating endothelial response to TNF-α. The data demonstrate the following.
1) TNF-α induces phosphorylation of all three ERM proteins on the conserved threonine residue critical for conformational changes and many functions of ERM. This phosphorylation requires TNF-α-induced signaling pathways that include activation of p38 MAPK, PKC isoforms, and PIP5K. 2) ERM proteins are required for TNF-α-induced increases in EC permeability. Phosphorylated ERM proteins concentrate along EC edges early after TNF-α treatment. Inhibition of p38 or PKC activation or ERM protein expression completely prevents TNF-α-induced F-actin reorganization, paracellular gap formation, and increases in fluxes of albumin and dextran across ECs. These data demonstrate that ERM proteins are integral signaling components essential for TNF-α-induced increases in EC permeability.

The phosphorylation state of this critical threonine residue of ERM is modulated by many stimuli. This study using primary human pulmonary microvascular ECs shows that phosphorylation is influenced by the confluence of the cultures and by treatment with TNF-α. The findings that ERM phosphorylation inversely correlates with the confluence state of cultured primary ECs in vitro led us to postulate that ERM phosphorylation likely occurs in ECs during pathological conditions such as wound healing in which ECs are actively proliferating and migrating in vivo. These proteins may in turn modulate endothelial responses during these conditions. Indeed, dynamic phosphorylation/dephosphorylation of ERM occurs during proliferation and migration of many cell types including ECs (23, 25, 26), and the blockade of ezrin enhances transplanted EC proliferation in vivo (26). Moreover, treatment of confluent ECs with TNF-α induced ERM phosphorylation on this threonine residue. Using siRNA to singly inhibit ERM proteins and an Ab that recognizes all ERM proteins (either phosphorylated or total), this study shows that these pulmonary microvascular ECs primarily express moesin and that moesin is the major phosphorylated ERM protein before TNF-α treatment. However, after TNF-α treatment, all three ERM proteins are similarly phosphorylated. Thus, all three proteins are targets of TNF-α-induced signaling pathways and likely contribute to downstream responses.

TNF-α-induced ERM phosphorylation requires TNF-α-induced signaling events. Activation of p38 and PKC plays important roles in modulating endothelial responses to TNF-α during inflammatory responses in vitro and in vivo. Consistent with previous reports (4–6), this study demonstrates that activation of p38 and PKC is indeed required for TNF-α-induced cytoskeletal changes and permeability increases, as measured by the effect of p38 inhibitor SB203580 and specific inhibitors of all PKCs or particular isoforms. In addition, activation of p38 and PKC is required for TNF-α-induced ERM phosphorylation, indicating that ERM proteins are downstream targets of the p38 pathway and the PKC.
pathway essential for TNF-α-induced cytoskeletal changes and paracellular gap formation.

The activation of p38 was not inhibited by the PKC inhibitory peptides, suggesting that the PKC pathway is either downstream of or independent of p38. TNF-α-induced activation of p38 MAPK peaks at 15 min, whereas ERM phosphorylation does not occur until 60 min after TNF-α treatment. This finding suggests that p38 activation is likely an upstream event and that there are many signaling intermediates leading to ERM phosphorylation. Moreover, the ERM threonine phosphorylation site does not contain the consensus sequence that can be recognized and phosphorylated by p38, further suggesting that p38 does not directly phosphorylate ERM. Whether the PKC pathway lies downstream of p38 has not been examined, although this seems unlikely because few reports indicate that p38 is an upstream regulator of PKC activity. Thus, our leading hypothesis is that the p38 pathway and the PKC pathway are independent of each other in regulating ERM phosphorylation. The fact that the p38 inhibitor or the PKC inhibitors can completely prevent ERM phosphorylation suggests that both pathways are required and that the activation of either pathway alone is insufficient for ERM phosphorylation. Thus, these two pathways likely converge into common pathways leading to ERM phosphorylation. The identity of these common pathways or the kinases that directly phosphorylate ERM remains to be determined.

ERM phosphorylation also required PIP5Kια, one of the key enzymes generating PIP2. PIP2 may modulate ERM phosphorylation through the following two mechanisms. First, it may modulate signaling pathways leading to ERM phosphorylation, in particular the activation of the classical (α, β, γ) and novel (δ, ε, θ, η) isoforms of PKC. PIP2 is cleaved by activated phospholipase C to generate diacylglycerol and inositol 1,4,5-trisphosphate. Activation of classical and novel isoforms of PKC requires diacylglycerol. Thus, PIP2 may be required for the activation of PKC isoforms essential for ERM phosphorylation. Second, PIP2 may influence ERM phosphorylation by directly binding to ERM. ERM proteins have at least three consensus phosphoinositide-binding sites. Binding to PIP2 causes conformational changes of ERM, and a mutant form of ERM incapable of binding to PIP2 cannot be phosphorylated on the threonine residue (27, 28).

The experiments demonstrating that ERM proteins are downstream targets of signaling pathways essential for TNF-α-induced cytoskeletal changes and permeability increases led to the studies examining the functions of ERM in modulating these changes. The role of ERM was examined by inhibiting their protein expression using siRNA. The individual ERM siRNAs are efficient in inhibiting their presumed targets and appear specific, although the limitations of RNA interference, most notably the off-target effects, should be considered. siRNA has been shown to activate the IFN pathways and may inhibit nontargeted proteins (29, 30). For these reasons, the effect of ERM siRNA is always compared with a control siRNA. Moreover, other approaches such as using dominant-negative constructs should be considered in future studies to further examine the functions of ERM proteins.
Studies directly comparing ECs treated with control siRNA and those treated with ERM siRNA show that ERM proteins are required for TNF-α-induced cytoskeletal changes, paracellular gap formation, and increases in the fluxes of albumin and dextran across ECs. Thus, ERM proteins are activated by p38 MAPK and PKC isoform-dependent mechanisms and are required for TNF-α-induced permeability increase, suggesting that these signaling events modulate endothelial responses to TNF-α through the activation of ERM proteins.

The underlying mechanisms through which ERM proteins regulate TNF-α-induced changes in the cytoskeleton and increased permeability remain to be determined. Two mechanisms are possible: 1) ERM proteins can directly promote de novo F-actin polymerization, as has been shown by studies using phagosomes (13); and 2) ERM proteins may modulate intermediate signaling events essential for cytoskeletal changes and permeability increase such as focal adhesion kinase and Rho GTPases (10, 11, 31–33). Although direct evidence is lacking, the observations that ERM...
FIGURE 8. The effect of pretreatment with SB203580 or RO-31-7549 on TNF-α-induced cytoskeletal changes (A) and permeability increases to fluxes of dextran (B) or albumin (C). ECs were pretreated with vehicle, 20 μM SB203580, or 10 μM RO-31-7549 before exposure to buffer (●, □, ○) or TNF-α (▲, ■, ▴). The distribution of VE-cadherin, F-actin, or nuclei and the fluxes of dextran or albumin across ECs were examined as described in Fig. 7 (n = 3–5 in each group; *, p < 0.05 when compared with controls that were not exposed to TNF-α).
proteins are phosphorylated on the threonine residue critical for many of ERK functions and that phosphorylated ERK proteins localize primarily along the EC periphery led us to postulate that phosphorylation of ERK may be required for modulating endothelial response to TNF-α. Studies using either nonphosphorylatable ERK mutants or mutants that mimic phosphorylated ERK proteins demonstrate that ERK phosphorylation is essential for oncogene-induced fibroblast transformation, association of ERM proteins with cytoskeletal changes and permeability increases.

Thus, the current working hypothesis is that TNF-α induces ERK phosphorylation along the EC periphery, where these activated ERK proteins modulate signaling events leading to cytoskeletal changes and permeability increases.

Together, this study presents data demonstrating the activation of ERK by TNF-α in primary human pulmonary microvascular ECs. These ERK proteins are downstream targets of TNF-α-induced signaling events and are required for TNF-α-induced cytoskeletal changes and permeability increases.

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Disclosures

The authors have no financial conflict of interest.

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