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Protein tyrosine kinase 6 mediates TNFα-induced endothelial barrier dysfunction

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ABSTRACT

A key event in the progression of systemic inflammation resulting from severe trauma or shock involves microvascular hyperpermeability, which leads to excessive plasma fluid and proteins accumulating in extravascular space resulting in tissue edema. The precise molecular mechanism of the hyperpermeability response is not completely understood. Protein tyrosine kinase 6 (PTK6, also known as breast tumor kinase BRK) is a non-receptor tyrosine kinase related to Src-family proteins. Although it has also been shown that PTK6 participates in regulating epithelial barrier function, the role of PTK6 in endothelial barrier function has not been reported. In this study, we hypothesized that PTK6 is (1) expressed in vascular endothelial cells, and (2) contributes to vascular endothelial hyperpermeability in response to TNFα. Results showed that PTK6 was detected in mouse endothelial cells at the level of protein and mRNA. In addition, PTK6 knockdown attenuated TNFα induced decrease in endothelial barrier function as measured by electric cell-substrate impedance sensing (ECIS) and *in vitro* transwell albumin-flux assays. Furthermore, we showed that TNFα treatment of endothelial cells increased active PTK6 association with p120-catenin at endothelial cell–cell junctions. Further analysis using immunocytochemistry and immunoprecipitation demonstrated that PTK6 knockdown attenuated TNFα induced VE-cadherin internalization as well as promoting its association with p120-catenin. Our study demonstrates a novel role of PTK6 in mediating endothelial barrier dysfunction.

1. Introduction

Exchange microvessels are composed of endothelium that forms an effective barrier to control movement of blood fluid and proteins across the vessel wall. Disruption of the endothelial barrier leads to microvascular leakage and tissue edema resulting in organ dysfunction [1,2]. This pathological process is involved in many diseases such as sepsis, ischemia–reperfusion injury, and systemic inflammation after trauma [3,4]. In particular, intestinal microvascular hyperpermeability resulting from severe trauma or burn contributes to intestinal hypoxia and tissue damage leading to breach of the intestinal mucosa and sepsis [5] as well as abdominal compartmental syndrome. Therefore, one of the clinical strategies in treating severe trauma is to restore endothelial barrier function of the microvasculature and prevent further tissue damage caused by edema [3].

Systemic inflammation activates leukocytes in a manner that causes the release of inflammatory mediators including histamine, tumor necrosis factor alpha (TNFα), and interleukins. These mediators trigger intracellular signaling events of the microvascular endothelium leading to changes of endothelial structure proteins which give rise to endothelial hyperpermeability [6,7]. TNFα is a cytokine released from activated leukocytes involved in mediating systemic inflammatory response syndrome (SIRS) [8]. In fact, intravenous injection of TNFα induces SIRS in rats [9]. In endothelial cells, TNFα induces hyperpermeability in part by tyrosine phosphorylation of VE-cadherin (Y658) followed by its internalization and degradation [10,11]. Tyrosine phosphorylation of VE-cadherin interrupts its association with p120-catenin, an association that is barrier protective in that p120-catenin promotes VE-cadherin retention at the plasma membrane [12]. It has been established that TNFα induces activation of the Src-family kinase Fyn which targets VE-cadherin and mediates its internalization in human lung endothelia [10]. Interestingly, the non-receptor tyrosine

Abbreviations: PTK6, protein tyrosine kinase 6; TNFα, tumor necrosis factor α; ECIS, electric cell-substrate impedance sensing; siRNA, silencing ribonucleic acid; E-cadherin, epithelial cadherin; VE-cadherin, vascular endothelial cadherin; mRNA, messenger ribonucleic acid.

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kinase evolutionarily related to Src-family kinases, protein tyrosine kinase 6 (PTK6) [13], activation and translocation to the plasma membrane was shown to be inversely related with E-cadherin downregulation in epithelial cells [14]. Furthermore, several key components of the cell–cell junction and cell-matrix adhesions are directly phosphorylated by PTK6, including paxillin [15], β-catenin [16], and focal adhesion kinase [17]. However the role of PTK6 in mediating VE-cadherin expression or internalization in endothelial cells has not been explored.

It is well known that the non-receptor protein tyrosine kinase Src and several Src-related kinases (Fyn, Yes, Lck, Lyn, Hck, Fgr, Blk) are involved in mediating vascular permeability in response to inflammation [18]. Although distinct from Src-family kinases, the intestinal kinase PTK6 is a non-receptor tyrosine kinase evolutionarily related to Src-family kinases. PTK6 shares common motifs with Src including SH2 and SH3 (Src-homology) domains [19,20], and is regulated in a similar manner in that a C-terminal tyrosine residue (Y447) negatively regulates its kinase activity by binding to the SH2 domain when phosphorylated [21]. In addition, PTK6 autophosphorylates residue Y342 which activates the kinase [13]. Although few studies have addressed the pathogenic role of PTK6 in a nononcogenic context, Whitehead et al. showed that PTK6−/− mice demonstrate robust colonic epithelial barrier function relative to their wild-type counterparts [22]. Considering that the PTK6 structure is similar to Src-family kinases and is involved in epithelial barrier function, we hypothesized that PTK6 is expressed in endothelial cells and may play a role in TNFα-mediated endothelial barrier dysfunction.

2. Methods

2.1. Cell culture and treatment

Primary mouse intestinal endothelial cells isolated from small intestine vasculature (Cell Biologics) were grown to confluence as described by vendor in endothelial medium supplemented with 10% FBS. For experiments, *cells between passage 3 and 6* were grown to 2–3 days post-confluence then were treated with vehicle control (0.1% BSA in PBS) or TNFα (Sigma, 60 ng/ml) for indicated time-points after serum starvation for 18 h. Cells were lysed with assay specific lysis buffer followed by RNA extraction and quantification or BCA protein assay (Pierce) for protein determination prior to qPCR, Western blotting, immunoprecipitation, and cellular fractionation experiments.

2.2. Quantitative PCR

Treated endothelial cells were lysed in RNAzol (Molecular Research Center, Inc) followed by RNA extraction as described by manufacturer. The mRNA fraction was quantified and equal masses of RNA were reverse transcribed to cDNA using random hexamers prior to qPCR. Equal volumes of cDNA were used to quantify PTK6 message using specific primers (sequence of primers is proprietary, Bio-Rad) and SYBR master mix (Bio-Rad) according to manufacturer's instructions. Samples were assayed in triplicate followed by analysis comparing relative expression levels using GAPDH as the housekeeping gene via CFX96 Touch Real-Time PCR detection system and software (Bio-Rad).

2.3. Nuclear fractionation

Endothelial cells were lysed in fractionation buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1× phosphatase inhibitor cocktail (Pierce)) followed by several passages through 25 G needle. After nuclear extraction (3000 RPM, 4 °C, 5 min), the membrane pellet was harvested by ultracentrifugation (40000 RPM, 4 °C, 1 h) with the supernatant retained as the cytosolic fraction. The fractions were subjected to BCA protein assay (Pierce) prior to Western blot analysis to assess changes in protein localization relative to treatment. Equal masses of protein were loaded to 4–20% polyacrylamide gels (Bio-Rad) for SDS–PAGE followed by Western blotting using PTK6 antibody (Santa Cruz), or Caveolin-1 antibody (BD biosciences) to determine membrane isolation efficiency.

2.4. Electric cell-substrate impedance sensing (ECIS)

ECIS was performed as described previously [23]. Briefly, endothelial cells were transfected with siRNA (IDT) directed against PTK6 or vehicle control (TE) via electroporation in electroporation solution (Mirus) using a Bio-Rad Gene Pulser Xcell according to manufacturer's instructions. Cells were then plated on fibronectin coated, gold plated ECIS chamber slides (Applied Biophysics, 8W10E+) at 5×10^5 cells/ml and allowed to express siRNA for 72 h followed by treatment with vehicle control (0.1% BSA in PBS) or TNFα (30 ng/ml) for indicated time-points. Resistance measurements taken from the endothelial monolayers were recorded every 90 s and normalized to time-point zero. After ECIS measurements were complete, cells were lysed in RNAzol and PTK6 was assayed by qPCR to confirm knockdown.

2.5. Monolayer albumin-flux assay

Albumin-flux assays were conducted as previously described [24]. Endothelial cells were transfected with siRNA directed against PTK6 or vehicle control (TE) as described above. Cells were plated on fibronectin coated polycarbonate 24-well transwell inserts (0.4 μM pore size, Corning) and allowed to express siRNA for 72 h followed by treatment with vehicle control (0.1% BSA in PBS) or TNFα (30 ng/ml) for indicated time-points. Following treatment, the luminal (top) compartment was treated with FITC-labeled albumin (2.5 mg/ml) for 45 min, then 100 μl aliquots were obtained from each abluminal (bottom) compartment and assayed for FITC fluorescence. Permeability coefficients were calculated using the formula $Pa = [Ab]/t \times 1/A \times V/[Lu]$, where [Ab] is the abluminal concentration of FITC-albumin, t is time in seconds for FITC-albumin incubation, A is area of membrane in cm^2, V is the volume of the abluminal chamber, and [Lu] is the luminal concentration of FITC-albumin.

2.6. Immunoprecipitation

Endothelial cells were grown to confluence then electroporated with siRNA directed against PTK6 or vehicle control (TE) as described above. Once cells reached 2–3 days post-confluence, cells were serum starved overnight then treated with TNFα or vehicle control (0.1% BSA in PBS) for 6 h. Cells were lysed with immunoprecipitation buffer followed by pre-clearing with agarose beads. Antibodies directed against proteins of interest were conjugated to agarose beads then added to pre-cleared lysates (500 μg protein) and allowed to incubate with rotisserie motion at 4 °C overnight. Beads were harvested by centrifugation and washed with PBS 5 times followed by target elution with elution buffer. Co-immunoprecipitation was assessed by SDS–PAGE of eluted proteins followed by Western blotting for proteins of interest.

2.7. VE-cadherin internalization assay

Experiment was conducted as described by Malik et al with modifications [11]. Briefly, mock or PTK6 siRNA electroporated cells were grown to confluence on fibronectin coated slides

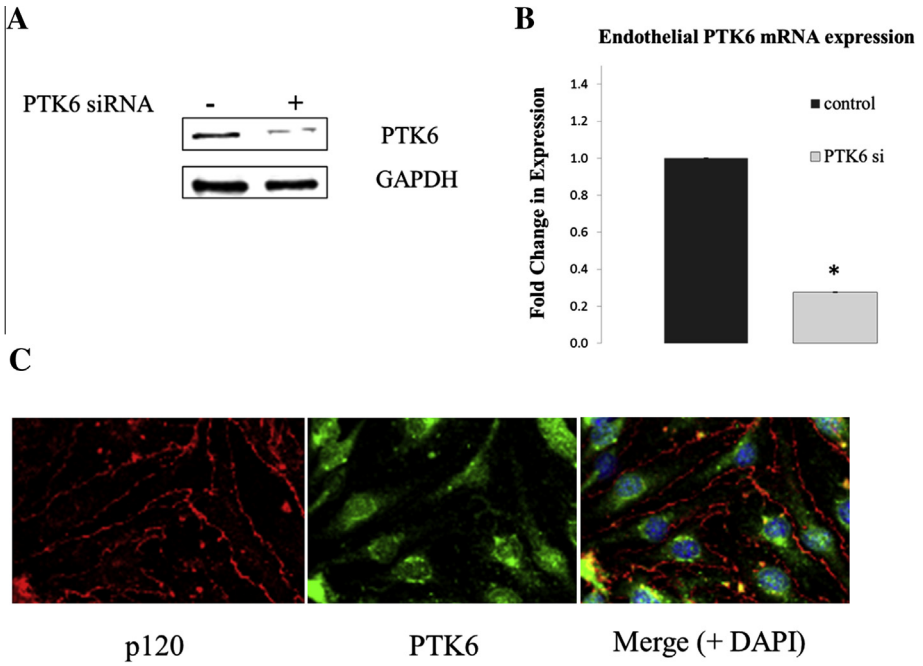

Fig. 1. PTK6 is expressed in endothelial cells. (A and B) Endothelial cells were either mock transfected or transfected with PTK6 siRNA and allowed to express siRNA for 48 h. Cells were lysed followed by protein and mRNA extraction then analyzed by Western blot (A), and qPCR (B). (C) Endothelial cells were probed with antibodies against p120-catenin and PTK6 antibody then detected with Alexafluor secondary antibodies (p120-catenin 568 nm (red), PTK6 488 nm (green)) and confocal microscopy. Results are representative of 3 experiments. $^{*}p < 0.05$.

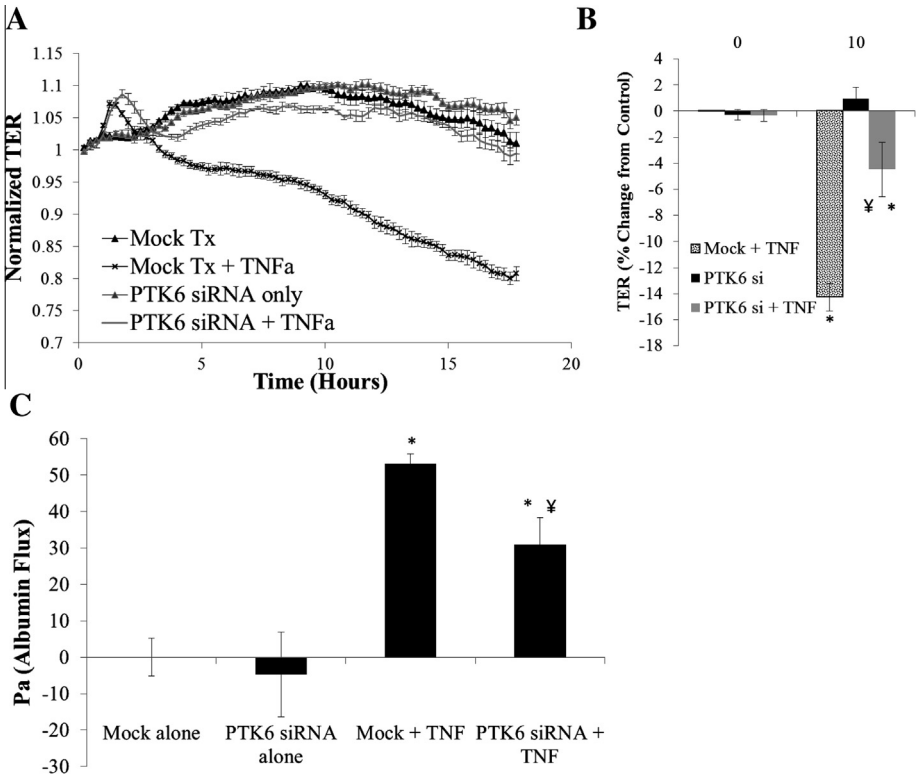

Fig. 2. PTK6 knockdown rescued TNFα mediated decrease in transendothelial electrical resistance (TER) and *attenuated TNFα induced albumin flux.* (A) Endothelial cells were either mock transfected or transfected with PTK6 siRNA then seeded on ECIS electrodes and treated with 60 ng/ml TNFα. TER values were measured and normalized to time-point zero. (B) Quantitative analysis of TER values at timepoints 0 and 10 h. Results are representative of 4 experiments. *$^{*}p < 0.05$ (Mock + TNF) and (PTK6 siRNA + TNF) vs. control , $^{¥}p < 0.05$ (Mock + TNF) vs.(PTK6siRNA + TNF).* (C) Endothelial cells were either mock transfected or transfected with siRNA directed against PTK6 then seeded on 0.4 μM transwell inserts then treated with TNFα (60 ng/ml) for 15 h. FITC-labeled albumin (0.25 mg/ml) was loaded to the apical chamber for 45 min then abluminal chamber was analyzed for presence of fluorescence. Results are representative of 4 experiments. *$^{*}p < 0.05$ (Mock + TNF) and (PTK6 siRNA + TNF) vs. control , $^{¥}p < 0.05$ (PTK6 siRNA + TNF) vs. (Mock + TNF).*

followed by serum starvation in 3% BSA containing media. Cells were allowed to incubate with antibody directed against VE-cadherin extracellular domain at 4 °C for 1 h, followed by treatment with either TNFα or vehicle control at 37 °C overnight in media containing 3% BSA and 100 μM chloroquine (prevents intracellular degradation of VE-cadherin). In order to enhance visualization of intracellular VE-cadherin, extracellular VE-cadherin was removed by washing cells in low pH buffer (PBS pH 2.7). Cells were then fixed with ice cold methanol then permeabilized with 0.3% triton X followed by blocking in 0.5% BSA in PBS. Incubation with antibody against p120-catenin (Santa Cruz) was conducted to determine co-localization with VE-cadherin relative to treatment. Detection of primary antibodies was performed by incubating cells with goat anti-mouse alexafluor 488 for VE-cadherin and goat anti-rabbit alexafluor 568 (Life Technologies) for p120-catenin for 1 h. ProLong Gold Anti-fade with DAPI (Life Technologies) was used to stain nuclei and mount coverslips. Images were acquired using the Olympus FV1000 MPE Multiphoton Laser Scanning Microscope.

2.8. Statistics

One-way ANOVA was used to identify significant difference among groups ($p < 0.05$). The Student's *t*-test was used to determine significant difference ($p < 0.05$) between indicated groups noted by (∗) or (¥).

3. Results

3.1. PTK6 is expressed in endothelial cells

Considering the significance of PTK6 in basal epithelial barrier function [22], we first sought to determine whether PTK6 is expressed in endothelial cells. Primary microvascular endothelial cells were treated with siRNA directed against PTK6 then assayed for expression at the level of mRNA and protein. As shown in Fig. 1A and B, cells treated with siRNA against PTK6 showed diminished PTK6 expression at the level of mRNA and protein when compared to mock transfected controls. Furthermore, confocal microscopy showed that PTK6 was detected in the perinuclear and cytosolic regions of untreated endothelial cells (Fig. 1C).

3.2. PTK6 knockdown rescued TNFα mediated endothelial barrier dysfunction

Next, we hypothesized that PTK6 plays a role in TNFα induced endothelial barrier dysfunction. *We tested this by assessing barrier*

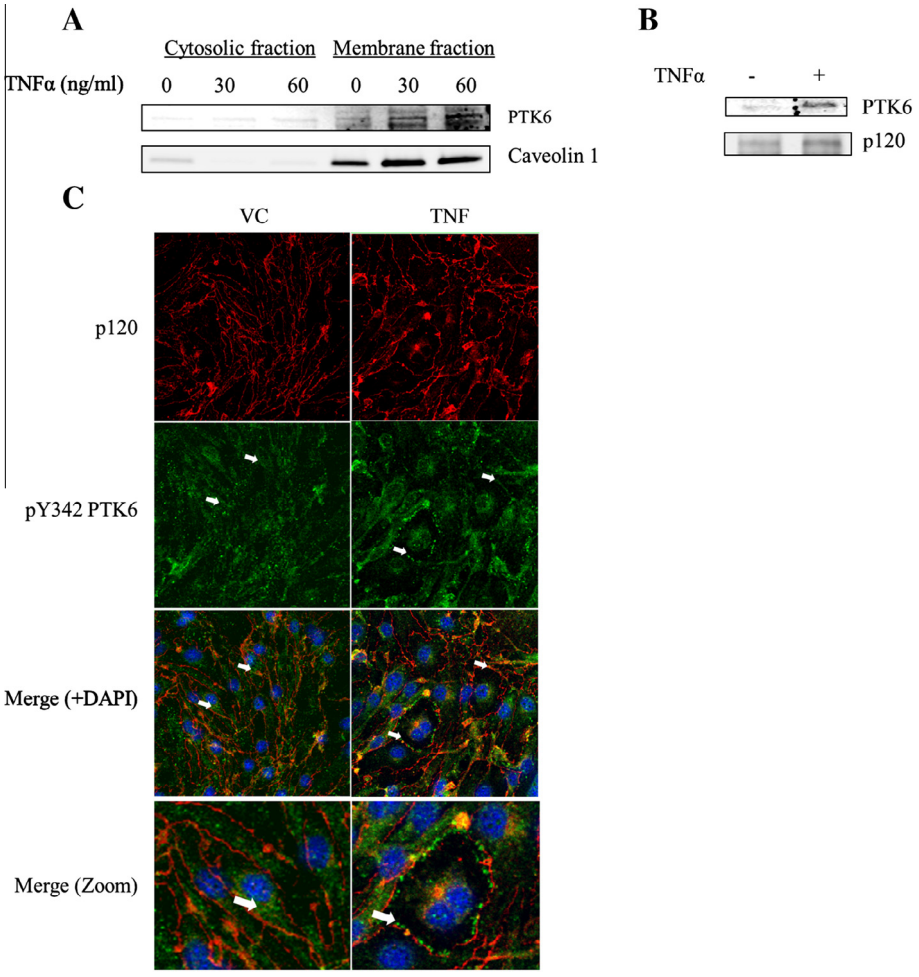

Fig. 3. TNFα induced PTK6 translocation to the plasma membrane and cell–cell junctions. Endothelial cells were treated with vehicle control (VC) or TNFα (60 ng/ml unless otherwise stated) for 3 h. (A) Cells were subjected to subcellular fractionation via sucrose gradient centrifugation followed by Western blot to determine PTK6 localization. Caveolin-1 was probed to confirm enrichment of membrane proteins. (B) p120-Catenin was immunoprecipitated then PTK6 was detected in co-eluted proteins. p120-Catenin immunodetection was assayed to ensure equal loading. (C) Confocal microscopy showed enhanced cell–cell junction localization of active PTK6 (pY342) upon TNFα treatment. Cell junctions are indicated by enhanced detection of p120-catenin. Arrows indicate areas of cytosolic pY342 PTK6 (left) vs. cell-junction localized pY342 PTK6 (right). Results are representative of 3 experiments.

function in two ways. First, we transfected endothelial cells with siRNA directed against PTK6 or mock transfected cells then plated on ECIS arrays and recorded resistance measurements of cells treated with TNFα or vehicle control. As shown in Fig. 2A and B, PTK6 knockdown significantly attenuated TNFα mediated barrier dysfunction by approximately 75%. *Next, we utilized* endothelial albumin-flux assays which showed that PTK6 knockdown significantly attenuated TNFα induced endothelial albumin-flux by approximately 50% of TNFα treated mock transfected controls (Fig. 2C).

3.3. TNFα induced PTK6 translocation to the plasma membrane and cell–cell junctions

In order to determine localization changes of PTK6 with respect to treatment, we treated endothelial cells with increasing doses of TNFα and harvested the membrane fraction using sucrose fractionation. As shown in Fig. 3A, PTK6 detection in the plasma membrane fraction was positively correlated with increased doses of TNFα. Next, since PTK6 contains SH2 and SH3 domains that may allow it to associate with the adherens junction protein p120-catenin [13], an important endothelial membrane scaffolding protein involved in maintaining VE-cadherin stability, we hypothesized that PTK6 associated with p120-catenin. Cells were treated with TNFα or vehicle control then p120-catenin was immunoprecipitated. Proteins co-eluted with p120-catenin were probed for presence of PTK6. As demonstrated in Fig. 3B, TNFα induced PTK6 association with p120-catenin in endothelial cells. To further analyze TNFα induced PTK6 association with p120-catenin, we utilized immunocytochemistry to examine any co-localization events between active PTK6 (pY342 PTK6) and p120-catenin upon TNFα treatment. As indicated in Fig. 3C, TNFα promoted p120-catenin targeting of pY342 PTK6 at cell–cell junctions.

3.4. PTK6 inhibition improved VE-cadherin stabilization in presence of TNFα

Since the Src-family kinase Fyn is known to phosphorylate VE-cadherin at Y658 and mediate its internalization in response to TNFα [10], and PTK6 has common protein domains with Fyn, we hypothesized that PTK6 may be involved in VE-cadherin phosphorylation upon TNFα stimulation. Results showed that PTK6 knockdown prevented TNFα induced phosphorylation of VE-cadherin at Y658 (Fig. 4A). Next, immunocytochemistry showed that PTK6 knockdown deterred VE-cadherin internalization resulting from TNFα treatment (Fig. 4B). Since it has been established that Y658 phosphorylation interferes with VE-cadherin association with p120-catenin [25], and this interference allows internalization of cadherins [26,27] to promote endothelial permeability, we sought to determine whether PTK6 inhibition with siRNA restored p120-catenin association with VE-cadherin with TNFα treatment. As indicated in Fig. 4C, VE-cadherin detection was improved when p120-catenin was immunoprecipitated from TNFα treated cells expressing PTK6 siRNA relative to TNFα treated mock transfected cells, suggesting that PTK6 may function in modulating p120-catenin association with VE-cadherin.

4. Discussion

Considering that the kinase PTK6 plays a role in epithelial barrier function, we sought to determine whether PTK6 modulates

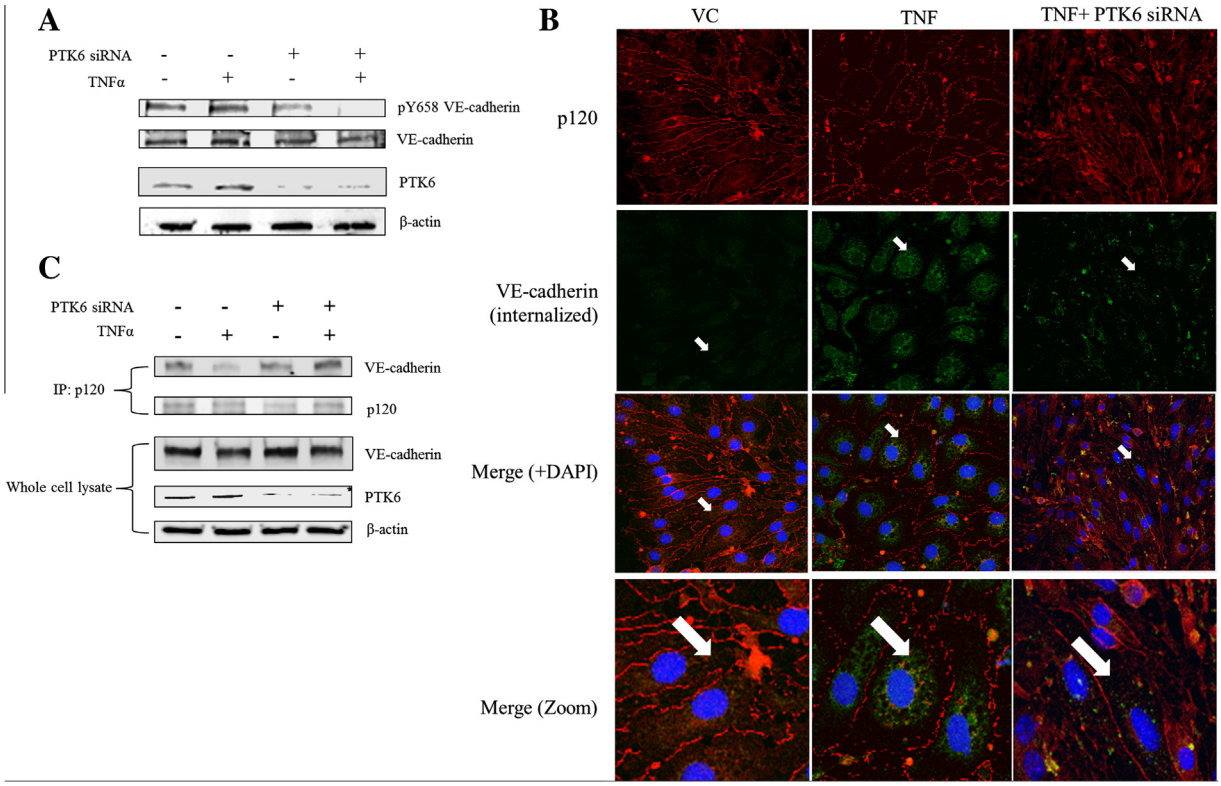

Fig. 4. PTK6 inhibition improved VE-cadherin stabilization in presence of TNFα. endothelial cells were either mock transfected or transfected with siRNA directed against PTK6 then treated with vehicle control (VC) or TNFα (60 ng/ml) for 3 h. (A) Whole cell lysates were analyzed by Western for VE-cadherin Y658 phosphorylation and total expression. β-Actin was probed as loading control. (B) Internalized VE-cadherin was immunodetected using antibodies directed against the external region of the molecule. Non-internalized VE-cadherin was removed with a low pH wash. Arrows indicate areas of impaired VE-cadherin detection (first and last vertical panels) or enhanced VE-cadherin detection (middle panel). p120-Catenin was probed to identify cell junctions for orientation. (C) VE-cadherin immunodetection was assayed in co-eluted proteins from equal masses of protein cell lysates immunoprecipitated with p120-catenin antibody. Total PTK6, VE-cadherin and β-actin were assayed to indicate efficient knockdown and equal loading, respectively. All results are representative of 3 experiments.

TNFα induced endothelial hyperpermeability. After first identifying the presence of PTK6 in endothelial cells at the level of protein and mRNA, we then explored the functional role of PTK6 in the context of inflammation by stimulating endothelial cells with TNFα. Results showed that decreasing PTK6 expression attenuated TNFα induced barrier dysfunction and albumin-flux, suggesting that PTK6 indeed contributes to impaired barrier function resulting from TNFα. *In addition, we found that PTK6 protein expression was elevated in lung tissue harvested from mice that underwent cecal ligation and puncture (CLP) to mimic sepsis (data not shown), suggesting PTK6 may play a role in mediating inflammation in vivo.* We then assessed the spatial regulation of PTK6 and found that PTK6 translocated to the endothelial cell–cell junctions and immunoprecipitated with the junction scaffolding protein, p120-catenin, upon stimulation with TNFα, further strengthening the idea that PTK6 plays a role in regulating endothelial junction dynamics. We further demonstrated that PTK6 is involved in adherens junction regulation by showing PTK6 co-eluted with p120-catenin in lysates of TNFα treated cells, but not untreated cells, suggesting that TNFα promotes p120-catenin PTK6 co-localization.

Src-related kinases are activated by inflammatory cytokines including TNFα [28]. *Our previous study first linked activated neutrophil-mediated changes in VE-cadherin tyrosine phosphorylation, internalization and barrier dysfunction in cultured microvascular endothelial cells* [29]. *Others reported VE cadherin internalization as a key step in disrupting endothelial barrier function by Src-dependent mechanisms in response to VEGF* [30,31]. *Most recently, Dejana's group has demonstrated that Src activation in veins resulted in increased VE-cadherin phosphorylation at Y658 and subsequent internalization in vivo. Inhibiting this phosphorylation event prevented VE-cadherin internalization and improved vascular leakage induced by inflammatory mediators such as bradykinin or histamine* [32]. Tyrosine phosphorylation of VE-cadherin leads to internalization and degradation of VE-cadherin, followed by increased intercellular gap formation thus contributing to permeability to plasma proteins and leukocytes [29,32]. In our study, we showed that TNFα induced cell-junction localization of endothelial PTK6, a Src-related kinase, indicating that TNFα places PTK6 in the vicinity of cell–cell junctions and therefore may also target junction proteins as do other Src-family kinases. Since TNFα is known to induce tyrosine phosphorylation (VE-cadherin pY658) and subsequent interference of the barrier protective p120-catenin:VE-cadherin association [25], we hypothesized that a possible mechanism for PTK6 induced increase in endothelial permeability may involve VE-cadherin. Indeed, we showed that PTK6 knockdown diminished pY658 phosphorylation of VE-cadherin induced by TNFα. The next logical question was to determine whether PTK6 knockdown prevented TNFα induced VE-cadherin internalization. Consistent with aforementioned experiments, immunodetection of internalized VE-cadherin was attenuated in TNFα treated cells transfected with PTK6 siRNA, suggesting that PTK6 plays a role in the internalization of VE-cadherin. Since VE-cadherin tyrosine phosphorylation and internalization is alleviated when associated with p120-catenin [1,25], showing that knocking down PTK6 in TNFα treated cells increased p120-catenin association with VE-cadherin when compared to mock transfected TNFα treated cells suggested that PTK6 may be associated with regulating p120-catenin interaction with VE-cadherin in a manner that promotes VE-cadherin internalization. Taken together, these results demonstrate a previously unrecognized role for PTK6 as a novel signaling molecule in mechanisms underlying endothelial barrier dysfunction resulting from inflammation.

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Disclosures

None.

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