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Vasodilator-Stimulated Phosphoprotein Deficiency Potentiates PAR-1-induced Increase in Endothelial Permeability in Mouse Lungs

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Abstract

Vasodilator-stimulated phosphoprotein (VASP) is implicated in the protection of the endothelial barrier *in vitro* and *in vivo*. VASP function in thrombin signaling in the endothelial cells (ECs) is not known. For the first time we studied the effects of VASP deficiency on EC permeability and pulmonary vascular permeability in response to thrombin receptor stimulation. We provided the evidence that VASP deficiency potentiates the increase in endothelial permeability induced by activation of thrombin receptor in cultured human umbilical vein endothelial cells (HUVECs) and isolated mouse lungs. Using transendothelial resistance measurement, we showed that siRNA-mediated VASP downregulation in HUVECs leads to a potentiation of thrombin- and protease-activated receptor 1 (PAR-1) agonist-induced increase in endothelial permeability. Compared to control cells, VASP-deficient HUVECs had delayed endothelial junctional reassembly and abrogated VE-cadherin cytoskeletal anchoring in the recovery phase after thrombin stimulation, as demonstrated by immunofluorescence studies and cell fractionation analysis, respectively. Measurement of the capillary filtration coefficient in isolated mouse lungs demonstrated that VASP^{-/-} mice have increased microvascular permeability in response to infusion with PAR-1 agonist compared to wild type mice. Lack of VASP led to decreased Rac1 activation both in VASP-deficient HUVECs after thrombin stimulation and VASP^{-/-} mouse lungs after PAR-1 agonist infusion, indicating that VASP effects on thrombin signaling may correlated with changes in Rac1 activity. This study demonstrates that VASP may play critical and complex role in the regulation of thrombin-dependent disruption of the endothelial barrier function.

Keywords

vasodilator-stimulated phosphoprotein; thrombin; endothelial permeability; lung perfusion

Introduction

The intima or the endothelial lining of the blood vessels has multiple functions. One of the most prominent functions is maintaining the barrier semi-permeable for passage of the macromolecules and liquids between the interstitium and blood (Mehta and Malik, 2006). This is particularly important in pulmonary vasculature due to its size and physiological

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function (Dudek and Garcia, 2001). Inflammatory conditions such as acute lung injury and sepsis are characterized by an increased endothelial permeability that represents loss of the endothelial barrier function (Dudek and Garcia, 2001). Inflammatory mediators such as thrombin and lipopolysaccharide (LPS) stimulate their receptors on the endothelial cells (ECs) leading to the activation of multiple signaling pathways. This results in cytoskeletal changes and disruption of the endothelial intercellular junctions leading to an increase in endothelial permeability and loss of the endothelial barrier function (Vandenbroucke et al., 2008).

In ECs, thrombin initiates the downstream signaling predominantly by activating the protease-activated receptor 1 (PAR-1). Activation of PAR-1 leads to stress fiber formation, cell contraction and disassembly of adherens junctions causing an increase in endothelial permeability and dysregulation of the endothelial barrier function (reviewed in (Mehta and Malik, 2006)). These effects of thrombin on the endothelium are reversible. The recovery of the adherens junctions and restoration of the endothelial barrier function occur within 2 h (Moy et al., 2002; Rabiet et al., 1996; Tiruppathi et al., 1992). Several studies have indicated that PAR-1 may initiate barrier-protective mechanisms (Feistritzer and Riewald, 2005) by activating complex signaling events leading to activation of Rac1 and Cdc42 and stabilization of the endothelial barrier function *in vitro* and *in vivo* (Konstantoulaki et al., 2003; Kouklis et al., 2004; Waschke et al., 2006). Expression of the dominant-negative Rac1 in HUVECs enhances thrombin-induced increase in permeability, indicating an essential role of Rac1 in thrombin signaling (Wojciak-Stothard et al., 2001).

Vasodilator-stimulated phosphoprotein (VASP) is a member of Ena/VASP family of proteins, comprised of VASP, Drosophila Enabled (Ena), a mammalian Ena homolog Mena, and Ena-VASP-like protein EVL (Gertler et al., 1995; Gertler et al., 1996; Haffner et al., 1995). Absence of all three Ena/VASP proteins in mice results in pronounced edema, hemorrhage and embryonic lethality (Furman et al., 2007). It was demonstrated that VASP^{-/-} mice exhibit increased vascular permeability in response to inflammatory agent bradykinin (Benz et al., 2008). These mice also have increased lung permeability after inhalation of LPS (Henes et al., 2009).

At resting conditions, siRNA-mediated downregulation of VASP does not affect transendothelial resistance (TER) (Rentsendorj et al., 2008) but increases permeability to FITC-dextran (Rosenberger et al., 2007). Similarly, murine microvascular myocardial endothelial (MyEnd) cells derived from VASP^{-/-} mice show no difference in TER when compared to control MyEnd cells (Schlegel and Waschke, 2009b), but exhibit an increase in permeability to FITC-dextran under resting conditions (Schlegel et al., 2008; Schlegel and Waschke, 2009a). Reconstitution of VASP in VASP^{-/-} cells leads to an increase in TER (Schlegel and Waschke, 2009b). In response to stimuli such as hypoxia, H₂O₂ and LPS, VASP is implicated in protection of the endothelial barrier function of cultured ECs (Bogatcheva et al., 2009; Rentsendorj et al., 2008; Rosenberger et al., 2007). However, siRNA-mediated downregulation of VASP potentiated ATP-induced increase in TER, suggesting VASP as a negative regulator of the endothelial barrier (Kolossova et al., 2005).

Here, for the first time, we showed that VASP may be critically involved in the protection of the endothelial barrier challenged with thrombin. We demonstrated that siRNA-mediated VASP downregulation in ECs leads to a potentiation of thrombin-induced increase in endothelial permeability. These findings were confirmed in the lung perfusion animal model, which showed that VASP^{-/-} mice exhibit increased microvascular permeability in response to perfusion with PAR-1 agonist. This study demonstrates that VASP may play critical and complex role in the regulation of intact and compromised endothelial barrier and provides possible mechanistic basis for these findings.

Materials and Methods

Reagents

Polyclonal anti-VASP antibodies were purchased from EMD (Gibbstown, NJ). Thrombin was purchased from Enzyme Research Laboratories Inc (South Bend, IN). Fetal bovine serum (FBS), RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were from Invitrogen (Carlsbad, CA). Endothelial growth medium (EGM-2) BulletKit and EBM-2 basal medium were purchased from Lonza (Walkersville, MD). Anti-VE-cadherin antibodies, VASP-specific siRNA duplexes, siRNA transfection reagent and siRNA transfection medium were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Non-silencing control siRNA was from Qiagen (Valencia, CA). SiGLO RISC-Free Control siRNA was purchased from Dharmacon (Thermo Fisher Scientific, Waltham, MA). Rac1 monoclonal antibody was from BD Biosciences (San Jose, CA). PAR-1 peptide (SFLLRN) was from Biosource (Invitrogen Carlsbad, CA). Ketamine and xylazine were purchased from Abbott Laboratories (Abbott Park, IL). Protease inhibitor cocktail for mammalian cells was from Sigma-Aldrich (St. Louis, MO).

Cell culture and small interfering RNA (siRNA) transfection

The human umbilical vein endothelial cells (HUVECs), purchased from Lonza (Walkersville, MD), were cultured for up to 8 passages in EGM-2 BulletKit. HUVECs were maintained in EBM-2 medium supplemented with 0.2% serum ~2 h before the experiment. The cells were stimulated with 25 nM thrombin, 6 μ M or 30 μ M PAR-1 peptide agonist. Transfection of siRNA was performed using siRNA transfection reagent and siRNA transfection medium according to the manufacturer's protocol. We empirically found that transfection of confluent culture produces the best results for VASP downregulation. The final siRNA concentration was 30 nM unless indicated otherwise.

Immunoblotting

The cells were lysed by incubation with lysis buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM dithiothreitol, and 5 μ L/mL mammalian protease inhibitor cocktail) on ice for 15 min. The insoluble material was removed from the lysates by centrifugation at 15,000 $\times$ g for 10 min, cleared lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane, and analyzed by immunoblotting with appropriate primary antibodies and horseradish peroxidase-linked secondary antibodies (GE Healthcare Biosciences, Pittsburgh, PA). The Western blot signal was developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) and detected using Kodak® BioMax™ MS film (Sigma, St. Louis, MO). ImageJ program (U. S. National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>) was used for densitometry analysis of VASP bands detected with anti-VASP antibody and Hsp90 bands detected by anti-Hsp90 antibody from scanned Western blot images. The intensities of VASP bands were normalized to intensities of corresponding Hsp90 bands and used to calculate the percentage of VASP downregulation.

Transendothelial electrical resistance (TER) measurement

The electrical resistance across the EC monolayer was measured using an electric cell substrate impedance sensor (ECIS, Applied BioPhysics, Inc. Troy, NY) as described before (Andreeva et al., 2005; Giaever and Keese, 1991; Tiruppathi et al., 1992). HUVECs were grown on the small gold electrode until they reached confluence. Cells were maintained in the EBM-2 medium supplemented with 0.2% FBS for 2 h before the experiment and the electrodes were connected to a phase-sensitive lock-in amplifier. A constant current of 1 μ A

was applied between the small electrodes and the large counter electrode using a 1-V, 4,000-Hz AC signal supplied through a 1-M Ω resistor. The voltage changes between small electrodes and large electrode were recorded every 2 min and stored. The data are presented as the change in the normalized resistance.

Immunostaining and confocal microscopy

Confluent HUVECs were transfected for 48 h and maintained in the EGM-2 medium supplemented with 10% FBS. Two hours before the stimulation with thrombin, the cells were maintained in EBM-2 basal medium supplemented with 0.2% FBS. Thereafter, the cells were washed with HBSS, fixed with 2% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Nonspecific binding was blocked with blocking solution containing 0.2% fish skin gelatin (Sigma, St. Louis, MO) and 1% bovine serum albumin in HBSS followed by incubation with appropriate primary and secondary antibodies dissolved in the blocking solution. The coverslips were mounted using ProLong antifade kit (Molecular Probes, Invitrogen, Carlsbad, CA). Confocal images were captured on a Zeiss LSM 510 microscope equipped with 63 $\times$ water-immersion objective or 20 $\times$ objective with laser excitations at 488 and 543 nm. The gap surface area was measured using UTHSCSA ImageTool Version 3.0 program (University of Texas Health Science Center, San Antonio, TX, <http://ddsdx.uthscsa.edu/dig/itdesc.html>) in five randomly picked microscopic fields for each time point and expressed as the percentage of the total area of the analyzed field.

Preparation of cell membrane Triton-soluble and Triton-insoluble fractions

The fractions were isolated using modified fractionation protocol (Predescu et al., 2001). Briefly, HUVECs transfected with control and VASP siRNA for ~48 h were stimulated with 25 nM thrombin, washed with PBS, incubated 10 min in the homogenizing buffer (50 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 5 μ L/mL mammalian protease inhibitor cocktail) and homogenized by passing the cell lysates 10–12 times through 27-G needle. At this point, an aliquot of total lysate was taken. Thereafter, the cell lysates were centrifuged at 5,000 $\times$ g at 4 $^{\circ}$ C for 5 min to remove cell nuclei and debris. The resulting postnuclear supernatants were centrifuged at 100,000 $\times$ g at 4 $^{\circ}$ C for 45 min using polyallomer tubes in TLA-110 rotor (Beckman instruments, Palo Alto, CA). After removal of supernatant (cytosol fraction), the pellet (membrane fraction) was incubated for 30 min in the buffer (1% Triton X-100, 20 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, and 5 μ L/mL mammalian protease inhibitor cocktail) followed by centrifugation at 100,000 $\times$ g at 4 $^{\circ}$ C for 45 min. The resulting Triton-soluble fraction and Triton-insoluble fraction (cytoskeletal fraction) were supplemented with Laemmli's sample buffer, boiled for 5 min at 95 $^{\circ}$ C, and subjected to SDS-PAGE. The densities of scanned protein bands were analyzed using ImageJ program.

Rac1 GTPase activation assay

Activation of Rac1 GTPase was determined in HUVECs and mouse lungs lysates using pull-down assay (Rac1 assay) as described before (Niu et al., 2003).

HUVEC lysates were prepared as follows. Briefly, HUVECs seeded onto gelatin-coated 60-mm tissue culture plates were transfected with 30 nM control siRNA or VASP specific siRNA for 48 h. Thereafter, the cells were maintained in EBM-2 medium supplemented with 0.2% FBS for 2 h, stimulated with thrombin for different time intervals, washed with ice-cold PBS and lysed in the lysis buffer (50 mM Tris pH 7.5, 0.5% Triton X-100, 100 mM NaCl, 2 mM MgCl₂, 10 mM EGTA and 10 μ L/mL protease inhibitor cocktail for mammalian cells). Insoluble material was removed by centrifugation at 15,000 $\times$ g at 4 $^{\circ}$ C for 10 min. Mouse lung lysates were prepared as follows. Lungs of wild-type (WT) and VASP^{-/-} mice were perfused with RPMI 1640 medium for 10 min followed by perfusion

with either RPMI 1640 medium or RPMI 1640 medium containing 6 μ M PAR-1 agonist for 30 min. Thereafter, the lungs were extracted and snap frozen in liquid nitrogen. The lungs were thawed on ice, homogenized in the lysis buffer described above using PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT), and centrifuged at 15,000 $\times$ g at 4 °C for 10 min. Cleared HUVEC and lung lysates were used for Rac1 assay.

The lysates were incubated with aliquots of PAK1-GST protein beads containing Rac1-binding domain of Rac1 effector PAK1 (Cytoskeleton Inc, Denver, CO) at 4 °C for 90 min. Subsequently, beads were separated from the lysates by centrifugation at 1,000 $\times$ g at 4 °C for 2 min, washed three times with the lysis buffer, and boiled in the Leammli sample buffer. The total lysates and samples eluted from the beads after boiling were subjected to 12% SDS-polyacrylamide gel electrophoresis and Rac1 protein was detected by immunoblotting with anti-Rac1 antibody. The densities of scanned protein bands were analyzed using ImageJ program. The amount of the Rac1 bound to the beads was normalized to the total amount of Rac1 in the cell lysates.

Mice studies

The detailed methodology for generation and characterization of the VASP $^{-/-}$ mice was described by Hauser et al. (Hauser et al., 1999). Mice were on mixed 129sv/C57BL background. The mice were backcrossed with F1 hybrids about every 8 generations. WT mice and VASP $^{-/-}$ mice were age-matched littermates obtained by heterozygous breeding. The colonies were maintained at the University of Illinois College of Medicine Research Building animal facility. The studies involving animal models were approved by the University of Illinois Institutional Animal Care and Use Committee.

Genotyping and immunoblotting of mouse tissue

For genotyping, about 10 mm of tail tips were digested in 500 μ L buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.1 g/L gelatin, 0.45% v/v Nonident P40, 0.45% v/v Tween 20, and 2.5 μ L Proteinase K (Invitrogen, Carlsbad, CA)) at 55 °C overnight, heated for 10 min at 95 °C, centrifuged for 5 min at 15,000 $\times$ g, and cleared extracts were used for PCR. Primers used for the detection of Neomycin Cassette were as follows: sense primer, 5'-CGA ATA GCC TCT CCA CCC AAG CGG CCG GAG AAC - 3'; antisense primer, 5'-GGC CAG CAG AAC AGT ATT GGA GAA CAT CCA GG - 3'. Primers used for the detection of VASP WT gene were as follows: sense primer, 5'-TTA GCT TGG TTT GGG GAC TGA ACC AGC CTC CTT TC - 3'; antisense primer, 5'-CAG CCA CTC CCT GGT ACT TCC TTA CCT TGC TCA C - 3'. PCR reaction was performed for 1 cycle of 5 min at 95 °C, followed by 33 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, and 1 cycle of 10 min at 72 °C. PCR products were analyzed by 1.5% agarose gel electrophoresis. The size of PCR products was ~650 bp and 450 bp for Neomycin Cassette and VASP WT gene, respectively.

To analyze VASP protein expression in mouse lungs, the lungs were perfused with RPMI 1640, and homogenized using PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT) in the homogenization buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM dithiothreitol (DTT), and 5 μ L/mL mammalian protease inhibitor cocktail. Lung extracts were centrifuged at 15,000 $\times$ g at 4 °C for 10 min and cleared lung extracts were subjected to SDS-PAGE and immunoblotting with anti-VASP and anti-Hsp90 antibodies.

Determination of pulmonary microvascular permeability to liquids

Mouse lung preparations were isolated and maintained as described previously (Gao et al., 2000; Gorovoy et al., 2007). Briefly, the mice were anesthetized by intraperitoneal injection

of ketamine (100 mg/kg) and xylazine (5 mg/kg), and ventilated through the tracheal cannula. The lungs were perfused with the RPMI 1640 medium through the pulmonary artery cannula at constant flow of 2 mL/min. Changes in the lung weight and pulmonary arterial pressure were monitored during the experiment. Data acquisition and storage was achieved using Notebook Pro for Windows software (Labtech, Andover, MA). The lungs were weighted immediately after the conclusion of the experiment and again after being dried for 3–4 days (until isogravimetric) at 60 °C.

Capillary filtration coefficient (K_{fc}) was determined as a measure of pulmonary microvascular permeability to liquids using similar procedures as described earlier (Gao et al., 2000; Gorovoy et al., 2007; Vogel et al., 2000). Isolated lung was perfused for 15 min followed by infusion of PAR-1 where indicated for 15 min. Subsequently, it was subjected to a step increase of 10 cm H₂O in venous pressure for 20 min. K_{fc} was determined from the slope of the recorded lung weight change normalized to the applied pressure change and the lung dry weight and expressed in units of mL $\times$ cm H₂O⁻¹ $\times$ min⁻¹ $\times$ g dry weight⁻¹.

Statistical analysis

The data for the two groups were analyzed using Student's t-test. The values are expressed as mean $\pm$ SD. Differences were considered statistically significant if $P < 0.05$.

Results

Downregulation of endogenous VASP delays the recovery of the endothelial barrier after thrombin stimulation in HUVECs

Our previous study suggested that VASP function may be regulated by thrombin-induced phosphorylation and translocation (Profirovic et al., 2005). In this study, we addressed the question of VASP involvement in thrombin signaling. Our goal was to investigate the role of VASP in thrombin receptor-mediated dysregulation of the endothelial barrier function.

We examined the effect of VASP deficiency on the cultured endothelial monolayer permeability using siRNA-mediated downregulation of endogenous VASP in HUVECs. We were able to reduce the level of VASP protein by up to 80% (Fig. 1A). For the experiments described below, the concentration of 30 nM was chosen as the lowest concentration that caused sufficient reduction of VASP level.

Transendothelial permeability of cell monolayers can be monitored by recording the changes in the TER using ECIS (Andreeva et al., 2005; Giaever and Keese, 1991; Tiruppathi et al., 1992). Downregulation of VASP had no effect on TER under the resting conditions over a period of 40 h (Fig. 1B). The baseline TER values were not significantly different in control and VASP siRNA-transfected cells ($12440 \pm 1126 \Omega$, $n=8$, and $10120 \pm 640 \Omega$, $n=8$, respectively). Thrombin stimulation of both control and VASP siRNA-transfected cells induced an initial decrease in TER indicating increased endothelial permeability followed by the recovery of the endothelial barrier function (Fig. 1C). In VASP-depleted cells the effect of thrombin was greatly potentiated. Fifteen min and 60 min after thrombin stimulation, the normalized resistance was significantly different in control and VASP-depleted cells (0.550 ± 0.023 and 0.414 ± 0.032 at 15 min and 0.815 ± 0.034 and 0.637 ± 0.073 at 60 min, respectively). These results indicated that the endothelial permeability-increasing effects of thrombin were more pronounced in VASP-depleted cells.

Because thrombin-mediated increase in endothelial permeability is mediated by PAR-1 (Vogel et al., 2000), we tested if similar effect may be observed after stimulation with specific PAR-1 peptide agonist (Fig. 2). Fifteen min after stimulation with 6 μ M PAR-1 peptide, we observed a significant difference in the normalized resistances between control

and VASP-depleted cells (0.760 ± 0.067 and 0.610 ± 0.065 , respectively, Fig. 2A). Significant difference in the normalized resistances between control and VASP-depleted cells was observed with $30 \mu\text{M}$ PAR-1 peptide stimulation (0.706 ± 0.025 and 0.537 ± 0.067 , respectively, Fig. 2B) as well. Sixty min after $6 \mu\text{M}$ PAR-1 peptide stimulation the normalized resistance was slightly, but not significantly greater in VASP-depleted cells (0.963 ± 0.103 and 1.060 ± 0.120 in control and VASP-depleted cells, respectively). At the same time point, after $30 \mu\text{M}$ PAR-1 peptide stimulation, it was significantly greater in control cells (0.831 ± 0.085 and 0.462 ± 0.069 in control and VASP-depleted cells, respectively). Thus, similarly to the effects observed with thrombin stimulation, VASP deficiency seems to potentiate increase in permeability induced by PAR-1 peptide.

Downregulation of endogenous VASP potentiates disruption of the interendothelial junctions and delays their recovery after thrombin stimulation in HUVECs

To examine the integrity of cell-cell contacts in the control cells and in the VASP-depleted cells after thrombin stimulation, we looked at the VE-cadherin localization using confocal microscopy. In non-stimulated cells, VE-cadherin formed a continuous border of the cells with no apparent intercellular gaps in both control and VASP siRNA-transfected cell monolayers (Fig. 3A). Fifteen min after thrombin stimulation, we observed pronounced adherens junction disruption with partial loss of VE-cadherin at the cell borders and wide intercellular gap formation (marked by arrows). These changes were partially reversed and the integrity of monolayers was improved 60 min after thrombin stimulation. As expected from the efficient VASP depletion (Fig. 1A), we were able to achieve high level of siRNA transfection efficiency as evidenced by red fluorescence of a marker of transfected cells, non-targeting siRNA siGLO (Fig. 3A, shown only for the cells stimulated with thrombin for 60 min). Fig. 3B depicts VE-cadherin staining of larger microscopic fields of control and VASP-depleted cells after thrombin stimulation. VASP-depleted cells showed more disturbance of the monolayer after thrombin stimulation and delayed recovery as revealed by quantitative analysis of the intercellular gaps. The gap surface area in VASP-depleted cells was significantly greater than that in control cells both 15 min ($12.69 \pm 2.10\%$ and $8.75 \pm 0.74\%$, respectively) and 60 min ($7.68 \pm 1.80\%$ and $3.67 \pm 1.49\%$, respectively) after thrombin stimulation (Fig. 3C).

To test if VE-cadherin cytoskeletal anchorage in response to thrombin stimulation is affected by VASP deficiency, we isolated Triton-soluble and Triton-insoluble membrane fractions. The latter contained cytoskeleton and cytoskeleton-associated proteins. Our data demonstrated reduced level of VE-cadherin in the Triton-insoluble fraction in VASP-depleted compared to control cells 60 min after thrombin stimulation (Fig. 4A). To quantify the results, we determined the ratio between the corresponding Triton-insoluble to Triton-soluble band densities and found that it was significantly different between VASP-depleted and control cells (0.48 ± 0.07 and 0.86 ± 0.08 , respectively) stimulated with thrombin (Fig. 4B).

Downregulation of endogenous VASP abrogates activation of Rac1 after thrombin stimulation in HUVECs

Rac1 activation was reduced in VASP-deficient MyEnd cells (Schlegel et al., 2008). It was shown that inhibition of Rac1 enhanced thrombin-induced permeability in ECs (Wojciak-Stothard et al., 2001). Therefore, we addressed the question if VASP-deficiency might affect Rac1 activation after thrombin stimulation.

The level of activated Rac1 was similar in control siRNA- and VASP siRNA-transfected cells at baseline conditions and 15 min after thrombin stimulation (Fig. 4C and 4D). It was decreased in both control and VASP-depleted cells (0.67 ± 0.08 and 0.52 ± 0.09 ,

respectively) 15 min after thrombin stimulation. Sixty min after thrombin stimulation, the level of Rac1 activation was increased in the control cells (1.40 ± 0.19), but decreased by more than 40% in VASP-deficient cells (0.58 ± 0.14) compared to non-stimulated cells. These data suggest that there is a correlation between Rac1 activation and recovery of thrombin-induced increase in endothelial permeability in VASP-deficient cells.

Increased lung microvascular permeability in VASP^{-/-} mice

To address the question if VASP is involved in thrombin receptor-mediated dysregulation of endothelial barrier function *in vivo*, we employed VASP^{-/-} mouse described earlier (Hauser et al., 1999).

The disruption of VASP gene was confirmed by PCR analysis using genomic DNA obtained from the mouse tails (Fig. 5A), and the absence of VASP protein in the total lung extracts was confirmed by immunoblotting analysis (Fig. 5B). The expression levels of Mena and EVL, the mammalian members of Ena/VASP family, were not affected by the absence of VASP (Hauser et al., 1999).

To determine microvessel liquid permeability in the lungs, we measured the capillary filtration coefficient (K_{fc}) expressed as $\text{mL} \times \text{min}^{-1} \times \text{cmH}_2\text{O} \times \text{g dry weigh}^{-1}$ (U) in the perfused mouse lungs isolated from the WT and VASP^{-/-} animals. Baseline K_{fc} values were not significantly different in the WT and VASP^{-/-} mice (0.0056 ± 0.0043 U and 0.0077 ± 0.0028 U, respectively). Because thrombin-mediated increase in lung microvascular permeability is mediated by PAR-1 (Vogel et al., 2000), we used specific PAR-1 peptide agonist. Addition of PAR-1 peptide to the lung-perfusing fluid induced a marked increase in the K_{fc} values in both WT (0.0145 ± 0.0025 U) and VASP^{-/-} (0.0351 ± 0.0062 U) mouse lungs (Fig. 5C). Notably, the increase in K_{fc} in VASP^{-/-} mouse lungs was ~2.5 times greater than that in WT mouse lungs, suggesting that endothelial barrier disruption after stimulation of thrombin receptor is much greater in the absence of VASP.

Activation of Rac1 is impaired in VASP^{-/-} mouse lungs

Since we demonstrated that Rac1 activation is abrogated in HUVECs transfected with VASP siRNA after thrombin stimulation, we next addressed the question if Rac1 activation was affected in the VASP^{-/-} mouse lungs after PAR1 peptide infusion. At the baseline level, Rac1 activity was similar in VASP^{-/-} and WT mouse lungs (Fig. 6). After PAR-1 peptide infusion, the level of Rac1 activation was significantly reduced in VASP^{-/-} mouse lungs (1.50 ± 0.11) compared to WT mouse lungs (2.07 ± 0.23). When compared to the baseline levels, Rac1 activity doubled in WT but increased only by ~50% in VASP^{-/-} mouse lungs after PAR-1 peptide infusion. Together, these results suggest that Rac1 activation in response to PAR-1 activation in VASP^{-/-} mouse lungs is considerably impaired.

Discussion

Using two independent models, cultured HUVECs and perfused isolated mouse lungs, we demonstrated that VASP deficiency leads to a more profound endothelial barrier disruption and delayed recovery after activation of thrombin receptor. These results suggest that VASP is likely a critical factor that protects the endothelial barrier from thrombin receptor-mediated dysregulation.

Our study showed that downregulation of VASP in HUVECs potentiates thrombin- and PAR-1-induced decrease in TER with no effect on baseline TER. These findings are consistent with previous reports showing that VASP may protect the endothelial barrier under the conditions of increased endothelial permeability such as exposure to H_2O_2

(Rentsendorj et al., 2008), hypoxia (Rosenberger et al., 2007) and LPS (Bogatcheva et al., 2009; Rentsendorj et al., 2008; Rosenberger et al., 2007). Downregulation of VASP using VASP siRNA transfection in human pulmonary artery ECs (HPAEC) exacerbated H₂O₂-induced decrease in TER (Rentsendorj et al., 2008) whereas in human lung microvascular ECs (HMLVEC) it potentiated LPS-induced decrease in TER (Bogatcheva et al., 2009). Thus, VASP appears to be a common downstream effector for oxidants and inflammatory mediators such as thrombin and LPS. Notably, downregulation of VASP did not affect TER under our experimental conditions in HUVECs baseline conditions. Similar results were obtained in HPAECs (Rentsendorj et al., 2008) at baseline conditions. However, downregulation of VASP decreased TER in HMLVECs (Bogatcheva et al., 2009). The discrepancy between these and our results may be caused by the differences between endothelial cells of different origin (HUVECs vs. HLMVECs).

We observed that VASP-deficient cells have more pronounced gaps at an early time point (15 min), as well as in the recovery phase (60 min) after thrombin stimulation, which was consistent with the results of TER measurement. Reduced VE-cadherin cytoskeletal anchorage may be related to impaired cell-cell junctions in VASP deficient cells. Similar conclusions were drawn for increased endothelial permeability in MyEnd VASP^{-/-} cells (Schlegel and Waschke, 2009a).

Consistent with our *in vitro* findings, experiments with the isolated perfused mouse lungs demonstrated increased lung vascular permeability in VASP^{-/-} mice compared to the WT mice after stimulation with the PAR-1 peptide. Other studies have also demonstrated essential role of VASP in the maintenance of endothelial barrier function in *in vivo* models. Triple knockout mice, lacking all three members of Ena/VASP family of proteins, exhibit discontinuity of small blood vessels and defects in endothelial cell-cell contacts, which suggests a crucial role of these proteins in the development of blood vessels (Furman et al., 2007). Recent works using VASP^{-/-} mice suggested a prominent role of VASP in endothelial barrier protection. VASP^{-/-} mice have an increased vascular permeability in response to bradykinin as assessed by Evans Blue dye extravasation in skin vascular leakage model (Benz et al., 2008). Another report showed that lung vascular permeability to Evans Blue dye and lung water content of VASP^{-/-} mice were similar to those of WT mice under the baseline conditions but greatly increased after inhalation of LPS or mechanical injury (Henes et al., 2009). Both of these studies provided the evidence for the vascular leakage of dye-labeled proteins (Benz et al., 2008; Henes et al., 2009). The latter study showed the change in the water content of the lungs in response to inflammatory stimuli as well (Henes et al., 2009). Consistent with these findings, our work demonstrated that baseline K_{fc} did not differ between WT and VASP^{-/-} animals, but was significantly increased in VASP^{-/-} mice in response to stimulation with PAR-1 agonist. Complex response to LPS inhalation was observed over a relatively long period of time (4 h) when full inflammatory response could develop (Henes et al., 2009). In contrast, we have focused on understanding the specific response to thrombin receptor stimulation and changes in permeability to water. For the first time, this study demonstrated an increased pulmonary vascular permeability to liquids in ventilated VASP^{-/-} mouse lungs compared to WT mouse lungs in response to thrombin receptor stimulation.

Under our experimental conditions, Rac1 activity was decreased in both control siRNA- and VASP siRNA-transfected cells 15 min after thrombin stimulation. It was increased in control siRNA-transfected cells but decreased in VASP siRNA-transfected cells 60 min after thrombin stimulation. Furthermore, we demonstrated that PAR-1 peptide infusion reduced Rac1 activity by ~25% in VASP^{-/-} mouse lungs when compared to that in WT animals, thus supporting our *in vitro* data. These findings provide possible mechanism of VASP-dependent barrier protection after thrombin receptor activation. Previous studies showed an

essential role of Rac1 in the maintenance of the endothelial barrier function. The precise control of Rac1 activity seems to be critical for the integrity of adherens and tight junctions, as expression of either dominant-negative or constitutively active Rac1 increases endothelial permeability (van Wetering et al., 2002; Wojciak-Stothard et al., 2001). The dynamics of changes in Rac1 activation after thrombin stimulation correlates well with the recovery of endothelial barrier function. Rac1 has been shown to be inhibited in HUVECs at the early time points after thrombin stimulation, but its activity returns to initial value 30 min after stimulation (Vouret-Craviari et al., 2002). Several lines of evidence indicate that VASP may be involved in the regulation of Rac1 activity. VASP deficiency leads to a reduced Rac1 activity in the cultured MyEnd cells, which can be rescued by transfection of VASP (Schlegel et al., 2008). On the other hand, overexpression of VASP induces Rac1 activation (Wojciak-Stothard et al., 2009). In line with these studies, our work established the correlation between VASP deficiency and the decreased Rac1 activity after thrombin receptor stimulation.

How is Rac1 activity regulated by VASP? From the results of our work and the other studies, it may be concluded that Rac1 is downstream from VASP in the signaling pathway that regulates cell-cell junctions. It has been suggested that VASP may interfere with Rac1 GTP hydrolysis activity as it can interact with p120RasGAP (Garcia Arguinzonis et al., 2002; Schlegel et al., 2008), or with GDP-GTP exchange by opposing the effects of Rac1 GTP exchange factor Trio (Liebl et al., 2000; Schlegel et al., 2008). Additionally, VASP was shown to co-immunoprecipitate with active Rac1 in MyEnd cells (Schlegel and Waschke, 2009b), suggesting their possible interaction or co-existence in the common protein complexes. We were not able to detect VASP in the immunoprecipitates of active Rac1 from HUVECs or co-immunoprecipitate VASP and constitutively active Rac1 overexpressed in human embryonic kidney 293 cells (data not shown), which may reflect cell type-specific signaling between VASP and Rac1. As VASP may act directly on the actin cytoskeleton and bind junctional proteins, it is possible that Rac1 regulation by VASP serves as a feedback loop that may tune the cell responses to different stimuli. Further studies should identify precise mechanisms of VASP-Rac1 signaling and other possible proteins involved.

Since thrombin potently disrupts the endothelial barrier and contributes to the development of inflammatory conditions, it is essential to understand the mechanisms underlying these effects in the ECs. For the first time, we provide the evidence that VASP protects the endothelial barrier function and enhances its recovery after thrombin-dependent dysregulation *in vitro* and *in vivo*. Particularly interesting is the finding that VASP deficit significantly enhances permeability of the intact lung microvasculature in response to activation of thrombin receptor. Our results also suggest possible mechanism for these effects, which involves regulation of adherens junctions and Rac1. The precise mechanisms involved in VASP-dependent Rac1 activation remain to be elucidated. We have previously shown that thrombin may induce prolonged phosphorylation of VASP in HUVECs (Profirovic et al., 2005). As it is now clear that VASP may be critical in thrombin-dependent increase in endothelial permeability and recovery of endothelial barrier, next challenge would be to investigate if VASP phosphorylation is essential for these effects. The results of this and other studies related to the role of VASP in endothelial barrier function under inflammatory conditions may be of interest for development of efficient pharmacological treatment of these conditions.

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Figure 1.

Downregulation of VASP in ECs potentiates thrombin-induced increase in endothelial permeability. A, VASP specific siRNA downregulates endogenous VASP level in HUVECs up to 80%. Confluent HUVECs, seeded on gelatin-coated 24-well plates, were transfected with control non-silencing or VASP-specific siRNA in the indicated concentrations for 48 h followed by Western blot analysis. B, Downregulation of VASP does not affect baseline EC permeability. HUVECs were seeded onto the gold electrodes and transfected with either control siRNA or VASP siRNA. Three hours after the transfection, the electrodes were connected to ECIS instrument and the resistance through the endothelial monolayer was recorded for 40 h. C, Downregulation of VASP potentiates an increase in permeability after thrombin stimulation. About 48 h after transfection with either control siRNA or VASP siRNA, HUVECs were serum-starved for 2 h, and connected to ECIS instrument for recording. At the indicated time point, the cells were stimulated with thrombin. The recorded values were normalized to the initial resistances at the corresponding electrodes. The tracings are representative of two experiments performed in triplicates. The normalized TER values, presented as means $\pm$ SD, are shown for the indicated time points after stimulation (*, $P<0.05$, $n=3$).

Figure 2.

Downregulation of VASP potentiates an increase in permeability after PAR-1 peptide stimulation. The cells treated as in Fig. 1C were stimulated with PAR-1 peptide in the concentration of 6 μ M or PAR-1 peptide (2A) or 30 μ M (2B). The tracings are representative of two experiments performed in triplicates. The normalized TER values, presented as means $\pm$ SD, are shown for the indicated time points after stimulation (*, $P<0.01$; *, $P<0.05$, $n=3$).

Figure 3.

Downregulation of endogenous VASP by VASP specific siRNA abrogates the sealing of intercellular gaps after thrombin stimulation in HUVECs. A, Confluent HUVECs, grown on gelatin-coated glass coverslips, were transfected with control siRNA or VASP siRNA together with the equal amount of non-targeting siGLO siRNA. Forty-eight hours after the transfection, the cells were serum-starved for 2 h, stimulated with thrombin for 15 or 60 min, fixed and stained with mouse anti-VE-cadherin antibody followed by anti-mouse Alexa fluor 488. Red color and green color represent siGLO fluorescence and VE-cadherin, respectively. Arrows are pointing to the intercellular gaps. Images were taken using confocal microscope with 63× water-immersion objective. Scale bars, 20 μ m. B, Larger microscopic fields of control siRNA- and VASP siRNA-transfected cells after stimulation with thrombin and VE-cadherin staining. Images were taken using confocal microscope with 20× objective. Scale bars, 50 μ m. C, Intercellular gap formation is potentiated in VASP-deficient cells after thrombin stimulation. Gap area was analyzed in five microscopic fields for each time point. It is expressed as mean $\pm$ SD percentage of the total area of each field (**, $P < 0.01$, $n = 5$). The experiment was repeated two times with the similar results.

Figure 4.

A and B, VASP deficiency reduces VE-cadherin cytoskeletal anchorage after thrombin stimulation. A, VE-cadherin level is reduced in the Triton-insoluble fraction of VASP-deficient cells compared to the control cells after thrombin stimulation. Western blot analysis of Triton-soluble and Triton-insoluble membrane fraction and total lysates of HUVECs transfected with control or VASP siRNA stimulated with thrombin. The experiment was repeated three times with similar results. B, Quantitative analysis of the protein bands is shown as mean $\pm$ SD of the ratio between VE-cadherin levels in Triton-insoluble and Triton-soluble fraction (**, $P < 0.01$, $n = 3$). C and D, Downregulation of endogenous VASP in HUVECs reduces activation of Rac1 after thrombin stimulation. C, The activity of Rac1 was determined in control siRNA- or VASP siRNA-transfected HUVECs after stimulation with thrombin as described in "Materials and Methods". The experiment was repeated three times with the similar results. D, Quantitative analysis of the protein band densities expressed as mean $\pm$ SD of the level of Rac1-GTP over total Rac1 normalized to control (**, $P < 0.01$; *, $P < 0.05$ compared to non-stimulated VASP siRNA-transfected cells; $n = 3$).

Figure 5.

Microvascular permeability is increased in VASP-deficient mouse lungs in response to treatment with PAR-1 agonist. A, Analysis of the genomic DNA of heterozygous, WT and VASP^{-/-} mice. Genomic DNA from the mouse tails was analyzed by PCR for the presence of the Neomycin selection cassette (~650 bp) and VASP DNA fragment (~450 bp). B, Analysis of VASP expression in the total lung extracts of WT and VASP^{-/-} mice. Total lung extracts obtained from the extracted and homogenized lungs were analyzed by electrophoresis on SDS-PAGE followed by immunoblotting with anti-VASP and anti-Hsp90 antibody. C, Pulmonary microvessel permeability expressed as K_{fc} value in the WT and VASP^{-/-} mice under the baseline conditions and after perfusion with PAR-1 peptide solution. There was no significant difference in K_{fc} values under the baseline conditions. After infusion of PAR-1 peptide, K_{fc} was significantly greater in VASP^{-/-} than in WT mouse (**, $P < 0.01$; ***, $P < 0.001$). K_{fc} values represent mean $\pm$ SD, n=5 lung preparations.

Figure 6. Activation of Rac1 is abrogated in VASP^{-/-} mouse lungs after PAR-1 peptide infusion. A, Total lung extracts were prepared and subjected to the Rac1 pull-down assay. The experiment was performed three times and the representative result is shown. B, Quantitative analysis of the protein band densities expressed as mean ± SD of the level of Rac1-GTP over total Rac1 normalized to control (*, $P < 0.05$, $n=3$).