SH2 Domain-Containing Protein Tyrosine Phosphatase 2 and Focal Adhesion Kinase Protein Interactions Regulate Pulmonary Endothelium Barrier Function

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Abstract

Enhanced protein tyrosine phosphorylation is associated with changes in vascular permeability through formation and dissolution of adherens junctions and regulation of stress fiber formation. Inhibition of the protein tyrosine phosphatase SH2 domain-containing protein tyrosine phosphatase 2 (SHP2) increases tyrosine phosphorylation of vascular endothelial cadherin and β -catenin, resulting in disruption of the endothelial monolayer and edema formation in the pulmonary endothelium. Vascular permeability is a hallmark of acute lung injury (ALI); thus, enhanced SHP2 activity offers potential therapeutic value for the pulmonary vasculature in diseases such as ALI, but this has not been characterized. To assess whether SHP2 activity mediates protection against edema in the endothelium, we assessed the effect of molecular activation of SHP2 on lung endothelial barrier function in response to the edemagenic agents LPS and thrombin. Both LPS and thrombin reduced SHP2 activity, correlated with decreased focal adhesion kinase (FAK) phosphorylation (Y³⁹⁷ and Y⁹²⁵) and diminished SHP2 protein–protein associations with FAK. Overexpression of constitutively active SHP2 (SHP2^{D61A}) enhanced baseline endothelial monolayer resistance and completely blocked LPS- and thrombin-induced permeability *in vitro* and significantly blunted pulmonary edema formation induced by either endotoxin

(LPS) or *Pseudomonas aeruginosa* exposure *in vivo*. Chemical inhibition of FAK decreased SHP2 protein–protein interactions with FAK concomitant with increased permeability; however, overexpression of SHP2^{D61A} rescued the endothelium and maintained FAK activity and FAK–SHP2 protein interactions. Our data suggest that SHP2 activation offers the pulmonary endothelium protection against barrier permeability mediators downstream of the FAK signaling pathway. We postulate that further studies into the promotion of SHP2 activation in the pulmonary endothelium may offer a therapeutic approach for patients suffering from ALI.

Keywords: endothelium; SH2 domain-containing protein tyrosine phosphatase 2; pulmonary edema; acute lung injury; focal adhesion kinase

Clinical Relevance

This work studies the mechanism through which SHP2 activation protects the endothelial barrier against injury. In settings of acute lung injury, activation of SHP2 may offer a novel therapeutic approach to treat pulmonary edema.

Acute respiratory distress syndrome (ARDS) is a complex syndrome associated with severe arterial hypoxemia. Onset of

ARDS results from several predisposing factors, such as pneumonia, pancreatitis, sepsis, and trauma. At present, patients

suffering from ARDS are treated with mechanical ventilation; however, there are insufficient treatment options available, and

(Received in original form November 14, 2013; accepted in final form October 7, 2014)

This work was supported by Providence Veterans Affairs Medical Center and National Institutes of Health (NIH), National Heart, Lung, and Blood Institute (NHLBI) grant R01 HL-67795, American Heart Association grant 10GRNT4160055, COBRE National Institute of General Medical Sciences award 5P20 GM103652, the University Medicine Foundation of the Rhode Island Hospital (E.O.H.), American Heart Association Fellowship award 13POST16860031 (H.C.), and by the Leadership Alliance and the NIH NHLBI grant R25 HL088992.

Author Contributions: acquisition of data—H.C., J.B., and H.D.; conception and design—H.C. and E.O.H.; analysis and interpretation: H.C. and E.O.H.; drafting the manuscript for important intellectual content—H.C. and E.O.H.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 52, Iss 6, pp 695–707, Jun 2015

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Originally Published in Press as DOI: 10.1165/rcmb.2013-0489OC on October 15, 2014

Internet address: www.atsjournals.org

the mortality rate remains at roughly 40% (1, 2). Vascular barrier dysfunction is one cause of pulmonary edema formation observed in settings of acute lung injury (ALI). The integrity of the vascular barrier is disrupted after the breakdown of endothelial intercellular junctions, which occurs after dissolution of the adherens junction (AJ) and tight junction protein complexes, as well as altered contractile forces and centripetal tensions (3, 4). Thus, understanding signaling mechanisms that regulate the endothelial monolayer may provide therapeutic targets for treatment of ALI.

SHP2 domain-containing protein tyrosine phosphatase 2 (SHP2) is a critical mediator in the maintenance of endothelial barrier function. Chemical or molecular inhibition of SHP2 promoted barrier disruption in both cultured microvascular endothelial monolayers and in the intact lung (5). SHP2 associates with vascular endothelial (VE)-cadherin via β -catenin, and regulates VE-cadherin levels at the AJ by maintaining β -catenin in a dephosphorylated state (6). After thrombin-induced loss of SHP2 from the AJ, tyrosine phosphorylation of VE-cadherin, β -catenin, γ -catenin, and p120-catenin is increased, thus altering the protein complex association with α -catenin, leading to AJ disassembly (7). Using substrate trapping studies, Hartman and colleagues (8) identified focal adhesion kinase (FAK) as a substrate of SHP2, with FAK tyrosine 397 (FAK^{Y397}) phosphorylation being a key event for the protein–protein interaction. In cardiomyocytes and breast carcinoma cells, SHP2 negatively regulates FAK, maintaining FAK in a dephosphorylated, inactive state (8, 9). The activity of FAK is important in regulation of endothelial barrier function through centripetal tensions, both at focal adhesion complexes (via interactions with the cytoplasmic tail of β -integrin) and through the stabilization of AJ protein complexes (10, 11). The latter is likely mediated through the interactions of key AJ component p120-catenin and/or by modulating the activity of p190RhoGAP through tyrosine phosphorylation (11, 12). Thus, the FAK–SHP2 protein–protein associations may represent a key mechanism regulating endothelial barrier function.

Tyrosine phosphorylation of FAK has been observed at six tyrosine (Y) residues (Y³⁹⁷, Y⁴⁰⁷, Y⁵⁷⁶, Y⁵⁷⁷, Y⁸⁶¹, and Y⁹²⁵), with the phosphorylation patterns at these sites differing in response to varying stimuli

(13). FAK^{Y397} phosphorylation, an autophosphorylation site of FAK, creates a high-affinity binding site for the SH2 domain of Src, an ubiquitously expressed proto-oncogene—interactions critical to downstream signaling events (14). The formation of a transient FAK–Src dual kinase signaling complex results in the phosphorylation of FAK at Y⁵⁷⁶, Y⁵⁷⁷, and Y⁹²⁵ residues, promoting maximal FAK activation (15, 16). FAK autophosphorylation occurs immediately after integrin clustering and associates with different signaling proteins, including Grb2-Sos, p130^{cas}, and paxillin (17, 18), leading to the stabilization of the focal adhesion complexes and enhancing the integrin–cytoskeletal interactions (19). FAK activation has been implicated as both a positive and negative regulator of endothelial barrier function (20). However, the relationship between SHP2 and FAK protein–protein interactions and activities, and the resulting influence on endothelial barrier function, are not well known.

Although SHP2 activity has been shown to influence barrier integrity *in vitro* and *in vivo* through regulating tyrosine phosphorylation of key AJ complex proteins (5), the mechanism through which SHP2 regulates FAK to maintain endothelial barrier function has not been described. In the present study, we used the edemagenic agents, LPS and thrombin, and *Pseudomonas aeruginosa* (strain PA103), and the barrier-enhancing mediator, sphingosine-1-phosphate (S1P), to investigate the link between SHP2, FAK, and barrier function within the pulmonary vasculature. We show that barrier-disrupting agents diminished the activities and protein–protein interactions of FAK and SHP2, whereas barrier-enhancing agents did the opposite. We further show that constitutive activation of SHP2 blocked LPS- or thrombin-induced barrier dysfunction *in vitro* and *in vivo* and blunted *P. aeruginosa*-induced ALI *in vivo*. Finally, constitutively active SHP2 protected against FAK inactivation or disruption of FAK–SHP2 protein–protein interactions by the chemical inhibitor of FAK, PF-573228. Thus, this work suggests that SHP2 activation protects against pulmonary edema formation through the maintenance of cellular centripetal tensions through interactions with FAK.

Materials and Methods

Cell Lines and Reagents

Rat lung microvascular endothelial cells (LMVECs; Vec Technologies, Rensselaer, NY) as previously described (21).

LPS (endotoxin) from *Escherichia coli* serotype 055:B5 was obtained from Enzo Life Sciences (Plymouth Meeting, PA); S1P was purchased from Cayman Chemicals (Ann Arbor, MI); 8-Hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877) was purchased from Calbiochem (San Diego, CA); and thrombin (from human plasma) and FAK inhibitor PF-573228 were purchased from Sigma (St. Louis, MO). Polyjet was obtained from SignaGen (Gaithersburg, MD).

The vectors encoding activated SHP2 (PJ3-SHP2^{D61A}) and non-kinase C-terminal (0–2186 amino acid region) of FAK (green fluorescent protein [GFP]-FAK-related nonkinase) were purchased from Addgene (Cambridge, MA), and phosphorylated GFP (pGFP-C1) was obtained from Clontech (Mountain View, CA). Antibodies directed against phospho-SHP2 (Y⁵⁸⁰ and Y⁵⁴²) and phospho-FAK (Y^{576/577} and Y⁹²⁵) were obtained from Cell Signaling (Beverly, MA). Phospho-FAK (Y³⁹⁷) and FAK (immunogen at amino acids 354–533) antibodies were obtained from Invitrogen (Carlsbad, CA) and BD transduction (San Jose, CA), respectively. Rabbit IgG, VE-cadherin, and SHP2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). *P. aeruginosa* strain PA103 bacteria was a kind gift from Dr. Troy Stevens (University of South Alabama, Mobile, AL).

Liposome Preparation

For production of liposomes, dimethyldioctadecyl-ammonium bromide and cholesterol were mixed in chloroform, as described elsewhere (22, 23). Liposomes dissolved in 5% glucose solution were combined with GFP or SHP2^{D61A} complementary DNA (cDNA; 50 μ g) and injected into the retrobulbar sinus of the orbit of anesthetized C57 BL/6 mice. Confirmation of cDNA overexpression within the pulmonary vasculature was assessed by endothelial cell isolation and immunoblot analysis of GFP, SHP2, and VE-cadherin, as previously described (24). Transfection

efficiency was assessed by densitometry of SHP2.

In Vivo Models of ALI

LPS was administered to adult C57 BL/6 mice via single intraperitoneal injection (5 mg/kg). *P. aeruginosa* strain PA103 was grown on solid tryptic soy agar and resuspended in sterile PBS to a concentration of 1×10^8 CFU/ml. Under light isoflurane anesthesia, mice were administered 100 μ l (containing 10^7 CFU of *P. aeruginosa*) intratracheally. At 4 hours (*P. aeruginosa*) and 24 hours (LPS) after administration, mice were given a lethal dose of anesthesia. Time course studies demonstrated 4-hour *P. aeruginosa* exposure to cause a significant induction of pulmonary edema. Longer exposure times (> 4 h) resulted in animal discomfort and mortality; thus, this was avoided by using the earlier time points.

Bronchoalveolar lavage (BAL) was performed and BAL cell counts and protein concentrations were determined as previously described (25). In additional experiments, at 24 hours after intraperitoneal injection of LPS or vehicle into mice, lung permeability was determined by *ex vivo* perfusion of lungs, as previously described (24, 25). Representative plots for lung filtration coefficients experiments are included in the online supplement.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committees of the Providence Veterans Affairs Medical Center and Brown University, and comply with the Health Research Extension Act and U.S. Public Health Service policy.

Endothelial Monolayer Permeability

Changes in endothelial monolayer permeability of transiently transfected LMVECs were assessed using the electrical cell impedance sensor technique (Applied Biophysics, Troy, NY), as previously described (24, 25). Transfection efficiency was assessed by densitometry of phosphorylated SHP2 (Y⁵⁴² and Y⁵⁸⁰) and total SHP2 or GFP.

Immunoprecipitations and Immunoblot Analyses

Confluent LMVECs were pretreated with NSC-87877 or vehicle (H₂O) for 3 hours followed by treatment with S1P (1 μ M,

30 min), thrombin (1 U/ml, 15 min), LPS (1 μ g/ml, 2 h), or PF-573228 (0.1 μ M, 3 h). Immunoprecipitations and immunoblot analyses were performed as previously described (24), using IgG and nonimmunoprecipitated whole-cell lysate as controls for immunoprecipitations. Immunoprecipitation data are presented as ratios of FAK relative to precipitated SHP2.

Statistical Analysis

Experimental number (*n*) is presented for each experiment. For three or more groups, variance was assessed using Bartlett's test, with data sets not reaching significance studied by Kruskal-Wallis test followed by Dunn's test. For all other data sets, differences among the means were tested for significance by ANOVA with Tukey's range significance difference test. For two groups, variance in data sets was assessed using the Mann-Whitney test followed by *t* test. Data are presented as means ($\pm$ SD). Significance was reached at a *P* value less than 0.05.

Results

Endothelial Edemagenic Agents Modulate SHP2 Activation

We have previously shown an increase in basal lung endothelial permeability upon either chemical or molecular inhibition of SHP2 (5). In the present study, we further investigated if activation of SHP2 could serve a protective role in the pulmonary circulation. Despite a previously reported median effective concentration (EC₅₀) of 0.32 μ M, the chemical inhibitor of SHP2, NSC-87877, was used at 100 μ M in the present study due to significant increases in interendothelial cell gapping previously observed by our group (5). We first assayed the activity levels of SHP2 by measuring the phosphorylation state of key tyrosine residues, tyrosine 542 (Y⁵⁴²) and tyrosine 580 (Y⁵⁸⁰), which reside within the C terminus and have been shown to correlate with PTPase enzymatic activity (26). To investigate the link between SHP2 activation and barrier integrity, we assessed the effect of the permeability agents, LPS and thrombin, and the barrier protective agent, S1P, on SHP2 phosphorylation at Y⁵⁴² and Y⁵⁸⁰. We noted a significant decrease in the levels of SHP2 Y⁵⁴² and Y⁵⁸⁰

phosphorylation after 15-minute exposure to thrombin or 2-hour exposure to LPS (Figures 1A and 1B). Conversely, S1P promoted an increase in SHP2 Y⁵⁴² and Y⁵⁸⁰ phosphorylation, although only Y⁵⁸⁰ phosphorylation reached significance (Figures 1C and 1D). Interestingly, exposure of the endothelial cells with SHP2 chemical inhibitor, NSC-87877, attenuated the level of SHP2 Y⁵⁸⁰ phosphorylation; however, S1P treatment ablated this effect (Figure 1D). These data suggest that SHP2 activity may correlate with the state of the barrier function within the pulmonary endothelium.

Constitutively Active SHP2 Protects Against the Effect of Pulmonary Endothelial Barrier Modulating Agents *In Vitro* and *In Vivo*

We next studied the effect of constitutively activated SHP2 on endothelial barrier function in settings of LPS, thrombin, or S1P exposure *in vitro* by overexpressing constitutively activated SHP2 (SHP2^{D61A}) in LMVECs. In parallel, LMVECs were transfected with cDNA encoding GFP, as a negative control. Overexpression of both SHP2 and GFP proteins was confirmed using Western blotting with antibodies specific to phosphorylated SHP2 (Y⁵⁴² and Y⁵⁸⁰), total SHP2, and GFP (Figure 2A, *inset*). Transfection efficiency, measured as percentage increase in SHP2 protein expression in SHP2^{D61A} overexpressing LMVECs relative to endogenous SHP2 protein expression in cells overexpressing GFP, was 84 ($\pm$ 11)% (*n* = 4; *P* < 0.05; Figure 2A, *inset*). As expected, SHP2 activity, measured by tyrosine phosphorylation, was significantly increased in LMVECs overexpressing SHP2^{D61A} compared with GFP-overexpressing cells at residues of Y⁵⁴² ($262 \pm 87.5\%$; *n* = 4; *P* < 0.05) and Y⁵⁸⁰ ($128.3 \pm 7.7\%$; *n* = 4; *P* < 0.05) (Figure 2A, *inset*). Under baseline conditions, SHP2^{D61A} significantly elevated endothelial monolayer resistance by $137.9 (\pm 22.7) \Omega$ (*P* $\leq$ 0.05; *n* = 4–7) relative to GFP overexpressing endothelial cells (Figure 2A). In keeping with the noted effect of SHP2 activity on endothelial barrier function, we observed that transient overexpression of SHP2^{D61A} blocked endothelial permeability induced by SHP2 inhibitor, NSC-87877

Figure 1. Endothelial edemagenic agents modulate SH2 domain-containing protein tyrosine phosphatase 2 (SHP2) activation. (A and B) Lung microvascular endothelial cells (LMVECs) were treated with LPS (1 μ g/ml, 2 h) or thrombin (1 U/ml, 15 min) or vehicle (PBS) for 2 hours ($n = 3$). (C and D) LMVECs were pretreated with SHP2 inhibitor, 8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877 [NSC]; 100 μ M), or vehicle (H_2O) for 3 hours, followed by treatment with sphingosine-1-phosphate (S1P; 1 μ M) or vehicle (methanol) for 30 minutes ($n = 6$). SHP2 activity was assayed by subjecting the lysates to immunoblot analysis and probing with antibodies directed against phosphorylated Y⁵⁴² (A and C) and Y⁵⁸⁰ (B and D) residues. Blots were stripped and reprobed for SHP2. Data are presented as a ratio of phosphorylated SHP2 to total SHP2, and as the mean ($\pm$ SD). * $P < 0.05$, † $P < 0.05$, versus vehicle for respective treatments. IB, immunoblot; Thr, thrombin; Veh, vehicle.

(Figure 2B). Next, we noted that transient overexpression of SHP2^{D61A} completely blocked LPS-induced increases in endothelial permeability as compared with cells transiently overexpressing cDNA encoding GFP (Figure 2C). Similarly, LMVECs overexpressing SHP2^{D61A} protein displayed no thrombin-induced increase in permeability compared with GFP-overexpressing cells (Figure 2D). In contrast, although S1P enhanced the barrier function of GFP-overexpressing LMVECs relative to vehicle, this was not noted in SHP2^{D61A}-overexpressing cells (Figure 2E). Increased permeability, induced by NSC-87877, was blunted after treatment with S1P (Figure 2F). Thus, SHP2 activity is alternatively modulated in settings of barrier dysfunction versus barrier enhancement.

We next sought to assess whether SHP2^{D61A}-mediated protection against endothelial barrier disruption *in vitro* is also observed in the pulmonary circulation of the intact lung. Mice were administered cationic liposomes containing GFP or SHP2^{D61A} cDNA, via retro-orbital injection, and, after 24 hours, LPS was administered. After a further 24 hours, lung permeability in the *ex vivo*, isolated, perfused lung was assessed, as previously described (24, 25). Transient overexpression of cDNA in the pulmonary vasculature was confirmed via immunological isolation of endothelial cells from the lung, and the homogenates were immunoblotted for SHP2 or GFP; the isolation of endothelial cells was confirmed via immunoblot analysis for VE-cadherin (Figure 3A, *inset*). Isolated endothelial cells from lungs of mice overexpressing SHP2^{D61A} cDNA exhibit a significant increase ($95.5 \pm 7\%$;

$n = 5$; $P < 0.05$) in SHP2 protein expression compared with those after overexpression of GFP cDNA (Figure 3A, *inset*). As previously shown (24, 25), LPS exposure increased the filtration coefficient in GFP-overexpressing mice, correlating with lung edema formation. However, mice overexpressing SHP2^{D61A} in the pulmonary vasculature demonstrated no edema formation in response to LPS (Figure 3A). Similar results were noted using the bacteria, *P. aeruginosa* (PA103), as a model for ALI (27, 28). For these studies, 44 hours after transfection of the SHP2^{D61A}, PA103 was administered intratracheally. At 4 hours after intratracheal injection, lung edema formation was measured. In GFP-overexpressing mice, we noted lung edema formation, as assessed by the increased wet-to-dry lung weight ratio, relative to mice exposed to vehicle. However, SHP2^{D61A}

Figure 2. Constitutively active SHP2 blocks the effect of pulmonary endothelial barrier-modulating agents *in vitro*. Equal numbers of LMVECs were transfected with eukaryotic vectors encoding constitutively active SHP2 (SHP2^{D61A}) or green fluorescent protein (GFP) cDNA. (A) The resistance across the monolayers was measured at 48 hours after transfection. SHP2 and GFP overexpression and SHP2 phosphorylation (Y⁵⁴² and Y⁵⁸⁰) were confirmed for each experiment by immunoblot analysis of the lysates of transiently transfected LMVECs (inset). Representative images are shown ($n = 4$). Electric cell impedance system data are presented as means ($\pm$ SD) ($n = 7$). $*P < 0.05$ versus GFP. (B–E) Monolayer resistance was assessed in LMVECs overexpressing SHP2^{D61A} (squares) or GFP (diamonds) in the presence (open symbols) and absence (closed symbols) of NSC-87877 (100 μ M; $n = 4$), LPS (100 ng/ml; $n = 11$), thrombin (1 U/ml; $n = 4$), or S1P (1 μ M; $n = 6$). Arrow indicates point of addition of modulating agents. Data are presented as means ($\pm$ SD). $*P < 0.05$ versus GFP treated with vehicle. (F) Monolayer resistance was assessed in LMVECs in the presence (open symbols) and absence (closed symbols) of NSC-87877 (100 μ M), followed by treatment with S1P (1 μ M; square symbols) or vehicle (circle symbols; $n = 4$). Arrows indicate points of addition of modulating agents. Dashed lines in D and E indicate treated samples (thrombin in D, S1P in E). Data are presented as means ($\pm$ SD). $*P < 0.05$ versus vehicle.

overexpression attenuated PA103-induced increase in lung wet-to-dry weight ratio (Figure 3B). Parallel measurements demonstrated that, in mice overexpressing

GFP, the protein content and number of cells infiltrated in the BAL fluid was significantly elevated after PA103 exposure (Figures 3C and 3D). Similar to the lung

filtration coefficients measurements, SHP2^{D61A}-overexpressing mice had significantly lower levels of BAL protein in PA103-exposed mice compared with

Figure 3. Constitutively active SHP2 blocks bacterial endotoxin (LPS)-induced barrier disruption and attenuates bacteria-induced barrier disruption. Mice were injected with liposomes encapsulating cDNA encoding SHP2^{D61A} or GFP. (A) At 24 hours after liposome injection, mice received an intraperitoneal injection of LPS (5 mg/kg). After an additional 24 hours, lung edema was measured by determining lung permeability in *ex vivo* isolated, perfused lungs. *Inset*: overexpression of SHP2 and GFP in the lung endothelium was confirmed by immunological isolation of endothelial cells. The lysates from isolated lung endothelial cells were immunoblotted for SHP2 and GFP. The membranes were stripped and reprobed for vascular endothelial-cadherin expression to confirm that the harvested lung cells were endothelial cells. Level of SHP2 protein expression (in arbitrary units) is indicated. Data are presented as means ($\pm$ SD). GFP, $n = 6$; SHP2^{D61A}, $n = 7$. * $P < 0.05$ versus GFP, vehicle. (B–D) At 44 hours after liposome injection, mice were administered *P. aeruginosa* strain PA103 (10^7 CFU) via intratracheal injection. After an additional 4 hours, lungs were harvested for wet-to-dry weight measurements (B), or bronchoalveolar lavage (BAL) fluid was collected and analyzed for protein concentration (C) and cell infiltration (D). Data are presented as means ($\pm$ SD). (B) $n = 6$; (C and D) $n = 4$. * $P < 0.05$ PA103-treated versus vehicle-treated mouse lungs; $^\dagger P < 0.05$ versus GFP and PA103. k_f , lung filtration coefficient.

GFP-overexpressing mice exposed to PA103 (Figures 3C). In addition, after PA103 exposure, overexpression of SHP2^{D61A} in mice displayed no increase in cell number in the BAL compared with GFP-overexpressing mice (Figure 3D). Thus, the data strongly suggest that the enzymatic activity of SHP2 is a key component in protection of the pulmonary vasculature against barrier dysfunction and edema formation both *in vitro* and *in vivo*.

SHP2 Activity Increases FAK Phosphorylation and FAK–SHP2 Association

FAK is a tyrosine kinase known to be important in regulating endothelial barrier function through action at both the focal adhesion complex and AJ complexes

(29). SHP2 has been implicated in the regulation of FAK phosphorylation, and thus activation (9, 30). Thrombin, LPS, and S1P have all been previously observed to differentially alter FAK phosphorylation status (31, 32). Thus, we next assessed whether FAK plays a role in the mechanism through which SHP2 exerts protection of endothelial barrier function. We assessed the phosphorylation status of FAK at the autophosphorylation residue, Y³⁹⁷, within the activation loop at residue Y^{576/577}, and at Y⁹²⁵, a residue located within the focal adhesion-targeting (FAT) domain and strongly associated with intrinsic FAK activity. In response to both LPS and thrombin exposure, we noted significant decreases in FAK phosphorylation at Y³⁹⁷ (Figure 4A) and Y⁹²⁵ (Figure 4C);

however, FAK phosphorylation at residue Y^{576/577} was unchanged (Figure 4B). Phosphorylation of FAK at Y³⁹⁷ and Y⁹²⁵ was increased by S1P treatment (Figures 4D and 4F), although only increases in FAK Y³⁹⁷ phosphorylation reached significance. Pretreatment with NSC-87877 attenuated S1P-induced phosphorylation at Y³⁹⁷ and Y⁹²⁵ (Figures 4D and 4F). Interestingly, similar to the effects of thrombin and LPS, SHP2 inhibition by NSC-87877 treatment caused significantly decreased phosphorylation of FAK at Y³⁹⁷ and Y⁹²⁵ (Figures 4D and 4F), but exerted no effect on FAK phosphorylation at Y^{576/577} (Figure 4E).

FAK phosphorylation at Y³⁹⁷ is essential for FAK–SHP2 association (8). Thus, we next studied whether the

Figure 4. SHP2 activity increases focal adhesion kinase (FAK) phosphorylation on select residues. (A–C) LMVECs were treated with LPS (1 μ g/ml, 2 h) or thrombin (1 U/ml, 15 min). (D–F) LMVECs were pretreated with SHP2 inhibitor, NSC-87877 (100 μ M), or vehicle (H_2O) for 3 hours, followed by treatment with S1P (1 μ M, 30 min) or methanol vehicle. FAK phosphorylation status was assayed by subjecting the lysates to immunoblot analysis, probing with antibodies directed against phosphorylated Y³⁹⁷ (A and D), Y⁵⁷⁶ (B and E), and Y⁹²⁵ (C and F) residues. Membranes were then stripped and re probed for total FAK protein. Data are presented as a ratio of phosphorylated FAK to total FAK, mean ($\pm$ SD). (A–C) $n = 4$; (D–F) $n = 5$. Insets are representative immunoblots for each experiment. * $P < 0.05$ treatment versus respective vehicle; † $P < 0.05$ S1P versus vehicle.

alterations in FAK phosphorylation profile, by exposure to LPS, thrombin, and S1P, regulate the direct protein–protein association between SHP2 and FAK. We

immunoprecipitated SHP2 from LMVEC lysates treated with LPS or thrombin. We observed a significant decrease in SHP2 association to FAK after treatment with

both LPS and thrombin (Figure 5A), similar to the effect of SHP2 chemical inhibition (Figure 5B). In parallel studies, we immunoprecipitated SHP2 from

Figure 5. SHP2 activity increases FAK-SHP2 association. (A) LMVECs were treated with LPS (1 μ g/ml, 2 h), thrombin (1 U/ml, 15 min), or vehicle (PBS). (B) LMVECs were pretreated with NSC-87877 (100 μ M) or vehicle (H_2O) for 3 hours, followed by treatment with S1P (1 μ M) or vehicle (methanol) for 30 minutes. Equivalent amounts of lysate were immunoprecipitated for SHP2 using an antibody derived from rabbit or rabbit IgG antibody. In parallel, nonimmunoprecipitated lysate (whole-cell lysate) was resolved by SDS-PAGE and immunoblotted with an FAK antibody derived from mice. Membranes were then stripped and reprobed with a SHP2 antibody, and FAK-SHP2 association was determined as the ratio of FAK bound to total immunoprecipitated SHP2. *Inset:* representative immunoblot. Data are presented as means ($\pm$ SD). (A) $n = 4$; (B) $n = 5$. * $P < 0.05$ versus respective vehicle; † $P < 0.05$ S1P versus vehicle. IP, intraperitoneal.

LMVEC lysates treated with S1P in the presence and absence of SHP2 inhibitor, NSC-87877. S1P significantly elevated FAK-SHP2 binding, an effect that was partially reduced ($42.5 \pm 8.9\%$) by SHP2 inhibition (Figure 5B). Taken together, these studies imply that FAK dephosphorylation mediated by SHP2 inhibition, and the resulting changes in FAK-SHP2 binding, may be responsible

for the alterations in endothelial barrier permeability.

Effect of FAK Inhibition on Pulmonary Endothelium Is Attenuated by Constitutive Activation of SHP2

Finally, we sought to understand the effect of FAK inhibition on SHP2 activity and endothelial barrier function. Treatment of LMVECs with a FAK chemical inhibitor,

PF-573228, showed a diminished level of FAK Y³⁹⁷ phosphorylation, thus decreased FAK activity (Figure 6A). Overexpression of the catalytically deficient FAK-related nonkinase in LMVECs resulted in increased endothelial monolayer permeability under baseline conditions, similar to the level of decreased resistance observed after PF-573228 treatment in GFP-overexpressing cells (Figure 6B). In addition to dephosphorylation of FAK Y³⁹⁷ and increased monolayer permeability, we observed a significant decrease in FAK-SHP2 binding in LMVECs treated with PF-573228 (Figure 6C). Interestingly, overexpression of SHP2^{D61A} abrogated PF-573228-induced inhibition of FAK phosphorylation (Figure 6A) and attenuated endothelial barrier dysfunction (Figure 6E). We further noted that endothelial cells overexpressing SHP2^{D61A} maintained FAK-SHP2 protein-protein interactions in settings of PF-573228 exposure (Figure 6D). Thus, SHP2 activity can override FAK dephosphorylation and inactivation and preserve coassociation, resulting in maintenance of endothelial barrier function.

Taken together, the data presented here indicate that S1P, LPS, and thrombin modulate the pulmonary vasculature through a FAK-SHP2-dependent pathway. We demonstrate a key role for SHP2 activation on FAK activity, FAK-SHP2 protein associations, and in the regulation of endothelial barrier function. Thus, we propose that the activation of SHP2 represents an interesting therapeutic target in the treatment of ALI.

Discussion

In the current study, we demonstrate that SHP2 activation offers significant protection against barrier disruption through activation of FAK and the maintenance of protein interactions with FAK. We further implicate the effect of the barrier-enhancing agent, S1P, and edemagenic agents, LPS and thrombin, on endothelial barrier function to be mediated via FAK-SHP2-dependent mechanisms. We note that barrier-enhancing agents promoted the tyrosine phosphorylation and activation of FAK and SHP2 and FAK-SHP2 protein-protein interactions. Conversely, LPS and thrombin exposure caused diminished tyrosine phosphorylation of each protein, inhibition

Figure 6. FAK regulates endothelial barrier function through SHP2-mediated mechanism. (A, B, D, and E) Equal numbers of LMVECs were transfected with eukaryotic vectors encoding catalytically active SHP2 (SHP2^{D61A}), GFP-FAK-related nonkinase (FRNK), or GFP cDNAs. (A) At 48 hours, LMVECs were treated with FAK inhibitor, PF-573228 (0.1 μ M), or vehicle (H₂O) for 3 h. FAK phosphorylation status was assayed by subjecting the lysates to immunoblot analysis, probing with antibodies directed against phosphorylated Y³⁹⁷ residue. Membranes were then stripped and reprobed for total FAK protein. Representative blot is shown ($n = 4$). (B) Representative plot of changes in monolayer resistance were measured in LMVECs overexpressing FRNK (square symbols) or GFP (diamond symbols) cDNA, in the presence (open symbols) and absence (closed symbols) of PF-573228 (0.1 μ M). Arrow indicates point of addition ($n = 3$). FRNK protein overexpression was confirmed by immunoblot analysis of the lysates of transiently transfected LMVECs with an antibody raised against FAK (inset). Representative images are shown ($n = 3$). (C) LMVECs were treated with FAK inhibitor, PF-573228, or vehicle (H₂O) for 3 h. Equivalent amounts of lysate were immunoprecipitated for SHP2 using an antibody derived from rabbits. Immunocomplexes were resolved with total lysate via SDS-PAGE and immunoblotted with a FAK antibody derived from mice. Membranes were then stripped and reprobed with an SHP2 antibody, and FAK-SHP2 association was determined by the ratio of FAK bound to total immunoprecipitated SHP2. Inset: representative immunoblots. Data are presented as means ($\pm$ SD); $n = 3$. $*P < 0.05$ versus vehicle. (D) At 48 hours, LMVECs were treated with FAK inhibitor, PF-573228, or vehicle (H₂O) for 3 hours. SHP2 was immunoprecipitated from lysates and subjected to FAK immunoblot analysis as for (C). Inset: representative immunoblots. Data are presented as means ($\pm$ SD). $*P < 0.05$ versus vehicle. (E) Changes in monolayer resistance were measured in LMVECs overexpressing SHP2^{D61A} (squares; $n = 4$) or GFP (diamonds; $n = 5$) cDNA, in the presence (open symbols) and absence (closed symbols) of PF-573228 (0.1 μ M). Arrow indicates point of addition. Data are presented as means ($\pm$ SD). $*P < 0.05$ versus GFP vehicle.

of FAK–SHP2 associations, and disruption of endothelial barrier function. We further show that constitutively active SHP2 protein protects against pulmonary endothelial barrier disruption in settings of FAK inhibition by maintaining FAK autophosphorylation and FAK–SHP2 protein interactions. These findings suggest that SHP2 regulates pulmonary endothelial barrier function via maintenance of FAK–SHP2 protein–protein associations.

Activation of SHP2 is dependent on phosphorylation at the C-terminal tyrosine residues, Y⁵⁴² and Y⁵⁸⁰. Our findings show that barrier-enhancing S1P increases SHP2 phosphorylation, whereas barrier-disruptive agents, LPS and thrombin, cause dephosphorylation of SHP2 at both these residues. Phosphorylation of Y⁵⁴² creates an interaction in the N-terminal SH2 domain and appears to relieve basal inhibition of the PTPase, whereas phosphorylation at Y⁵⁸⁰ causes an interaction at the C terminus that stimulates PTPase activity (26). PTPase activity plays a key role in maintenance of pulmonary endothelial barrier function. In a recent study, we had shown SHP2 inhibition to promote pulmonary barrier dysfunction through the enhanced tyrosine phosphorylation of AJ protein constituents, VE-cadherin, β -catenin, and p190RhoGAP, as well as activation of RhoA (5). Our previous findings suggested SHP2 to act at the level of endothelial intercellular junctions to regulate endothelial monolayer permeability (5). In the present study, we observe SHP2 dephosphorylation at Y⁵⁴² and Y⁵⁸⁰ residues concomitant with FAK dephosphorylation at Y³⁹⁷ site upon exposure to the SHP2 chemical inhibitor, NSC-87877. Furthermore, we observe that NSC-87877-induced endothelial cell permeability is blocked by overexpression of SHP2^{D61A}. Thus, we propose that NSC-87877 compound exhibits a dramatic effect on FAK phosphorylation, specifically through inhibition of SHP2.

Although FAK is well defined as a key regulator of focal adhesion complexes, recent data have demonstrated an additional important function in regulating AJ complexes. There remains controversy regarding the effect of FAK activity on endothelial barrier function (33); however, in the present study, we observe that both chemical and molecular inhibition of FAK, via inhibition of FAK Y³⁹⁷ phosphorylation, increased pulmonary endothelial cell permeability. Many other studies have

similarly observed FAK activity to play an important role in maintaining barrier integrity. Schmidt and colleagues (34) showed that the knockdown of FAK in the mouse endothelium promoted lung edema and cell infiltration through the impairment of VE-cadherin surface expression and AJ formation. Further studies demonstrated that disruption of FAK signaling caused dysfunction of AJs (12, 29, 35), and have implicated the kinase activity of FAK as vital in maintaining endothelial cell barrier function and localization of VE-cadherin at AJs via select phosphorylation of VE-cadherin Y⁶⁵⁸ (36). Indeed, Lu and colleagues (37) demonstrated attenuated pulmonary endothelial barrier dysfunction and AJ disruption upon cigarette smoke exposure in cells overexpressing FAK. FAK null cells are not viable (38, 39), and cells overexpressing dominant negative SHP2 form focal adhesions, but do not spread or migrate (40). Thus, it is likely that SHP2 regulates a pool of FAK that functions to establish and/or maintain the intercellular junctions.

Embryonic fibroblasts isolated from SHP2^{-/-} mice exhibit FAK hyperphosphorylation (40). In cardiomyocytes, SHP2 was observed to negatively regulate FAK, maintaining FAK in a tyrosine dephosphorylated, inactive state; events that correlated with increased FAK–SHP2 interactions and cell apoptosis or hypertrophy (9, 30). Similarly, in Sertoli cells, SHP2 activity correlated with reduced tight junction integrity (41). We observed the opposite of these findings, with SHP2 activation correlating with enhanced interendothelial cell junctions. In pulmonary endothelial cells, we showed enhanced FAK Y³⁹⁷ phosphorylation and sustained FAK–SHP2 interactions in response to barrier-enhancing S1P or constitutive SHP2 activation, and the converse was noted with barrier disruption upon thrombin or LPS exposure. It is possible that FAK–SHP2 associations may invoke a conformational change resulting in autophosphorylation of FAK or phosphorylation via another kinase, such as c-Src, leading to the stabilization of AJ. Alternatively, SHP2 association with FAK may act as an anchor or associate with other scaffolding platform proteins, such as receptor of activated C kinase 1, to control FAK phosphorylation (42, 43). Further studies are needed to examine if disruption of FAK–SHP2 interactions

affects endothelial barrier function through disruption of intercellular junctions.

Tyrosine phosphorylation of FAK is central to mediating signal transduction in focal adhesions. Data from the present study indicate that SHP2 activity is directly related to FAK phosphorylation status, suggesting that SHP2 mediates FAK activity. Indeed, FAK Y³⁹⁷ phosphorylation promotes a conformational change, revealing sites within the kinase for binding with various signaling mediators, including the tyrosine kinase, Src, phosphatidylinositol-4,5-bisphosphate-3-kinase, and phosphatase and tensin homolog (44, 45). Furthermore, protein interactions with FAK can lead to subsequent phosphorylation, as is the case with FAK–SHP2 binding. SHP2-deficient fibroblasts display increased recruitment of the inhibitory Src kinase, C-terminal Src kinase, to the focal adhesion, resulting in reduced Src activity (46). Likewise, Laird and colleagues (47) showed that chemical inhibition of Src caused FAK dephosphorylation at a putative Src autophosphorylation epitope. Thus, the activation of Src, upon SHP2 activation, may provide a pathway through which SHP2 activity increases FAK phosphorylation and mediates protection of the endothelial barrier function. Several members of the Src family kinase have been observed to increase vascular permeability concomitant with FAK phosphorylation. Indeed, *in vitro* and *in vivo* knockdown of Src family kinases, Yamaguchi sarcoma virus oncogene or src-related homolog, Fyn, attenuates vascular permeability associated with dephosphorylation of FAK (15). However, a recent study by Han and colleagues (48) demonstrated the c-Src kinase homolog, Lyn, enhances pulmonary vascular barrier function by promoting tyrosine phosphorylation of FAK at residues Y⁵⁷⁶ and Y⁹²⁵, but not Y³⁹⁷, and suggested these sites as crucial in stabilizing interendothelial cellular junctions. However, we also observe that SHP2 activation attenuated disruption of the endothelial barrier caused by FAK inhibition, indicating that SHP2 promotes the pulmonary endothelium downstream of FAK signaling. Therefore, the effect of FAK phosphorylation on endothelial barrier function may be dependent on both upstream and downstream tyrosine kinase and phosphatase signaling.

In the present study, we did not assess the localization of phosphorylated FAK after exposure to LPS, thrombin, or S1P or SHP2^{D61A} overexpression. Wang and colleagues (49) demonstrated increased FAK activity in response to the pharmacological S1P analog, FTY720, to enhance barrier function through elevated formation and stabilization of the focal adhesions. G protein $\beta\gamma$ -dependent FAK activation localized the kinase to the AJ to promote resealing of the junctions and restore barrier function (22). Likewise, Han and colleagues (48) observed that small interfering RNA knockdown of FAK in human umbilical vein endothelial cells resulted in VE-cadherin breakdown at the endothelial cell border. Thus, the protective effect of FAK activation on the pulmonary vasculature, after SHP2 activation, may be mediated by the localization of FAK–SHP2 protein–protein interactions at the focal adhesion complexes, as well as AJs, in the endothelial cell.

To assess enhanced barrier function after overexpression of SHP2^{D61A} *in vitro*, thrombin and LPS were used to induce monolayer permeability. Both these agents have been previously observed *in vitro* to increase endothelial permeability (50–52). Conversely, *in vivo* studies using these agents causes varying results. Thrombin increases weight gain in isolated perfused lungs as a result of increased capillary pressure (53, 54). LPS increases vascular and airway resistance and leukocyte leak; however, a lack of LPS-induced edema

formation in isolated, blood-free, perfused lung preparation suggests a key role for circulating leukocytes in the role of endotoxin-induced vascular permeability (55, 56). However despite the potential differences between *in vitro* and *in vivo* mechanisms of vascular permeability, both models increase endothelial permeability and, thus, were used in the current studies to elucidate the protective role of SHP2 activation. In the present study, we induced ALI using both endotoxin (LPS) and bacteria (*P. aeruginosa*) to study the effect of SHP2 activation on pulmonary edema formation. In addition to permeability of the alveolar epithelium and endothelium, dysregulated inflammation and accumulation of activated leukocytes and platelets are observed in settings of ALI (57). We observed increased SHP2 expression within the pulmonary vasculature after retro-orbital administration of SHP2^{D61A} cDNA, concomitant with attenuation of pulmonary edema formation. Given the ubiquitous distribution of SHP2 within the lung (58–60), we cannot discount that the protective role of SHP2 activation may be mediated by other cell types within the lung, which had taken up the injected cDNA, such as via tightening of epithelial junctions or diminishing the inflammatory response (61, 62). However, given that overexpression of SHP2^{D61A} resulted in enhanced endothelial barrier function *in vitro*, and that we demonstrate SHP2^{D61A} overexpression in lung endothelial cells *in vivo*, our studies support the notion that

SHP2 is protective against edema formation in the lung vasculature. Thus, we propose that, although *in vivo* activation of SHP2 may modulate other pathways in settings of ALI, enhanced endothelial barrier function is a key mechanism through which SHP2 activation attenuates pulmonary edema formation.

In conclusion, the present study demonstrates the key role of SHP2 activity in modulating endothelial barrier function *in vitro* and pulmonary edema formation *in vivo* through regulation of FAK activity and protein–protein interactions between FAK and SHP2. We further show that constitutive activation of SHP2 in the pulmonary endothelium attenuates the effect of barrier-modulating agents, thereby maintaining a stabilized vasculature. We demonstrate that activation of SHP2 protects FAK against inactivation, thus maintaining activated FAK associated with SHP2, and therefore protecting the pulmonary endothelium from barrier-disruptive agents. Thus, we propose that further study on the FAK-dependent protective mechanisms mediated by SHP2 may allow development of therapeutic agents to restore pulmonary vascular function in ALI. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Katie Grinnell and Milinda Thompson for their contributions to the studies.

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