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A critical role for phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 1 in endothelial junction disruption and vascular hyperpermeability

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Abstract

Rationale—The small GTPase Rac is critical to vascular endothelial functions, yet its regulation in endothelial cells remains unclear. Understanding the upstream pathway may delineate Rac activation mechanisms and its role in maintaining vascular endothelial barrier integrity.

Objective—By investigating P-Rex1, one of the Rac-specific guanine nucleotide exchange factors (GEFs) previously known for G protein-coupled receptor (GPCR) signaling, we sought to determine whether Rac-GEF is a nodal for signal integration and potential target for drug intervention.

Methods and Results—Using gene deletion and siRNA silencing approach, we investigated the role of P-Rex1 in lung microvascular endothelial cells (HLMVECs). TNF-α exposure led to disruption of endothelial junctions, and silencing P-Rex1 protected junction integrity. TNF-α stimulated Rac activation and ROS production in a P-Rex1-dependent manner. Removal of P-Rex1 significantly reduced ICAM-1 expression, PMN transendothelial migration and leukocyte sequestration in TNF-α challenged mouse lungs. The P-Rex1 knockout mice were also refractory to lung vascular hyper-permeability and edema in a LPS-induced sepsis model.

Conclusions—These results demonstrate for the first time that P-Rex1 expressed in endothelial cells is activated downstream of TNF-α, which is not a GPCR agonist. Our data identify P-Rex1 as a critical mediator of vascular barrier disruption. Targeting P-Rex1 may effectively protect against TNF-α and LPS-induced endothelial junction disruption and vascular hyper-permeability.

Keywords

Vascular permeability; acute lung injury; reactive oxygen species; small GTPases; guanine nucleotide exchange factors; pulmonary edema; endothelial dysfunction

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DISCLOSURES
None.

Introduction

Vascular endothelial cells form the lining of blood vessels and separate the underlying tissue from circulating blood. Disruption of the endothelial barrier leads to increased vascular permeability to plasma proteins and inflammatory cells, resulting in edema as seen in acute lung injury and in its more severe form, acute respiratory distress syndrome (ARDS)[1]. Vascular permeability can be transcellular or paracellular. Transcellular permeability involves the formation of transport vesicles whereas paracellular permeability requires disruption of the adherens junctions between two adjacent endothelial cells. VE-cadherin is an endothelial specific marker of adherens junctions[2,3] and a determinant of integrity of endothelial junctions. Increased vascular permeability is associated with extravasation of leukocytes into the underlying tissue[4], and VE-cadherin has been proposed to play a role in leukocyte transmigration[5].

Several pro-inflammatory factors are released into the blood stream during an inflammatory response, among which thrombin, TNF-α, IL-1β and histamine are known to disrupt the endothelial barrier. TNF-α is one of the most commonly encountered proinflammatory cytokines in pathological conditions such as sepsis[6]. Elevated TNF-α levels are found in the bronchoalveolar fluid[7] and plasma[8] of ARDS patients. TNF-α increases the permeability of pulmonary microvessel endothelial barrier[9,10] and causes edema in animals[11]. However, the mechanisms of TNF-α-induced endothelial barrier dysfunction are not clearly understood. Tyrosine phosphorylation of VE-cadherin[12,13], production of reactive oxygen species (ROS)[14,15], and activation of the small GTPase Rac[16] have been associated with TNF-α-induced endothelial barrier dysfunction.

Rac is a monomeric GTPase of ~21 kDa[17]. In endothelial cells, Rac activation downstream of GPCRs, such as the thrombin receptor PAR1, induces re-annealing of endothelial junctions during endothelial barrier repair phase[18]. However, it was also reported that introduction of a constitutively activated Rac led to endothelial barrier dysfunction[19]. Similar findings were reported in endothelial cells stimulated with VEGF[20] and platelet-activating factor[21]. In phagocytes, Rac is known for its role in NADPH oxidase activation and superoxide production[22]. Genetic deletion or silencing of the Rac gene showed that Rac is also essential for NADPH oxidase-dependent ROS production in endothelial cells[20]. Despite these observations, the mechanism by which ROS regulates vascular permeability remains unclear. TNF-α has been shown to induce ROS production and vascular permeability, but the role for Rac in ROS production downstream of the TNF-α pathway has not been established. There are approximately 70 Dbl family guanine nucleotide exchange factors (GEFs)[23], yet much fewer Rho family small GTPases (to which Rac belongs), suggesting that Rho GEFs confer specificity of Rho GTPase activation in tissues where the GEFs are expressed. To date, Tiam-1 and Vav-2 are the only GEFs implicated in regulating Rac activation in endothelial cells[21,24,25].

In this study, we examined phosphatidylinositol (3,4,5)-trisphosphate (PIP3)-dependent Rac Exchanger 1 (P-Rex1) for its possible involvement in TNF-α-induced endothelial barrier dysfunction. P-Rex1 is a Rac-specific GEF regulated by PIP3 and G protein βγ subunits[26,27]. In neutrophils from P-Rex1-deficient mice, GPCR-induced activation and bactericidal functions are compromised[28,29]. P-Rex1 is primarily expressed in myeloid cells[26] and neuronal tissue[30]. Its biological functions range from neuron migration and neurite differentiation[30,31] to tumor metastasis[32–34]. In this study, we show that P-Rex1 is a key mediator of TNF-α-induced vascular permeability, which involves Rac activation and ROS production in a PI3K-dependent manner. In addition, we identified a novel role for endothelial P-Rex1 in regulating transendothelial migration of polymorphnuclear leukocytes (PMNs).

METHODS

Human lung microvascular endothelial cells (HLMVEC) were obtained from Lonza (Walkersville, MD). These cells were transfected with siRNA specific for P-Rex1 or with scrambled siRNA as negative controls. Experiments with mice were conducted using procedures approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. A detailed, expanded Methods section can be found in the Supplemental Materials.

RESULTS

Endothelial expression of P-Rex1 and its role in the regulation of endothelial barrier function

P-Rex1 was originally identified in neutrophils and neurons, and its function outside these cells remains unclear[26,30]. We examined P-Rex1 expression in endothelial cells and its potential involvement in endothelial barrier function. Using RT-PCR, P-Rex1 transcript was found in 3 different types of endothelial cells tested, including human lung microvascular endothelial cells (HLMVEC), human pulmonary artery endothelial cells (HPAEC), and human umbilical vein endothelial cells (HUVEC) (Online Figure I-A). The expression level of P-Rex1 protein in these endothelial cells was comparable to that in bone marrow-derived macrophages (Online Figure I-B).

To assess the role for P-Rex1 in endothelial barrier function, HLMVECs were treated with P-Rex1-specific siRNA to reduce P-Rex1 expression. Scrambled (sc) siRNA was used as a negative control. A ~80% reduction in P-Rex1 protein level was obtained (Online Figure I-C, I-D). The siRNA-transfected cells were then subjected to measurement of changes in transendothelial electrical resistance (TER) following TNF-α stimulation, which increases vascular permeability. As expected, the control (sc-siRNA transfected) cells showed a decrease in TER culminating 4–5 h post stimulation, suggesting barrier disruption. In comparison, P-Rex1 siRNA transfected cells showed much less barrier disruption (Figure 1A). Based on quantification of barrier disruption against absolute resistance values (Figure 1B), P-Rex1 is a necessary component for TNF-α-induced loss of TER, which reflects endothelial cell barrier dysfunction. TNF-α-induced barrier dysfunction is not a consequence of endothelial cell apoptosis, because the majority of transfected HLMVECs remained healthy after TNF-α treatment in DNA fragmentation assay (Online Figure II).

Fluorescent imaging analysis was conducted to examine barrier dysfunction of HLMVECs, characterized by intercellular gap formation[14] (Figure 1C). The endothelial adherens junctions were detected with an anti-VE-cadherin antibody (green). After stimulation with TNF-α, sc-siRNA transfected endothelial cells displayed discontinuities between neighboring cells (marked with arrows). In comparison, HLMVECs receiving P-Rex1 siRNA showed minimal alteration of barrier integrity. The area of inter-endothelial gaps was quantified (Figure 1D), and the changes were significant ($p<0.05$). Thus, data from both TER and imaging analysis support a role for P-Rex1 in TNF-α-induced disruption of endothelial barrier.

P-Rex1 is essential for TNF-α-induced Rac activation

Recent studies have shown that TNF-α, at concentrations that induce opening of inter-endothelial junctions, activates the small GTPase Rac[16]. However the GEF responsible for TNF-α-induced Rac activation in endothelial cells remains unidentified. To explore the signal transduction pathway downstream of TNF-α stimulation, we examined possible involvement of P-Rex1 in Rac activation. TNF-α induced a rapid and transient Rac activation in HLMVECs that peaked within 1 min and continued for 2 min before it began to

decrease (Figure 2A). Based on Rac-GTP pull-down assay, TNF-α induced up to an 8-fold increase in Rac activation compared to unstimulated cells (Figure 2B). In HLMVECs receiving P-Rex1 siRNA, Rac activation was significantly reduced, indicating that P-Rex1 is required for TNF-α-induced Rac activation. In addition, the Rac inhibitor NSC23766 prevented TNF-α-induced endothelial permeability as measured by TER (Online Figure III). We also transfected HLMVECs with a dominant negative Rac (T17NRac) to exclude non-specific effects of NSC23766. The dominant negative Rac ablated TNF-α-induced barrier dysfunction (Online Figure IV). These results strongly suggest that Rac is required for TNF-α-induced endothelial barrier dysfunction and P-Rex1 is an essential Rac GEF regulating TNF–α-induced Rac activation.

A number of molecules were examined in order to exclude several possibilities that might have affected the outcome of our experiments. GEF-H1 is not only a Rac GEF but also a Rho GEF[35] and has been implicated in TNF-α-induced epithelial barrier integrity[36]. To determine whether it plays a role in our experiments, we used siRNA to knock down GEF-H1 in HLMVECs and performed TER. Our results indicate that, unlike P-Rex1, GEF-H1 removal did not reverse barrier dysfunction induced by TNF-α (Online Figure V). We also considered potential involvement of Rho, known to be responsible for endothelial barrier dysfunction[37]. To exclude the involvement of Rho downstream of the TNF-α and P-Rex1 pathway, we performed Rho pull-down assay as detailed in the *Methods*. The absence of P-Rex1 did not alter Rho activation (Online Figure VI). Therefore, even though TNF-α has the ability to activate Rho[16], it does not require P-Rex1.

TNF-α-induced ROS production is P-Rex1-dependent

NADPH oxidase (Nox) has been implicated in TNF-α-induced endothelial barrier dysfunction[14,15]. However, it is unclear how TNF-α regulates ROS production. In phagocytes, Rac is required for NADPH oxidase activation leading to ROS production[38,39]. Therefore, we determined whether P-Rex1 is involved in endothelial ROS production through Rac activation. HLMVECs plated on gelatin-coated glass dishes were treated with either sc-siRNA or P-Rex1 siRNA and then stimulated with TNF-α, ROS production was measured by dihydrorhodamine 123 (DHR123). As shown in Figure 3A and quantified in Figure 3B, siRNA-mediated silencing of P-Rex1 led to a significant reduction in TNF-α-stimulated ROS production. Diphenyleneiodonium (DPI), a flavocytochrome inhibitor, diminished TNF-α-induced ROS production, suggesting that inducible activation of the NADPH oxidase is required (Figure 3C, 3D). Likewise, NSC23766 reduced ROS production, supporting the notion that Rac is involved in TNF-α-induced NADPH oxidase activation in HLMVECs (Figure 3C, 3D). We next determined whether suppression of ROS production could alter the integrity of the HLMVEC monolayer. DPI-treated cells were refractory to TNF-α-induced decrease in TER (Figure 3E, 3F). These results demonstrate a correlation between endothelial P-Rex1 and TNF-α-induced ROS production leading to a loss of barrier function.

Silencing P-Rex1 prevents TNF-α-induced Src activation and VE-cadherin phosphorylation

We examined the role for P-Rex1 in regulating TNF-α-induced tyrosine phosphorylation of VE-cadherin since this has been reported to be critical to the loss of vascular integrity[13]. In sc-siRNA transfected HLMVECs, TNF-α treatment led to phosphorylation of VE-cadherin at 10 min and peaked at 20 min (Figure 4A, 4B). TNF-α-induced VE-cadherin phosphorylation was diminished in HLMVECs transfected with P-Rex1 siRNA (Figure 4A, 4B), suggesting that VE-cadherin was phosphorylated in a P-Rex1-dependent manner. It was reported that the Src family protein tyrosine kinases undergo ROS-dependent phosphorylation that is required for VE-cadherin phosphorylation[12,14]. To test whether P-Rex1 is required for Src activation downstream of TNF-α stimulation, HLMVECs

transfected with sc-siRNA or P-Rex1 siRNA were stimulated with TNF-α for the indicated time points, and phosphorylation of c-Src at Tyr416 was determined. HLMVECs receiving P-Rex1 siRNA displayed more than 70% reduction in phosphorylation of Tyr416 at 5 and 10 min compared to cells transfected with sc-siRNA (Figure 4C, 4D), indicating that P-Rex1 is required for TNF-α-induced Src activation.

Signaling mechanism of TNF-α-induced P-Rex1 activation

P-Rex1 resides in the cytosol of resting neutrophils and translocates to membrane upon cell activation[40]. We determined whether P-Rex1 is translocated to plasma membrane in TNF-α stimulated endothelial cells. HLMVECs were stimulated with TNF-α for 0, 1 and 2 min, and P-Rex1 in the membrane and cytosolic fractions was determined. P-Rex1 underwent membrane translocation as early as 1 min and continued to increase at 2 min (Figure 5A, 5B, 5C). We also used an additional approach to test the membrane translocation of P-Rex1. HLMVECs were unstimulated or stimulated with TNF-α for 2 min. The cells were then permeabilized, fixed, and incubated with anti-P-Rex1 and an Alexafluor 488 conjugated secondary antibody. Images acquired by confocal microscopy showed accumulation of P-Rex1 to the membrane periphery after stimulation (Figure 5D). Pre-treatment of HLMVECs with the PI3K inhibitor LY294002 prevented membrane translocation of P-Rex1 (Figure 5D), suggesting that it is PI3K-dependent in TNF-α stimulated HLMVECs.

Earlier studies have shown that P-Rex1 activation downstream of GPCRs requires both PI3K and the βγ subunits of heterotrimeric G proteins[26,27]. However, TNF-α is not a GPCR agonist and how a non-GPCR activates P-Rex1 remains unclear. To determine whether Gβγ subunits are involved in TNF-α signaling, we pre-treated HLMVECs with Gβγ modulator II (Gallein; 3',4',5',6'-Tetrahydroxyspiro [isobenzofuran-1(3H),9'-(9H)xanthen]-3-one), which inhibits conformational changes of Gβγ subunits and blocks Gβγ-dependent activation of PI3K and Rac in HL-60 cells[41]. Pre-treatment with Gallein did not affect TNF-α-induced membrane translocation of P-Rex1 (Figure 5D, lower panels), whereas it significantly inhibited thrombin-induced calcium mobilization in HLMVECs (Figure 5E, 5F). These findings suggest that Gβγ is not indispensable in TNF-α-induced P-Rex1 activation, but PI3K is necessary. The requirement of PI3K for P-Rex1 activation in TNF-α-stimulated endothelial cells was also confirmed when HLMVECs pre-treated with the PI3K inhibitor LY294002 displayed reduced Rac activation by more than 50% (Online Figure VII-A, VII-B).

P-Rex1 knockout mice display reduced lung vascular permeability and edema

To determine an in vivo function of P-Rex1 in acute lung injury, WT and P-Rex1 knockout mice[29] were instilled with TNF-α intra-tracheally. Changes in lung vascular permeability were evaluated based on the accumulation of Evans blue albumin (EBA) after tail vein injection. Significantly less accumulation of EBA was seen in the lungs of mice lacking P-Rex1, compared to WT controls (Figure 6A). The in vivo role for P-Rex1 in lung edema was also evaluated based on lung wet-to-dry weight ratio after intra-tracheal instillation of TNF-α. Again, the P-Rex1 knockout mice had significantly less edema compared to WT controls (Figure 6B, 6C). These results demonstrate a critical role for P-Rex1 in the dynamic regulation of lung vascular permeability in vivo.

LPS-induced sepsis is a clinically relevant model of acute lung injury. We tested a potential role for P-Rex1 in this model where barrier dysfunction contributes to the pathological changes. WT and P-Rex1 knockout mice were intraperitoneally injected with LPS or PBS for 6 h, which produced septic signs such as decreases in leukocyte count and platelet count (Online Figure XI). The lungs were subjected for $K_{f,c}$ measurements (Figure 6D) and EBA dye leakage measurement (Figure 6E). Both the $K_{f,c}$ and EBA data showed that P-Rex1

knockout mice have significantly less lung microvascular capillary filtration and EBA leakage, indicating a role for P-Rex1 in LPS-induced barrier dysfunction during sepsis.

Role of endothelial P-Rex1 in PMN transmigration

In acute lung injury, there is marked infiltration of PMNs and macrophages in the lungs. To determine infiltration of phagocytes into bronchoalveolar (BAL) fluid, WT and P-Rex1 knockout mice were instilled intra-tracheally with PBS or 0.5 μg of murine recombinant TNF-α. After 24 h, mice were anesthetized and BAL fluid was collected. BAL fluid obtained from P-Rex1 knockout mice showed significantly less PMNs and macrophages compared to WT mice (Figure 7A and 7B). This result indicates that P-Rex1 is necessary for transendothelial migration of these leukocytes. In a parallel experiment, WT and P-Rex1 knockout mice were intratracheally injected with murine TNF-α for 24 h followed by collection of lungs for histological analysis. Haematoxylin and Eosin staining showed significantly less cellular infiltration and interstitial tissue thickening in P-Rex1 knockout mouse lungs compared to WT lungs (Online Figure VIII).

Since the knockout approach results in a loss of P-Rex1 in all tissues, we next determined the relative contribution of P-Rex1 in endothelial cells *vs* PMNs to the reduced PMN transendothelial migration. Figure 7C shows the effects of eliminating P-Rex1 from endothelial cells on transendothelial migration of WT and P-Rex1$^{-/-}$ PMNs (a more detailed version of the experiment, with ligand controls included, was shown in Online Figure IX). HLMVECs transfected with sc-siRNA or P-Rex1 siRNA were plated on 3 μm membrane pore inserts. PMNs were isolated concurrently from both WT and P-Rex1$^{-/-}$ mice and applied to the HLMVEC monolayer, which received sc-siRNA (Figure 7C, filled bars) or P-Rex1 siRNA (Figure 7C, open bars) and simulated with TNF-α for 4 h. Eliminating P-Rex1 from the endothelial cells caused a significant reduction in PMN transmigration, which applies to both the WT and P-Rex1$^{-/-}$ PMNs (Figure 7C). In comparison, removal of P-Rex1 from PMNs does not significantly impact cell migration in this experiment (ns, Figure 7C). Based on these findings, we concluded that endothelial P-Rex1 plays an important role in PMN transendothelial migration.

We have also taken an *ex vivo* approach to determine the effect of P-Rex1 in PMN transmigration into the lung tissue. Lungs from WT and P-Rex1 knockout mice were perfused to remove blood cells and then exposed to murine TNF-α. Freshly isolated PMNs from WT and P-Rex1$^{-/-}$ mice were radiolabeled with ^{111}Indium oxine and perfused through WT and P-Rex1$^{-/-}$ lungs, or *vice versa*. As shown in Figure 7D, the P-Rex1$^{-/-}$ lungs showed significantly less radioactivity accumulation than WT lungs, suggesting that absence of P-Rex1 in lung tissue could significantly reduced PMN transmigration. In contrast, no significant difference in radioactivity accumulation was observed in WT lungs perfused with either WT PMNs or P-Rex1$^{-/-}$ PMNs (Figure 7D). These findings suggest that endothelial P-Rex1 is highly important in PMN transmigration into the lung tissue.

P-Rex1 is important for TNF-α-induced ICAM-1 expression in endothelial cells

In the above experiments, we observed that TNF-α treatment of HLMVECs is necessary for PMN transmigration. A number of proteins are induced upon TNF-α stimulation of endothelial cells, among which ICAM-1 is required for efficient PMN transendothelial migration. We examined TNF-α-induced ICAM-1 expression in HLMVECs transfected with either sc-siRNA or P-Rex1 siRNA, and found that cells receiving P-Rex1 siRNA displayed significantly less ICAM-1 expression compared to cells treated with sc-siRNA (Online Figure IX). A significantly lower ICAM-1 induction may contribute to the decreased PMN transmigration across the P-Rex1 deficient HLMVEC monolayer.

DISCUSSION

The present study examines P-Rex1 expression in endothelial cells and its role in mediating TNF-α-induced increase in vascular endothelial permeability. A number of new findings were made. 1) P-Rex1 is highly expressed in vascular endothelial cells and plays important roles in these cells. 2) Our results show for the first time that P-Rex1 can be activated by a non-GPCR, in this case the TNF receptor, in endothelial cells. 3) This study reaffirms a role for Rac in TNF-α induced vascular endothelial dysfunction, which has been an unsettled issue. 4) P-Rex1 activation leads to ROS production in endothelial cells. 5) Endothelial P-Rex1 is important for PMN transmigration into the lung tissue. These findings are summarized schematically in a working model (Figure 8).

P-Rex1 expression and functions in lung vascular endothelial cells

In this model, P-Rex1 is a major Rho GEF downstream of TNF-α receptor in endothelial cells. Prior to this study, P-Rex1 is mainly known for its functions in the brain and in PMNs, where it was first discovered[26]. Thus, the finding of P-Rex1 in various endothelial cells suggests that this Dbl family Rho GEF is more broadly expressed than previously thought. Our data demonstrates that P-Rex1 is expressed in vascular endothelial cells, and it mediates TNF-α-induced vascular permeability as well as PMN infiltration into the lung tissue. These functions of P-Rex1 require Rac activation, which leads to NADPH oxidase-dependent ROS production, c-Src activation and VE-cadherin phosphorylation in HLMVECs. Our *in vitro* results are corroborated by data from P-Rex1 knockout mice, which are refractory to TNF-α-induced increase in vascular permeability in the lungs as demonstrated by reduced edema. Collectively, these results demonstrate that endothelial P-Rex1 is critical to TNF-α signaling that leads to increased vascular endothelial permeability.

P-Rex1 activation by a non-GPCR

Our model places P-Rex1 downstream of the TNF-α receptor, whereas published reports depicts P-Rex1 as a Rac-specific GEF activated by GPCRs[26]. In endothelial cells, P-Rex1 can be activated by a GPCR[42]. Our finding that P-Rex1 is activated by TNF-α is totally unexpected since TNF-α is not known to couple to G proteins. We observed rapid membrane translocation of P-Rex1 in endothelial cells, which is characteristic of its activation[40]. It is also evident that TNF-α stimulates P-Rex1-dependent Rac activation in HLMVECs. Since the time course of Rac activation and P-Rex1 membrane translocation is consistent with that of GPCR signaling, we examined the requirement for P-Rex1 activation in TNF-α stimulated cells. A reported feature of P-Rex1 is its dependence on PIP3 and Gβγ for activation[26,27]. Our results show that TNF-α-induced Rac activation is PI3K-dependent. However, we observed no effect for the Gβγ inhibitor Gallein to affect TNF-α-induced P-Rex1 membrane translocation, thus challenging the conventional view that Gβγ is required for P-Rex1 activation. It is notable that TNF-α signaling has not been associated with activation or transactivation of heterotrimeric G proteins, although TNF-α is known for its activation of PI3K[43], suggesting that TNF-α-induced PIP3 production might be sufficient to trigger P-Rex1 activation in HLMVECs.

A role for Rac in endothelial barrier dysfunction

In endothelial cells stimulated with GPCR agonists such as thrombin, a reversible endothelial barrier disruption occurs. While there are multiple pathways for disrupting the endothelial barrier, Rac has been associated with re-annealing of junctions in response to GPCR activation[18]. Contrary to this view, there is evidence supporting a role for Rac in endothelial barrier dysfunction. For instance, van Wetering *et al* reported that expression of a constitutively active Rac in HUVECs could cause changes leading to increased vascular permeability[19]. Rac has been implicated in TNF-α and VEGF-induced increase in vascular

permeability[16,20]. As shown in our model, GTP-bound Rac is required for TNF-α induced ROS production, and the Rac inhibitor NSC23766 abrogated TNF-α-induced vascular endothelial permeability in HLMVEC. Likewise, dominant negative RacT17N, when expressed in endothelial cells, prevented TNF-α-induced endothelial barrier dysfunction. Our data supports a role for Rac in TNF-α induced endothelial barrier dysfunction, which is mediated through P-Rex1 activation and ROS production. These findings connect a Rho GEF to previously reported functions of ROS in the regulation of vascular permeability[15,19,44–46].

Endothelial P-Rex1 and PMN transmigration

Disruption of endothelial barrier is a triggering factor for infiltration of PMNs into tissues[4,47]. We tested whether TNF-α-induced disruption of endothelial barrier aggravates PMN infiltration, and if so, whether blocking P-Rex1 expression in EC prevents PMN transmigration. We found that, in the absence of P-Rex1, PMN transmigration was significantly reduced. Much fewer PMNs and macrophages were present in BAL fluid of P-Rex1 knockout mice compared to WT mice following instillation of murine recombinant TNF-α into the airways. Silencing endothelial P-Rex1 expression resulted in significantly less PMN transmigration compared to sc-siRNA transfected endothelium. This phenomenon was also confirmed ex vivo by PMN sequestration studies where radiolabeled WT or P-Rex1 knockout PMNs were perfused into WT and P-Rex1 knockout mice respectively and vice versa. It appears that the crosstalk between PMN and EC is key to PMN transendothelial migration[4]. Our Western blot data showed that there is significantly less ICAM-1 expression in the absence of P-Rex1. As depicted in the model, P-Rex1 appears to play a role in the regulation of TNF-α-induced ICAM-1 expression, which involves NF-κB activation. The mechanism underlying P-Rex1 regulation of NF-κB activation has yet to be delineated. An increase in ICAM-1 expression may in turn affect PMN transendothelial migration.

A potential target for therapeutic intervention

Our findings strongly implicate P-Rex1 in regulating TNF-α-induced vascular permeability and lung edema. This function is mediated through the activation of Rac and generation of ROS, thus promoting endothelial barrier disruption and transendothelial PMN migration. These results demonstrate that down-regulation of P-Rex1 may affect multiple pro-inflammatory pathways, and P-Rex1 may be a new therapeutic target in controlling lung vascular injury and PMN-mediated lung inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

ARDS	Acute respiratory distress syndrome
DHR 123	Dihydrorhodamine 123
DPI	Diphenyleneiodonium
EBM2	Endothelial basal medium 2
EBA	Evans blue albumin
ECIS	Electric cell-substrate impedance sensing
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
HLMVEC	Human lung microvascular endothelial cells
HPAEC	Human pulmonary artery endothelial cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
NADPH	Nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
PIP3	Phosphatidylinositol (3,4,5)- triphosphate
PMN	Polymorpho nuclear leukocytes
PMSF	Phenylmethylsulfonyl fluoride
PTKs	Protein tyrosine kinases
PTPs	Protein tyrosine phosphotases
P-Rex1	PIP3 dependent Rac Exchanger 1
ROS	Reactive oxygen species
siRNA	Small interference RNA
Sc-siRNA	Scrambled small interference RNA
TER	Transendothelial electrical resistance
TIAM1	T-cell lymphoma invasion and metastasis 1
TNF-α	Tumor Necrosis Factor α
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

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Novelty and Significance

What Is Known?

- Disruption of the endothelial barrier contributes directly to the entry of inflammatory cells and other blood contents to the surrounding tissue, leading to edema and tissue injury.
- The small GTPase Rac plays a role in regulating cytoskeleton and cell signaling, factors that influence endothelial barrier functions.
- Rac is activated by guanine nucleotide exchange factors (GEFs), downstream of receptors that sense environmental cues.

What New Information Does This Article Contributes?

- Rac GEF and P-Rex1 are critical to TNF-α induced endothelial barrier disruption.
- Deleting the P-Rex1 gene or inhibiting its expression significantly reduces TNF-α and LPS induced acute lung injury.

A large number of GEFs exist for a relatively small number of the small GTPases, suggesting that GEFs confer tissue specificity. We sought to determine which GEF is responsible for Rac activation in endothelial cells. We found that in endothelial cells, GEF for Rac is regulated by P-Rex1, a PtdInsP3 and Gβγ. Using siRNA-mediated knockdown and gene deletion approaches, we identified P-Rex1 as being critical for Rac activation by TNF-α. Cells and mice lacking P-Rex1 produce less reactive oxygen species and are more resistant to TNF-α- and LPS-induced loss of endothelial barrier functions. Moreover, removal of P-Rex1 reduced ICAM-1 expression and transendothelial migration of neutrophils, thus attenuating inflammatory responses.. Our findings identify a previously unrecognized function of P-Rex1, which may be a target for therapeutic intervention aimed at reducing inflammation and tissue injury.

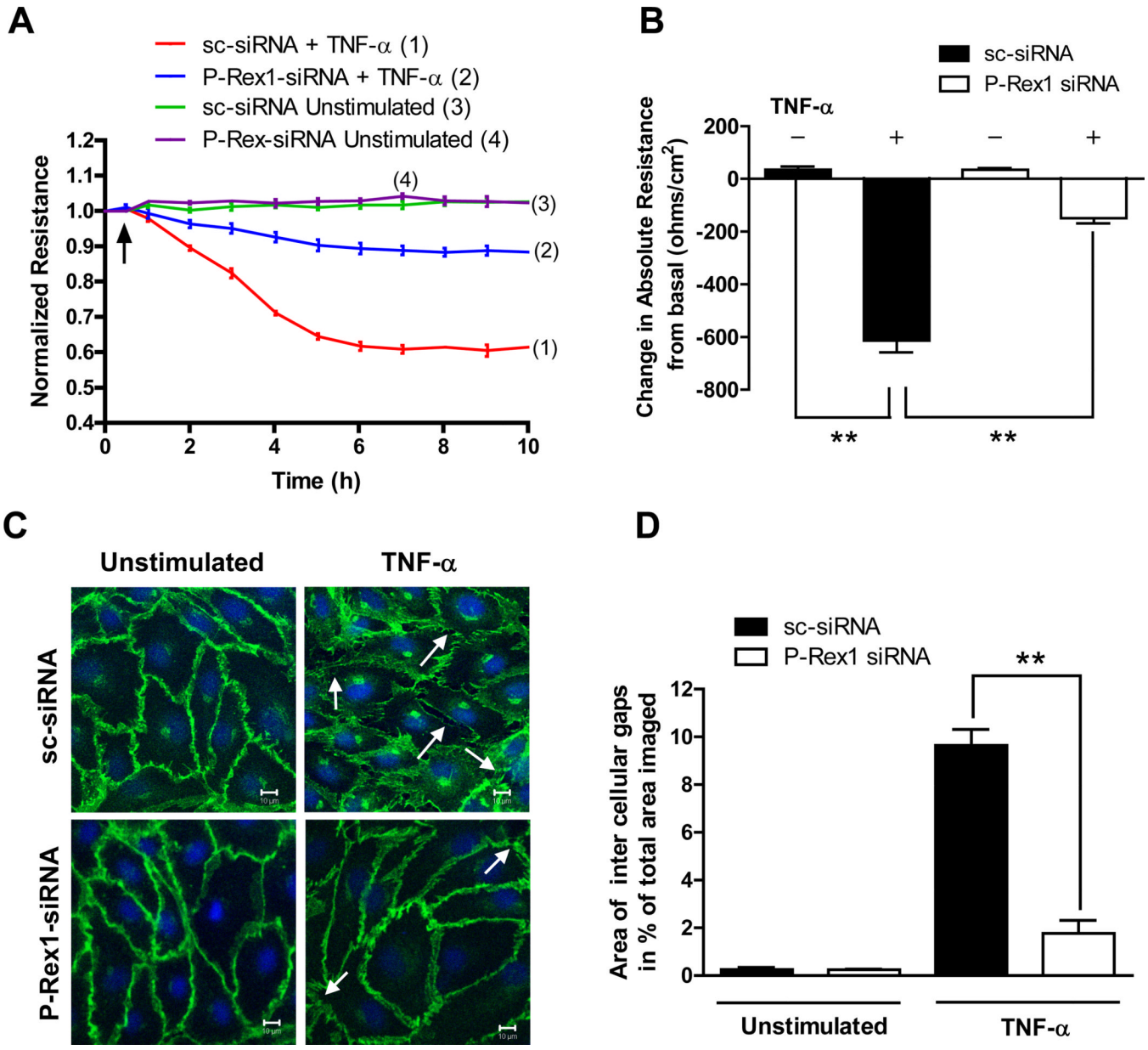

Figure 1. Effect of P-Rex1 knockdown on TNF-α-induced EC barrier dysfunction

(A)Transendothelial electrical resistance (TER) was measured across HLMVEC monolayers transfected with P-Rex1 specific siRNA or scrambled siRNA. Where indicated, cells were stimulated with TNF-α (25 ng/ml, or 500 units). Representative tracings from 5 experiments are shown. (B) Quantification of TER data showing changes in absolute resistance values over a 5 h period. Data shown are mean ± SEM based on 5 experiments. Asterisks (**), $p < 0.05$. (C) Fluorescent images showing the integrity of HLMVEC monolayers without or with TNF-α (25 ng/ml, 3.5 h). Green: Alexa fluor 488 conjugated anti-VE-cadherin; blue: DAPI. Arrows indicate gaps between cells; line scale: 10 μm (63 × magnification). (D) Quantification of area of intercellular gaps as percentage of total area imaged. Data shown are mean ± SEM. based on 5 experiments. Asterisks (**): $p < 0.01$.

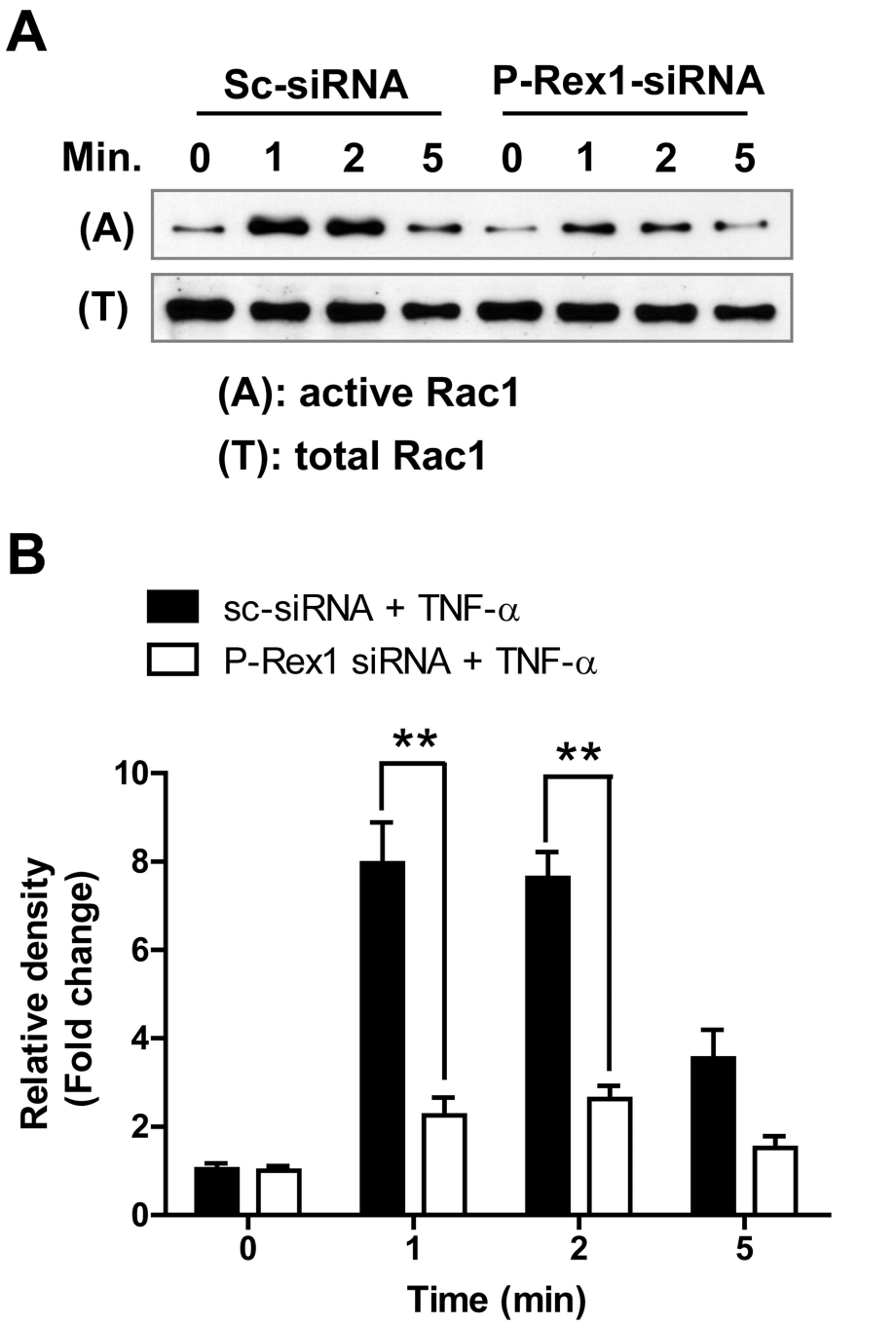

Figure 2. Role for P-Rex1 in TNF-α-induced Rac activation

(A) Scrambled (sc) and P-Rex1 siRNA transfected HLMVECs were serum starved and stimulated with 25 ng/ml of TNF-α for 1, 2, and 5 min. Rac pull-down with GST-PBD was performed, and active Rac1 was detected by Western blotting. Total Rac1 indicates loading control. (B) Bar graph showing quantification of Western blotting from 3 experiments. Data shown are mean ± SEM. Fold change is calculated based on ratio of active Rac1 to total Rac1. Asterisks (**): $p < 0.01$.

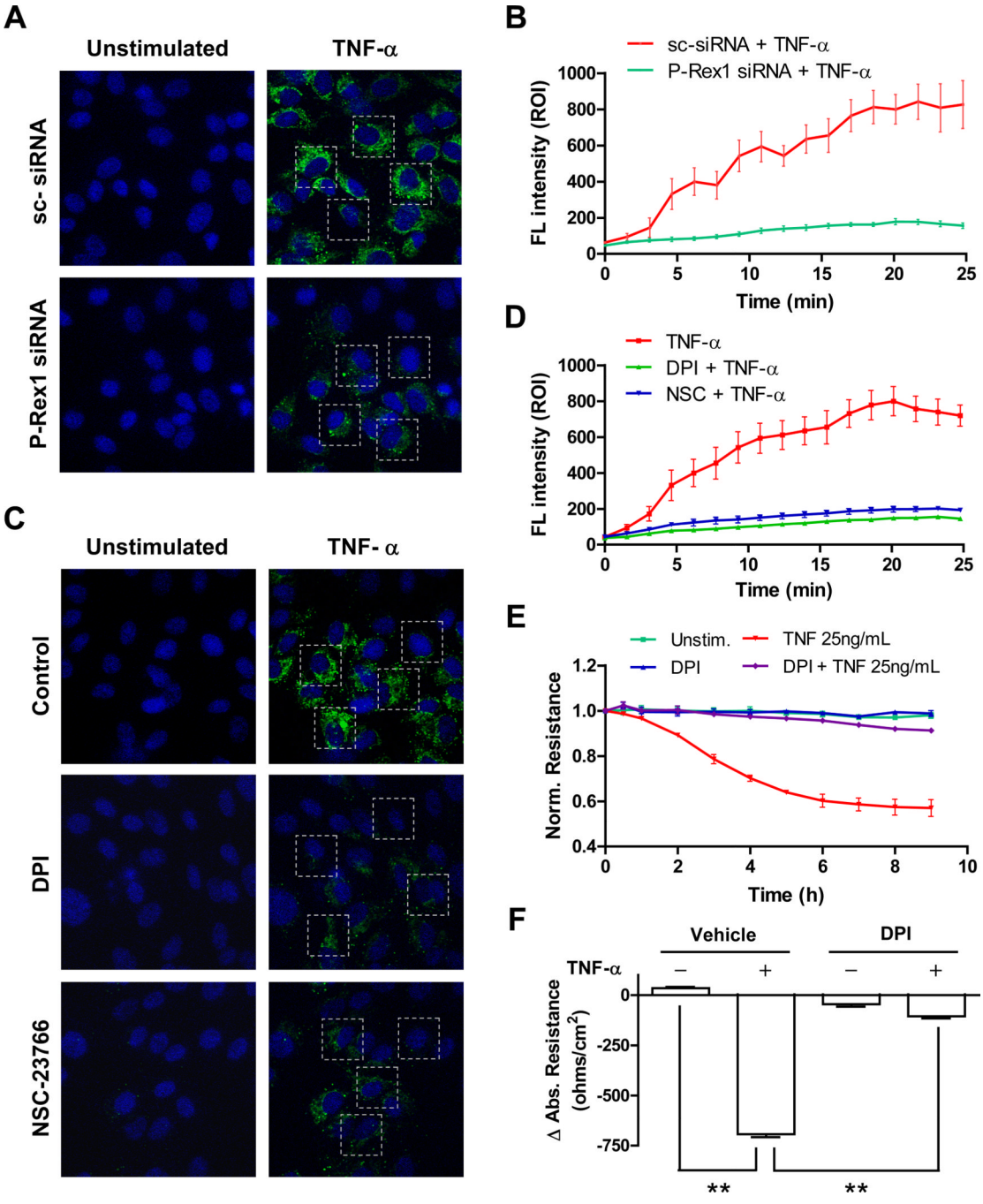

Figure 3. P-Rex1 regulates TNF-α-induced ROS production

(A) Fluorescent images showing ROS production in HLMVECs transfected with either scrambled (sc) siRNA or P-Rex1 siRNA. Rhodamine 123 (green) and Hoechst stain were used for detection of ROS and nuclei respectively. TNF-α stimulation was 25 ng/ml for 20 min. (B) Quantification of fluorescence intensity, shown as mean ± SEM of arbitrary light unit in randomly selected regions of interest (ROI), each containing a cell. A total of 4 ROIs each for sc and P-Rex1 siRNA groups were used for analysis, based on 4 experiments. (C) HLMVECs were untreated, pre-treated with DPI (2 μM, 1h) or the Rac inhibitor NSC-23766 (100 μM, 30 min), and ROS production was measured as in (A). Representative images from 5 experiments are shown. (D) Quantification of fluorescence intensity in (C), carried out as

described in (B) and based on 5 experiments. Data shown are mean ± SEM. (E) Effect of inhibition of ROS production on TER. HLMVECs monolayers were untreated or pre-treated with DPI (2 μM, 1 h) and stimulated with TNF-α (25 ng/ml) as indicated. Data shown are mean ± SEM based on 5 experiments. (F) Bar graph showing quantification of TER data in (E), expressed as change in absolute resistance at t=5 h from basal in ohms/cm2 from 5 experiments, each in duplicates. Data shown are mean ± SEM. Statistical analysis was carried out as described in *Methods*. Asterisks (**): $p < 0.01$.

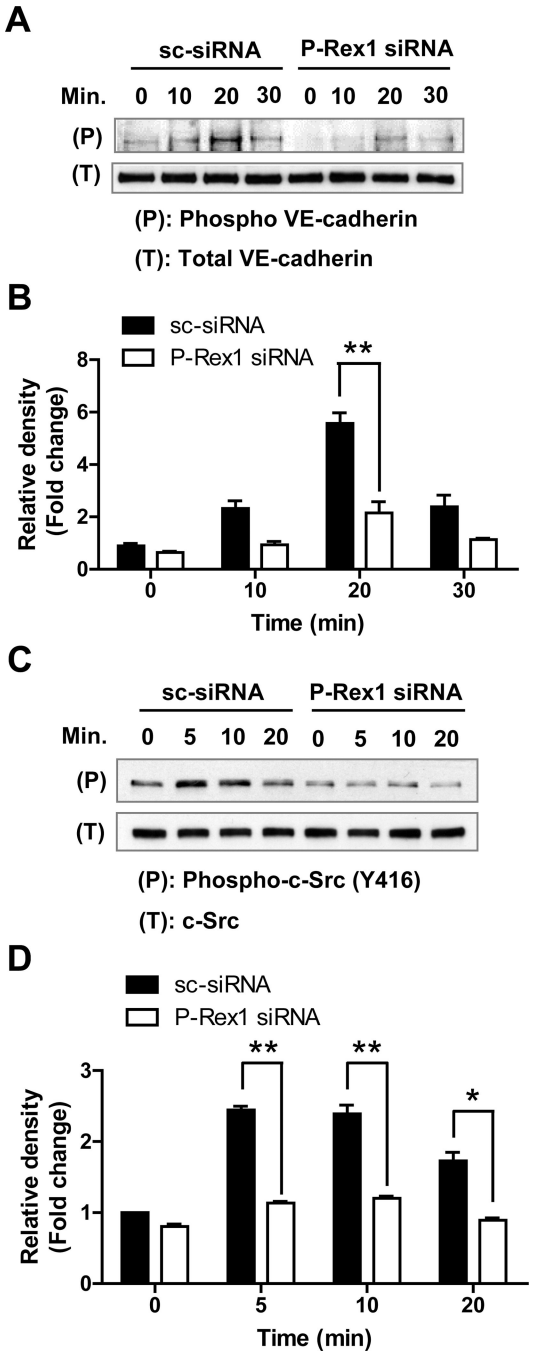

Figure 4. P-Rex1 regulates TNF-α-induced VE-cadherin phosphorylation

(A) Representative Western blots showing tyrosine phosphorylation of VE-cadherin in HLMVECs that were transfected with scrambled (sc) siRNA or P-Rex1-siRNA and stimulated with 25 ng/ml of TNF-α for 10, 20 and 30 min. The cell lysate was immunoprecipitated with a VE-cadherin antibody, and denatured samples were probed with an anti-phosphotyrosine antibody (PY20). Total VE-cadherin indicates loading control. (B) Quantification of Western blotting data by densitometry. Fold change is indicative of ratio of phosphorylated VE-cadherin to total VE-cadherin. Shown are mean ± SEM of relative density based on 3 experiments. Asterisks (**): $p < 0.01$. (C) Representative Western blots showing phosphorylation of c-src at Y416. HLMVECs transfected with scrambled or P-

Rex1 siRNA were serum starved for 2h followed by stimulation with 25 ng/ml of TNF-α for 5, 10 and 20 min or left unstimulated (0 min). Total c-Src was detected using a c-Src antibody. (D) Bar graph showing mean ± SEM of relative density based on 3 experiments. Fold change is indicative of ratio of phosphorylated c-Src to total c-Src. Asterisk (*): $p < 0.05$; (**): $p < 0.01$.

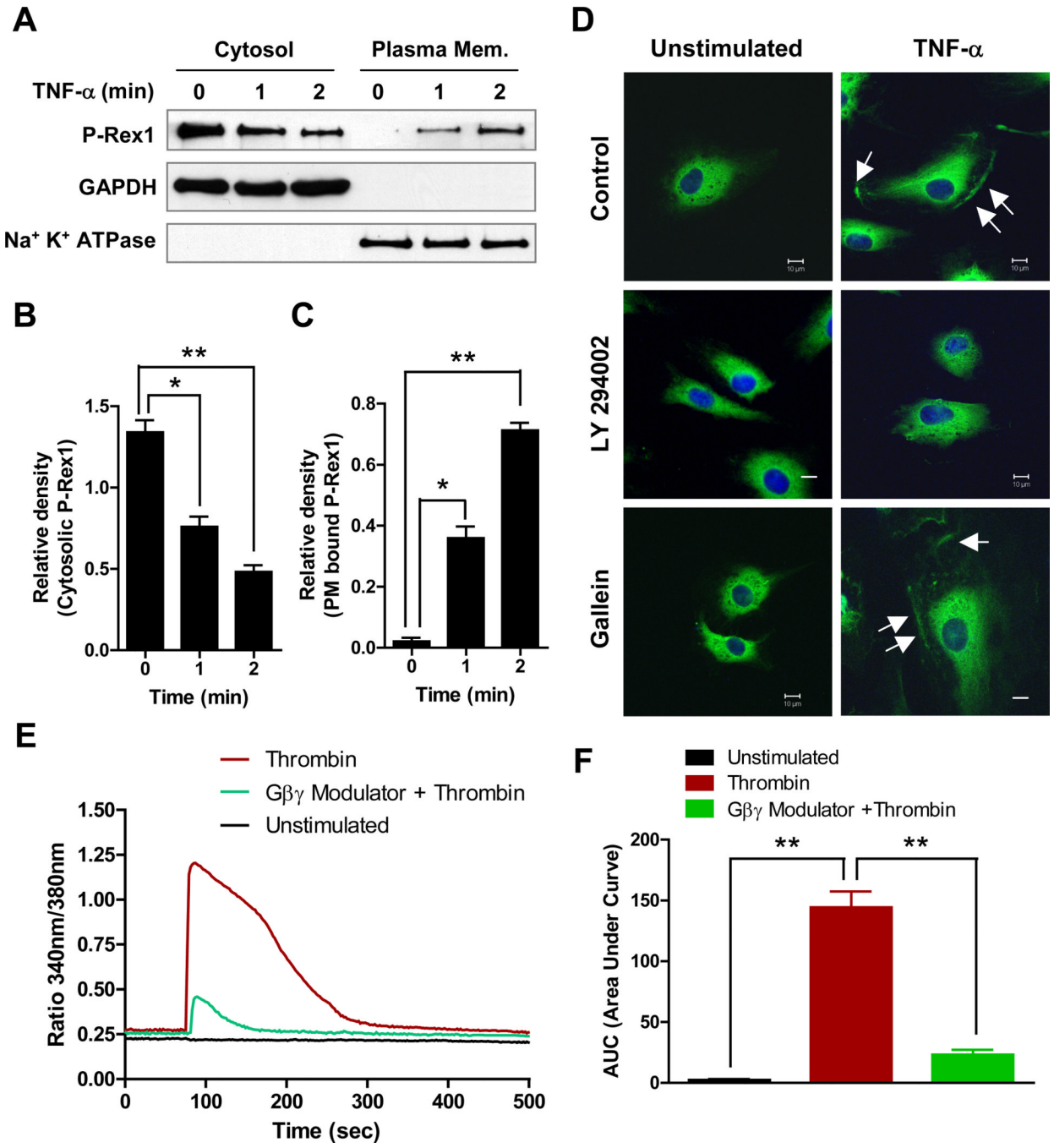

Figure 5. TNF-α induces PI3K-dependent membrane translocation of P-Rex1

(A) Western blots showing membrane translocation of P-Rex1. HLMVECs were stimulated with 25 ng/ml of TNF-α for 1 and 2 min or unstimulated (0 min) as indicated. Cytosolic and plasma membrane components were prepared, separated on SDS gel and blotted. P-Rex1 was detected using an anti-P-Rex1 antibody. GAPDH was used as cytosol marker and Na^+K^+ ATPase as a membrane marker. (B) For cytosolic fraction, P-Rex1 levels were calculated relative to GAPDH by densitometry from 3 experiments. (C) For membrane fractions, P-Rex1 levels were quantified relative to Na^+ K^+ ATPase by densitometry from 3 experiments. Data shown in (B) and (C) are mean ± SEM. Asterisk (*): $p < 0.05$; (**): $p < 0.01$. (D) Fluorescent images showing P-Rex1 membrane translocation. HLMVECs were

either untreated or treated with LY-294002 (20 μM, 30 min) or 10 μM of Gβγ modulator II (Gallein, 1h), and then stimulated with TNF-α (25 ng/ml) for 2 min as indicated. Cells were permeabilized, fixed and stained with anti-P-Rex1 and Alexa fluor 488 conjugated goat anti-mouse secondary antibody. Representative images from 3 experiments are shown. Arrows indicate P-Rex1 at membrane periphery (scale bar = 10 μm). (E) Calcium ratiometric assay showing the ability of Gβγ modulator II (Gallein) to inhibit thrombin-induced calcium release, using Fura 2. (F) Quantification of calcium release based on areas under the curve. Data shown (mean ± SEM) are based on 3 experiments. Asterisks (**): $p < 0.01$.

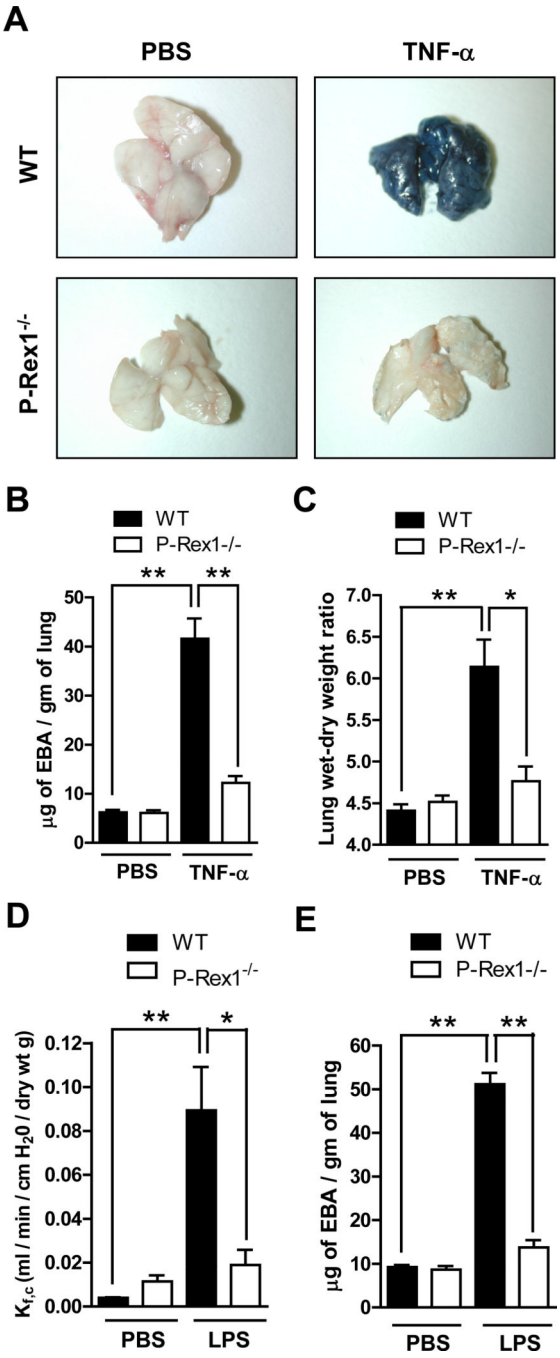

Figure 6. Lung microvascular permeability and edema formation in WT and P-Rex1$^{-/-}$ mice

(A) WT and P-Rex1$^{-/-}$ mice were intra-tracheally instilled with PBS or TNF-α (0.5 μg/mouse) for a total of 6 h. At 5.5 h, mice were intravenously injected with EBA (20 mg/kg body weight). Lungs were collected 30 min later for assessment of damage. Representative samples from a total of 5 experiments are shown. (B) Quantification of EBA accumulated in the lung (n=5) homogenates was conducted as described in *Methods*. (C) Lung wet weight to dry weight ratio was determined after intra-tracheally instillation of PBS or TNF-α (0.5 μg/mouse) for 6 h. (D) WT and P-Rex1$^{-/-}$ mice were intraperitoneally injected with PBS or LPS (5 μg/kg body weight) for 6 h and were assessed for lung microvascular permeability. *Ex vivo* lung perfusion was performed, and capillary filtration coefficient (Kf,c), indicative

of vascular leakiness was determined as described in *Methods*. N=3, where N is the number of mice that have undergone treatment in each group. (E) WT and P-Rex1$^{-/-}$ mice were given LPS or PBS as in (D), and EBA injection was performed as in (A). Quantification of dye accumulation in lungs was done as described in (B). A total of 5 mice were used in each group. For bar graphs (B to D), data shown are mean ± SEM, and statistical analysis was conducted as described in *Methods*. Multiple means comparison was performed by Tukey's method. Asterisk (*): $p < 0.05$.

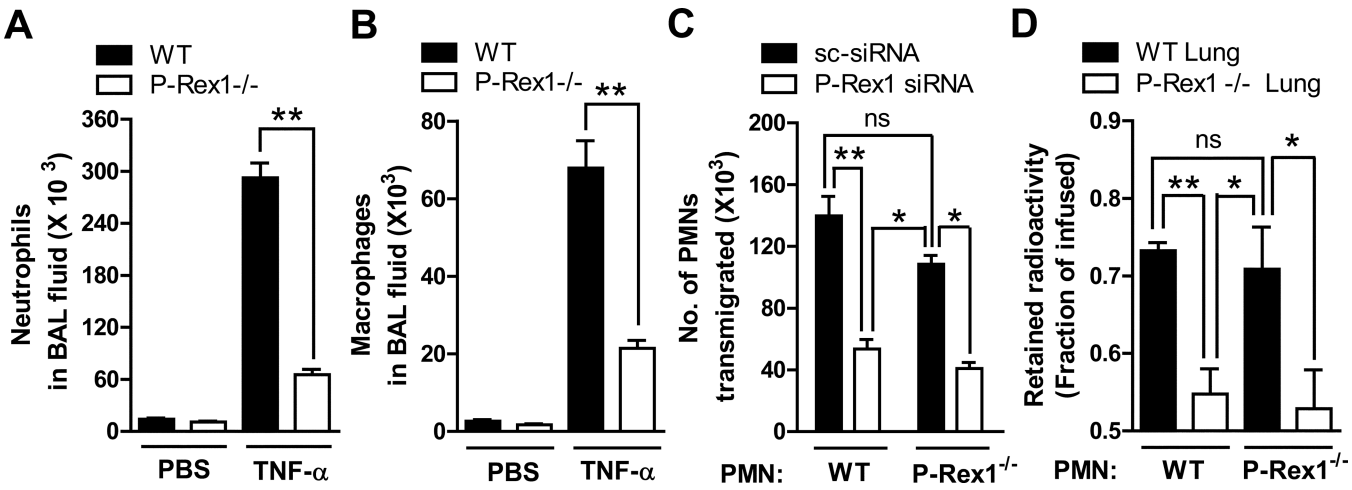

Figure 7. Role for P-Rex1 in leukocyte transmigration

WT and P-Rex1$^{-/-}$ mice were instilled intratracheally with PBS or 0.5 μg of murine recombinant TNF-α. After 24 h, mice were anesthetized, bronchoalveolar fluid (BAL) was collected, and total leukocytes, neutrophils and macrophages were counted. The number of neutrophils (A) and macrophages (B) in the BAL was shown, using 3 mice per group. (C) Transmigration of WT or P-Rex1$^{-/-}$ neutrophils across monolayers of HLMVECs. The endothelial cells were transfected with either sc siRNA or P-Rex1 siRNA, plated on gelatin-coated 3 mm pore filters, and treated with TNF-α (25 ng/ml) for 2 h. The formyl peptide fMIVIL (100 nM) was placed in bottom wells. A detailed figure set including all controls is shown in Supplemental Fig. S10. Data shown are mean ± SEM from 3 experiments. (D) Neutrophil sequestration assay was performed using ^{111}Indium oxine-labeled PMNs from WT or P-Rex1$^{-/-}$ mice, perfused across WT or P-Rex1$^{-/-}$ mouse lungs. The retained radioactivity was expressed as ratio of tissue cpm to infusate cpm. Data shown are mean ± SEM from 3 separate experiments with 3 mice in each group. Statistical analysis was done by two-factor ANOVA to analyze group mean comparison. Multiple means comparison was performed by Tukey's method. Asterisk (*): $p < 0.0.5$; (**): $p < 0.01$. “ns”: not significant.

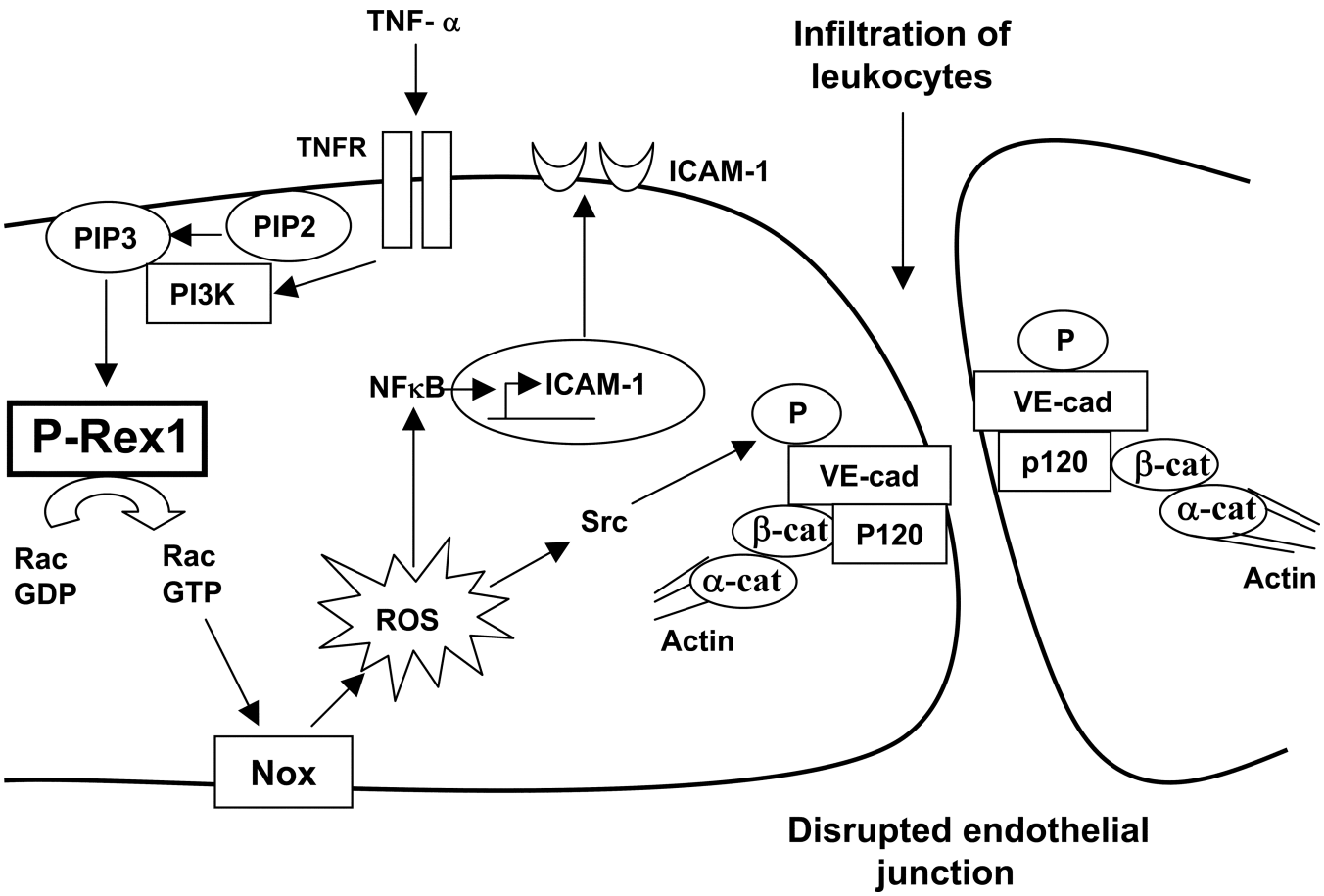

Figure 8. Schematic model showing the involvement of P-Rex1 in TNF-α-induced endothelial barrier dysfunction

See Discussion for a description of the role for P-Rex1 in TNF-α-induced Rac activation, ROS production, src-dependent tyrosine phosphorylation of VE-cadherin and ICAM-1 expression.