Protection against LPS-Induced Pulmonary Edema through the Attenuation of Protein Tyrosine Phosphatase-1B Oxidation

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One hallmark of acute lung injury is the disruption of the pulmonary endothelial barrier. Such disruption correlates with increased endothelial permeability, partly through the disruption of cell-cell contacts. Protein tyrosine phosphatases (PTPs) are known to affect the stability of both cell-extracellular matrix adhesions and intercellular adherens junctions (AJs). However, evidence for the role of select PTPs in regulating endothelial permeability is limited. Our investigations noted that the inhibition of PTP1B in cultured pulmonary endothelial cells (ECs), as well as in the vasculature of intact murine lungs via the transient overexpression of a catalytically inactive PTP1B, decreased the baseline resistance of cultured EC monolayers and increased the formation of edema in murine lungs, respectively. In addition, we observed that the overexpression of wild-type PTP1B enhanced basal barrier function *in vitro*. Immunohistochemical analyses of pulmonary ECs and the coimmunoprecipitation of murine lung homogenates demonstrated the association of PTP1B with the AJ proteins β -catenin, p120-catenin, and VE-cadherin both *in vitro* and *ex vivo*. Using LPS in a model of sepsis-induced acute lung injury, we showed that reactive oxygen species were generated in response to LPS, which correlated with enhanced PTP1B oxidation, inhibited phosphatase activity, and attenuation of the interactions between PTP1B and β -catenin, as well as enhanced β -catenin tyrosine phosphorylation. Finally, the overexpression of a cytosolic PTP1B fragment, shown to be resistant to nicotinamide adenine dinucleotide phosphate-reduced oxidase-4 (Nox4)-mediated oxidation, significantly attenuated LPS-induced endothelial barrier dysfunction and the formation of lung edema, and preserved the associations of PTP1B with AJ protein components, independent of PTP1B phosphatase activity. We conclude that PTP1B plays an important role in maintaining the pulmonary endothelial barrier, and PTP1B oxidation appears to contribute to sepsis-induced pulmonary vascular dysfunction, possibly through the disruption of AJs.

Keywords: endothelium; PTP1B; pulmonary edema; oxidation; acute lung injury

The nonreceptor protein tyrosine phosphatase (PTP), PTP1B, is a ubiquitously expressed protein that resides primarily on the cytoplasmic face of the endoplasmic reticulum (ER). This enzyme serves several functions critical for regulation of cellular

metabolism. Perhaps PTP1B has been best characterized as a negative regulator of insulin signaling. By dephosphorylating critical tyrosine residues within the insulin receptor kinase activation loop, PTP1B attenuates the downstream signaling of this receptor (1). Although normally serving as a negative feedback mechanism, an increase in PTP1B expression, above homeostatic levels, can lead to insulin resistance, as is the case in Type II diabetics (2–4). As a result, several clinical trials aimed at treating Type II diabetes centered upon suppressing PTP1B activity. The actions of PTP1B were also shown to increase food intake and adiposity through the negative regulation of the leptin receptor-associated Janus kinase 2 and signal transducer and activator of transcription 3 (Stat3) (5, 6).

Additional PTP1B substrates include the epidermal growth factor (EGF), insulin-like growth factor 1, and platelet-derived growth factor receptors (7), as well as the transmembrane cadherin proteins (designated N, E, and VE) (8–10). Several mechanisms have been proposed to explain how an ER-bound enzyme is capable of interacting with and dephosphorylating these membrane-bound targets. In the case of the EGF receptor, endocytosis was proposed to be necessary for its interaction with PTP1B (11). The calpain-mediated cleavage of PTP1B was also shown to promote the formation of a catalytically active cytosolic fragment that retains its enzymatic activity (12). This cytosolic PTP1B fragment was shown to bind to the cytoplasmic domain of the cadherin proteins, reducing the tyrosine phosphorylation of associated β -catenin and strengthening intercellular adherens junctions (AJs) (9). Consistent with this observation, Nakamura and colleagues showed that PTP1B expression was up-regulated after ischemia, and that increased PTP1B expression correlated with reduced VE-cadherin tyrosine phosphorylation and decreased endothelial permeability (8).

All of the PTP enzymes, including PTP1B, contain an oxidation-sensitive cysteine residue within the catalytic domain (13). The presence of this residue makes these enzymes susceptible to oxidation and subsequent inactivation by reactive oxygen species (ROS). Chen and colleagues demonstrated that another ER-resident enzyme, nicotinamide adenine dinucleotide phosphate-reduced oxidase-4 (Nox-4), is the primary source of ROS responsible for PTP1B oxidation (14). Interestingly, the calpain-mediated cytosolic cleavage fragment of PTP1B was not oxidized by Nox-4-generated ROS (14).

LPS is a major component of gram-negative bacterial cell walls, and induces the life-threatening inflammatory responses associated with bacterial sepsis. Plasma concentrations of LPS are commonly elevated in patients suffering from acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), as well as from gram-negative bacterial pneumonia (15, 16). A primary component of the pathogenicity of LPS within the lung involves the robust induction of ROS production within pulmonary endothelial cells (ECs) (17–19), leading to the disruption of intercellular AJs and resultant vascular dysfunction, through a series of intracellular signaling pathways. In the present study, we explored the role of PTP1B in the regulation of the pulmonary

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endothelium, under basal conditions and after LPS challenge. We investigated the hypothesis that LPS-induced pulmonary EC dysfunction is mediated, at least in part, by the generation of ROS, which in turn causes the oxidation of PTP1B (either to the reversible sulfenic acid, or to the irreversible sulfonic acid form) and the subsequent attenuation of its phosphatase activity. We observed that the inhibition of PTP1B led to an increase in pulmonary endothelial permeability *in vitro*, as well as an increase in pulmonary edema formation in the intact lung in the absence of any edemagenic agents. Consistent with previous studies of LPS-mediated endothelial injury, we noted an increase in pulmonary EC ROS generation in response to LPS treatment. This finding was coincident with increased PTP1B oxidation and decreased PTP1B phosphatase activation. We further showed that LPS increases the tyrosine phosphorylation of β -catenin, which correlated with diminished protein-protein associations between PTP1B and β -catenin. The overexpression of a PTP1B cleavage fragment resistant to Nox-4 oxidation protected against LPS-induced endothelial permeability *in vitro* and against lung edema formation *ex vivo*, and maintained interactions between PTP1B and β -catenin in the absence of elevated PTP1B phosphatase activity. The data presented here are the first, to the best of our knowledge, to demonstrate that the oxidation state of PTP1B contributes to the pathogenesis of LPS-induced acute lung injury, possibly through the modulation of AJs.

MATERIALS AND METHODS

Cell Lines and Reagents

Rat lung microvascular endothelial cells (LMVECs) were obtained from Vec Technologies (Rensselaer, NY), as described previously (20).

LPS was from Enzo (Plymouth Meeting, PA). We purchased 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein (DCF) from Sigma Chemical Co. (St. Louis, MO). PTP1B, p-Tyr (PY99), VE-cadherin, β -actin, and p120-catenin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). β -catenin and oxidized PTP antibodies were from BD Transduction (San Jose, CA) and R&D Systems (Minneapolis, MN). Texas Red-conjugated and Alexa-488-conjugated antibodies were from Molecular Probes (Eugene, OR).

The vector encoding the catalytically active PTP1B cleavage fragment (PTP1B^{A35}) was from Kai Chen (University of Massachusetts Medical School, Worcester, MA) (14). Wild-type (PTP1B^{WT}) and dominant negative (PTP1B^{C215S}) vectors were from Jack Lilien (University of Iowa, Iowa City, IA). The plasmid encoding green fluorescent protein (pGFP-C1) was from Clontech (Mountain View, CA).

Liposome Preparation

Liposomes were produced by mixing dimethyldioctadecyl-ammonium bromide and cholesterol in chloroform, as described elsewhere (21, 22), and rotating under constant vacuum pressure and temperature in a rotavaporator. Liposomes dissolved in 5% glucose solution were combined with cDNA (50 μ g), and injected into the retrobulbar sinus of the orbit of 8- to 10-week-old male C57/BL6 mice (Charles River, Wilmington, MA).

Capillary Filtration Coefficient

Ex vivo analyses of lung edema were performed as described (20, 23) on murine lungs 48 hours after the injection of liposomes.

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

Transfection

The transfection of LMVECs was performed using Polyjet reagent (SignaGen, Gaithersburg, MD). The overexpression of cDNA was confirmed at 48 hours after transfection.

Endothelial Monolayer Permeability

The permeability of LMVECs was assessed using the electrical cell impedance sensor technique (Applied Biophysics, Troy, NY), as described elsewhere (20).

Immunoblot Analyses

Proteins were resolved by SDS-PAGE and immunoblotted as described elsewhere (24).

Immunoprecipitations

Immunoprecipitations (IPs) were performed using 500 μ g of LMVEC lysate or 1.5 mg of lung homogenate. IPs of LMVEC lysates were processed as detailed elsewhere (25). Lung homogenates were incubated with CD31-conjugated magnetic beads, and immunocomplexes were separated from homogenate with a magnetic column. All purified immunocomplexes were subjected to immunoblot analysis.

Immunofluorescent Staining of LMVECs

LMVECs were prepared for staining as previously described (24).

Analysis of PTP1B Oxidation

LMVECs lysed in 100 mM iodoacetic acid, 20 mM Tris, 1% Nonidet P-40, 10% glycerol, 1 mM benzamidine, and 1% aprotinin were immunoprecipitated for PTP1B and immunoblotted for oxidized PTP. Membranes were stripped and reprobed for PTP1B.

Production of ROS

LMVECs in phenol red-free medium were preincubated with 10 μ M DCF for 30 minutes before the addition of LPS or vehicle. Changes in fluorescence intensity (λ_{Ex} , 492–495 nm; λ_{Em} , 517–527 nm) were monitored at 5-minute intervals for 2 hours after the addition of LPS. ROS concentrations at 1 hour and 2 hours after administering LPS were compared with the concentration of ROS before the addition of LPS.

PTP1B Activity

PTP1B immunoprecipitates were incubated with reaction buffer from the PTP1B Assay Kit (Calbiochem, San Diego, CA) at 37°C for 30 minutes, and activity was assessed by measuring at an optical density of 630 nm.

Statistical Analyses

For more than three groups, differences among means were tested for significance in all experiments, using ANOVA with the Fisher least significance difference test. For two groups, differences among means were tested for significance using the Student unpaired *t* test. Significance was achieved when $P < 0.05$. Data are presented as either the mean $\pm$ SD or the mean $\pm$ SE (*n* is indicated for each set of data).

RESULTS

PTP1B Inhibition Disrupts the Pulmonary Endothelial Barrier

PTP1B was previously shown to inhibit the binding of vascular endothelial growth factor (VEGF) to the VEGF receptor, and to aid in stabilizing interendothelial cell adhesions by reducing VE-cadherin tyrosine phosphorylation (8). The present study further investigated the role of PTP1B in the endothelium through an examination of its role in the regulation of the pulmonary vascular barrier. We first assessed how overexpression of either catalytically inactive (dominant negative) PTP1B or the wild-type enzyme affected the permeability of cultured pulmonary endothelial monolayers. Equivalent numbers of LMVECs were transiently transfected with eukaryotic vectors encoding dominant negative PTP1B (PTP1B^{C215S}), wild-type

PTP1B (PTP1B^{wt}), or green fluorescent protein (GFP). The overexpression of PTP1B in transfected endothelial cells was confirmed by immunoblot analysis of total PTP1B protein (Figure 1A, *inset*). In keeping with these data, we did not note an increase in PTP1B activity in PTP1B^{C215S} overexpressing cells (data not shown). The baseline resistance across monolayers overexpressing PTP1B^{C215S} was significantly reduced, compared with that observed in monolayers overexpressing GFP alone (Figure 1A). In contrast, we observed that PTP1B^{wt} overexpressing endothelial cells exhibited a significantly higher baseline resistance compared with GFP control cells (Figure 1A).

To assess how our *in vitro* findings correlated with pulmonary vascular function *in vivo*, we examined whether the inhibition of PTP1B within the intact lung correlated with increased pulmonary edema formation. C57/BL6 mice were injected with cationic liposomes encapsulating cDNA encoding PTP1B^{C215S} or GFP. At 48 hours after injection, the capillary filtration

coefficient (k_f) of the *ex vivo* isolated, perfused lung was determined. Overexpression after liposome-mediated transfection was confirmed by PTP1B and VE-cadherin immunoblot analysis of lysates of endothelial cells isolated from the transfected lungs (Figure 1B, *left*). The overexpression of PTP1B^{C215S} within the pulmonary vasculature led to an approximately 2.3-fold increase in k_f , compared with the overexpression of GFP alone (Figure 1B, *right*). Taken together with the *in vitro* findings, these data suggest that PTP1B is important in the regulation of the pulmonary endothelial barrier, and that the inhibition of this enzyme leads to increased vascular permeability and the formation of edema in the absence of edemagenic agents.

PTP1B Associates with Interendothelial AJ Proteins

Given the observed changes in endothelial permeability resulting from alterations in PTP1B, we next confirmed that PTP1B

Figure 1. The catalytically inactive protein tyrosine phosphatase-1B (PTP1B) mutant, C215S, confers changes in pulmonary endothelial permeability. (A) Equal numbers of lung microvascular endothelial cells (LMVECs) were transfected with eukaryotic vectors encoding wild-type PTP1B (PTP1B^{wt}), dominant negative PTP1B (PTP1B^{C215S}), or green fluorescent protein (GFP). *Main graph:* Beginning 48 hours after transfection, changes in trans-endothelial resistance were measured using electrical cell impedance sensing (ECIS). *Inset:* Overexpression of cDNA was measured by immunoblot (IB) analyses of the lysates of transiently transfected LMVECs, using an antibody specific to PTP1B. (B) Adult (8–10-week-old) C57/BL6 mice were injected with liposomes containing plasmid DNA encoding the dominant negative PTP1B (PTP1B^{C215S}) or GFP. At 48 hours after injection, lung vascular permeability in the intact lung was assessed by measuring the capillary filtration coefficient (k_f) of the lung (*right*). In parallel, to prove the overexpression of PTP1B cDNA in lung endothelium, endothelial cells were isolated from the lung homogenate of transfected mice, using CD31-conjugated magnetic beads purified through a magnetic column and measured by immunoblot analysis for PTP1B overexpression and VE-cadherin expression (*left*). Data are presented as means $\pm$ SD. (A) $n = 3$ –5. (B) $n = 4$. * $P < 0.05$, versus GFP.

Figure 2. PTP1B co-associates with adherens junction proteins. (A) Equivalent amounts of LMVEC lysate were immunoprecipitated (IP) with a rabbit-derived PTP1B antibody. The immunocomplexes were resolved with total lysate via SDS-PAGE and transferred to Immobilon-P membrane, and immunoblots were probed with antibodies specific to β -catenin (β -cat), VE-cadherin (VE-cad), and p120-catenin (p120^{ctn}). Membranes were then stripped and reprobed with a mouse-derived PTP1B antibody. Representative immunoblots are shown ($n = 3$). (B) LMVECs were grown to confluence on gelatin-coated glass coverslips and then fixed, permeabilized, and immunofluorescently costained for PTP1B and p120-catenin or their respective secondary antibodies only (inset). Images were obtained at $\times 1,000$ magnification. Nuclei are counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Scale bar = 20 μ m. Representative images are presented ($n = 3$). (C) Lung homogenates from adult C57/BL6 mice were immunoprecipitated for VE-cadherin, and the immunocomplexes were resolved with total lysate by SDS-PAGE and immunoblotted with an antibody for p120-catenin. Blots were then stripped and reprobed for PTP1B. The blots were stripped and reprobed for a second time for VE-cadherin. Representative immunoblots are shown ($n = 2$).

functions, at least in part, at the level of the interendothelial AJ. Equivalent quantities of LMVEC lysate were immunoprecipitated for PTP1B and subsequently immunoblotted for VE-cadherin, p120-catenin, or β -catenin. PTP1B coprecipitated with all three AJ-associated proteins (Figure 2A), supporting its role in maintaining interendothelial cell adherence. In addition, immunocytochemical analyses revealed the colocalization of PTP1B with p120-catenin (Figure 2B) and β -catenin (data not shown) at interendothelial cell borders. Similar to the *in vitro* data, in murine lung homogenates immunoprecipitated for VE-cadherin and immunoblotted for PTP1B, we demonstrated the co-association of PTP1B with AJ-associated proteins (Figure 2C).

LPS-Induced Disruption of the Pulmonary Endothelium Coincides with Increased Generation of ROS and PTP1B Oxidation and Decreased PTP1B Activation

LPS, a leading cause of sepsis and pneumonia, was shown to induce endothelial barrier dysfunction both directly and indirectly (16, 17, 26). In the present study, we demonstrated that LPS

Figure 3. LPS increases pulmonary endothelial permeability, coincident with an increase in the generation of pulmonary endothelial cell reactive oxygen species (ROS). (A) Resistance across confluent LMVEC monolayers was measured in the presence and absence of 1 μ g/ml LPS. Arrow indicates time of LPS addition. Data are presented as means $\pm$ SD ($n = 4$). * $P < 0.05$, versus vehicle. (B) LMVECs were preincubated with 10 μ M 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein (DCF) for 30 minutes, before the addition of LPS or vehicle. Negative control cells (black bars) were loaded with DCF alone, and not exposed to vehicle or LPS. Changes in fluorescence intensity (λ_{Ex} 492–495 nm; λ_{Em} 517–527 nm) were monitored up to 1 or 2 hours after the addition of LPS. Data are presented as means $\pm$ SD ($n = 3$). * $P < 0.05$, versus DCF alone (negative control).

applied directly to cultured LMVECs causes a significant increase in monolayer permeability (Figure 3A). Coincident with increased endothelial permeability, we observed an increase in the production of LMVEC ROS (Figure 3B). Oxidation is the primary mechanism by which the PTP family of enzymes is inhibited. Thus, we assessed the oxidation status of PTP1B after the treatment of LMVECs with LPS. We noted a significant increase in PTP1B oxidation at both 1 hour and 2 hours after the of LPS (Figures 4A and 4B). This increase in oxidation occurred concomitantly with a decrease in PTP1B catalytic activity (Figure 4C). Thus, LPS may function to increase pulmonary vascular permeability by inhibiting PTP1B, through PTP1B oxidation and subsequent inactivation.

LPS Causes Disruption of PTP1B- β -Catenin Interactions

Given the critical nature of the AJ proteins in maintaining interendothelial cell adherence and the observed colocalization of PTP1B with VE-cadherin-mediated AJ complexes, we examined the effects of LPS on PTP1B-AJ protein-protein interactions. At both 1 hour and 2 hours after LPS treatment, we noted a significant decrease in the co-association of PTP1B with β -catenin (Figures 5A and 5B). The protein-protein interactions of PTP1B with VE-cadherin and p120-catenin, however, were unaltered by LPS treatment (data not shown). Furthermore, at both 1 hour and 2 hours after LPS treatment, we observed an increase in the tyrosine phosphorylation of β -catenin (Figure 5C).

Overexpression of an Oxidation-Resistant Form of PTP1B Protects against LPS-Induced Endothelial Barrier Disruption, Independent of Phosphatase Activity

Within the pulmonary endothelium, Nox-2 and Nox-4 appear to be the key isoforms responsible for the generation of ROS (27). Chen and colleagues demonstrated that Nox-4 is able to inhibit PTP1B when the two proteins colocalize to the ER (14). We confirmed abundant Nox-4 expression by LMVECs (Figure 6A). We then overexpressed a cDNA encoding a truncated PTP1B protein lacking the C-terminal ER anchor, PTP1B ^{Δ 35}, in cultured pulmonary endothelial cells and in intact murine lungs. The loss of this motif, which tethers the protein within the outer ER membrane, was shown to correlate with the diminished oxidation of PTP1B by the ER-resident Nox-4, without affecting PTP1B catalytic activity (14). We found that the overexpression of this "oxidation-resistant" PTP1B significantly attenuated LPS-induced increases in endothelial monolayer permeability, beyond the effects noted with the overexpression of wild-type PTP1B (Figures 6B and 6C). In keeping with this observation, we demonstrated that the liposome-mediated overexpression of PTP1B ^{Δ 35} within the intact murine vasculature provided protection against LPS-induced pulmonary edema, significantly attenuating LPS-induced changes in the k_f values of isolated, perfused lungs (Figure 6D).

Overexpression of PTP1B^{wt} significantly elevated PTP1B activity; however, PTP1B ^{Δ 35}-overexpressing cells were found to display reduced basal PTP1B activity in the absence of LPS treatment (Figure 7A). Interestingly, PTP1B activity was significantly reduced in cells overexpressing either GFP or wild-type PTP1B cDNA, to levels noted in PTP1B ^{Δ 35}-overexpressing cells in the presence of LPS treatment (Figure 7A). We further noted a reduced association of PTP1B with β -catenin, in the presence of LPS, in GFP or PTP1B^{wt}-overexpressing cells. This effect was abolished in cells transiently transfected with PTP1B ^{Δ 35} (Figure 7B). Taken together, these findings strongly suggest that the prevention of PTP1B oxidation enables it to participate in

Figure 4. LPS induces the oxidation and inactivation of PTP1B. (A) Equal numbers of LMVECs were incubated with or without LPS for the indicated time or with 0.5 mM H₂O₂. Cells were lysed, and equivalent amounts of lysate were immunoprecipitated for PTP1B. The immunocomplexes were resolved via SDS-PAGE and immunoblotted with an antibody specific for oxidized PTP enzymes (oxPTP). Membranes were then stripped and reprobed with PTP1B antibody. Representative immunoblots are shown ($n = 3$). (B) Densitometric analyses of the IB were performed, and the data are presented as means $\pm$ SE of the concentration of oxidized PTP1B relative to total PTP1B ($n = 3$). * $P < 0.05$, versus vehicle. (C) LMVECs were incubated with or without LPS for the indicated times. Equivalent quantities of LMVEC lysate were then immunoprecipitated for PTP1B, and the resultant immunocomplexes were subjected to an *in vitro* PTP assay. PTP1B activity is presented as the absorbance at 630 nm, normalized to the activity measured in LMVECs cultured in complete medium (C). O.D., optical density. Data are presented as means $\pm$ SE ($n = 3$). * $P < 0.05$, versus untreated control sample.

Figure 5. LPS disrupts PTP1B- β -catenin protein-protein interactions and β -catenin phosphorylation status. Equal numbers of LMVECs were incubated with or without LPS for the indicated times. Equivalent amounts of lysate were immunoprecipitated for PTP1B, using an antibody derived from rabbit, or phosphorylated tyrosine residue. The immunocomplexes were resolved with total lysate via SDS-PAGE and immunoblotted with a β -catenin antibody. Membranes were then stripped and reprobed with a PTP1B antibody, derived from mouse. (A) Representative immunoblots for IP with PTP1B antibody are shown ($n = 3$). (B) Densitometric analyses of the IB were performed, and data are presented as means $\pm$ SD of the concentration of β -catenin-bound PTP1B relative to total PTP1B, relative to vehicle at respective time points ($n = 3$). * $P < 0.05$, versus vehicle. (C) Representative immunoblot for IP with PY99 (Y~P) antibody is shown.

pulmonary endothelial barrier regulation, a role not solely dependent on PTP1B activity.

DISCUSSION

In the present study, we demonstrated for the first time, to the best of our knowledge, that inhibition of the oxidation of PTP1B, but not phosphatase activity, plays a significant role in LPS-induced acute lung injury. We showed that PTP1B localizes to intercellular AJs, and that the inhibition of PTP1B activity led to significant increases in pulmonary endothelial permeability in the absence of edemagenic agents. In our model of sepsis-

induced pulmonary vascular injury, we showed that LPS-induced increases in the production of ROS resulted in PTP1B oxidation and inactivation. Additional experiments determined that PTP1B oxidation seemed to be the key determinant in modulating the edemagenic response to LPS-induced ALI, with the PTP1B oxidation-resistant mutant protecting against the formation of edema, possibly by preserving AJ protein-protein interactions.

PTP1B was the first protein tyrosine phosphatase to be discovered, and is expressed in a wide variety of tissues (28, 29). Much of the research focused on PTP1B has been aimed at elucidating its role in the regulation of metabolism (4–6, 30). Balsamo and colleagues first reported a role for PTP1B in the regulation of intercellular junctions, showing that PTP1B was able to regulate N-cadherin function through the dephosphorylation of β -catenin (31). Upon the inhibition of PTP1B, β -catenin became hyperphosphorylated, leading to disruption of the N-cadherin- β -catenin-actin cytoskeletal connection (9). The PTP1B- β -catenin relationship also appears to be critical for the regulation of E-cadherin-mediated epithelial junctions (10). After the treatment of Caco-2 cells with acetaldehyde, the concentration of activated PTP1B associated with E-cadherin and β -catenin was significantly reduced, coincident with increased β -catenin phosphorylation and the disruption of intercellular AJs (10).

The exact mechanism by which PTP1B oxidation/inhibition causes pulmonary vascular dysfunction remains unknown. LPS was shown to cause the disruption of interendothelial cell AJs, as measured by the dissociation of VE-cadherin and β -catenin (32). Several groups demonstrated the increased tyrosine phosphorylation of the AJ-associated cadherins and β -catenin after PTP1B inhibition (8–10). Tyrosine phosphorylation is a major regulator of intercellular junction formation. The increased tyrosine phosphorylation of AJ proteins correlates with junction disruption and disassembly (20, 33, 34). In the present study, we observed PTP1B localization at, and binding to, intercellular AJ proteins. LPS treatment resulted in the decreased association of PTP1B with β -catenin, but did not affect the binding of PTP1B with VE-cadherin. To explain this finding, we suggest that the oxidation of PTP1B at cysteine 215 results in a disruption of the β -catenin binding site, but leaves the VE-cadherin site unaltered. We observed elevated tyrosine phosphorylation of β -catenin after LPS treatment, thus opening the further possibility that the hyperphosphorylation of β -catenin, resulting from the LPS-mediated oxidation and inhibition of PTP1B, allows for the binding of IQ motif containing GTPase activating protein (IQGAP) to β -catenin, physically displacing PTP1B. PTP1B was also shown to mediate changes in the actin cytoskeleton, independent of AJ proteins. Murine embryonic fibroblasts deficient in PTP1B are unable to phosphorylate and activate Src, leading to an inhibition of integrin signaling and decreased cell spreading (35).

The disruption of AJs constitutes one of the defining features of pulmonary edema in settings of ALI. The disruption of intercellular AJ protein-protein interactions leads to gaps between adjacent endothelial cells, causing an increased movement of fluid and solutes from the vascular compartment into the lung interstitium and alveolar spaces. This, in turn, results in the formation of pulmonary edema (36–38). Given the data supporting the involvement of PTP1B in the regulation of AJs, this enzyme represents a potential player in the maintenance of pulmonary vascular barrier function. Nakamura and colleagues showed that the expression and activation of PTP1B are significantly elevated during post-ischemia angiogenesis, and that PTP1B was able to inhibit the VEGF-induced phosphorylation of VEGFR2 and extracellular regulated kinases 1 and 2 (8). Consistent with observations in neuronal and epithelial tissues,

Figure 6. Oxidation resistance of PTP1B mutant (PTP1B^{Δ35}) attenuates LPS-induced pulmonary endothelial barrier function, both *in vitro* and *in vivo*. (A) LMVEC lysate was resolved by SDS-PAGE and IB for nicotinamide adenine dinucleotide phosphate-reduced oxidase-4 (Nox-4) and β-actin. Representative immunoblot is shown ($n = 2$). (B) Equal numbers of LMVECs were transfected with eukaryotic vectors encoding the PTP1B cleavage fragment (PTP1B^{Δ35}), PTP1B^{wt}, or GFP. At 48 hours after transfection, transendothelial resistance was measured using ECIS, in the presence and absence of LPS. Arrow indicates point of addition. A representative tracing is shown ($n = 3-5$). (C) The percent drop in transendothelial resistance (TER), relative to that noted in untreated cells, is presented ($n = 3-5$). * $P < 0.05$, versus GFP-transfected LMVECs treated with LPS. (D) Adult C57/BL6 mice were injected with liposomes containing plasmid DNA encoding GFP or PTP1B^{Δ35}. At 24 hours after liposome injection, mice received an intraperitoneal injection of LPS (5 mg/kg). After an additional 24 hours, the capillary filtration coefficient (k_f) was assessed. Data are presented as means $\pm$ SE ($n = 3-5$). * $P < 0.05$, versus vehicle.

this study showed that PTP1B plays an important role in the regulation of VE-cadherin-mediated AJs. The overexpression of wild-type PTP1B led to a strengthening of VE-cadherin-mediated AJs, whereas small interfering RNA (siRNA)-mediated inhibition resulted in increased endothelial permeability, coincident with the increased tyrosine phosphorylation of VE-cadherin (8). In the present study, we showed that the overexpression of dominant negative PTP1B caused increased pulmonary endothelial permeability, both in cultured monolayers and in intact murine lungs. In contrast, the overexpression of the wild-type enzyme increased endothelial monolayer barrier function.

Although the data supporting a role for PTP1B in the regulation of endothelial barrier function are strong, little is known regarding the mechanisms by which it may be inhibited, leading to vascular dysfunction. All PTP enzymes possess an invariant cysteine residue within their catalytic domain, making them susceptible to oxidation by ROS (13, 39). This oxidation, either to

the reversible sulfenic acid form or to the irreversible sulfonic acid form, leads to a rapid inhibition of enzymatic activity. Although ROS are critical for a number of cell processes, their overproduction is associated with many disease states, including sepsis, ALI, and ARDS. Increased concentrations of ROS are routinely observed in the pulmonary edema and bronchoalveolar lavage fluid of patients with ALI or ARDS (40-41). Here, we demonstrate that in settings of LPS-induced pulmonary vascular dysfunction, the generation of pulmonary endothelial cell ROS is increased, correlating with a significant increase in PTP1B oxidation and an inhibition of PTP1B enzyme activity.

Several different forms of ROS are produced within the cell, including the superoxide anion, the hydroxyl radical, and hydrogen peroxide. These species are generated through a variety of systems, including the mitochondrial respiratory chain, endothelial nitric oxide synthases, cytochrome P450, xanthine oxidase, and the Nox enzymes (43, 44). Within the pulmonary vasculature, two Nox enzymes, Nox-2 and Nox-4, appear to make the

Figure 7. Effects of overexpression of an oxidation-resistant form of PTP1B on LPS-induced PTP1B activity and PTP1B associations with β -catenin. Equal numbers of LMVECs were transfected with eukaryotic vectors encoding the PTP1B cleavage fragment (PTP1B^{Δ35}), PTP1B^{wt}, or GFP. At 48 hours after transfection, LMVECs were incubated in the presence and absence of LPS for 2 hours. Equivalent quantities of LMVEC lysate were then immunoprecipitated for PTP1B, using an antibody derived from rabbit. (A) The resultant immunocomplexes were subjected to an *in vitro* PTP assay. PTP1B activity is presented as the absorbance at λ_{630nm} , normalized to the activity measured in LMVECs cultured in complete medium (C). Data are presented as means $\pm$ SE ($n = 3$). * $P < 0.05$, versus GFP-transfected control. ** $P < 0.01$, versus untreated control. (B) The resulting immunocomplexes were resolved via SDS-PAGE, and immunoblotted with a β -catenin antibody. Membranes were then stripped and reprobed with a PTP1B antibody derived from mouse. To confirm the overexpression of the PTP1B cDNA, equivalent amounts of LMVEC lysates, used for the immunoprecipitations, were resolved by SDS-PAGE and immunoblotted for PTP1B in parallel. A representative immunoblot is shown.

most significant contribution to the generation of ROS (27). Nox-4 was shown play a key role in the LPS-induced endothelial proinflammatory response (45). LPS binds to the cell through Toll-like receptor-4 (TLR4), leading to the subsequent activation of the NF- κ B and mitogen-activated protein kinase pathways (46). TLR4 was implicated as playing a role in the LPS-induced generation of ROS (26). The generation of ROS, in turn, was shown to be an important component of TLR4-associated NF- κ B activation (47). Consistent with these observations, Park and colleagues demonstrated a physical association of TLR4 and Nox-4, and showed that the siRNA silencing of Nox-4 led to a reduction in the LPS-induced production of ROS (45).

Like PTP1B, Nox-4 is an ER-resident protein. In a recent study, the Nox-4-mediated oxidation of PTP1B was observed in vascular endothelial cells (14). Activated PTP1B was shown to target the epidermal growth factor receptor (EGFR), leading

to the dephosphorylation and receptor-mediated endocytosis of the receptor. According to Chen and colleagues (14), the overexpression of Nox-4 attenuated the PTP1B-dependent inhibition of EGFR. This effect was shown to be dependent on the proximity of the two proteins at the ER membrane. When the N-terminal 35 amino acids of PTP1B, which serve to anchor the enzyme to the cytoplasmic face of the ER, were removed, the oxidation of PTP1B was significantly blocked. In addition, when Nox-4 was inhibited, the PTP1B-mediated suppression of EGFR was increased (14). In the present study, we showed that, despite lowering PTP1B activity, the overexpression of a mutant form of PTP1B that lacks the N-terminal 35 amino acids (PTP1B- $\Delta 35$) was able to attenuate the LPS-induced disruption of cultured pulmonary endothelial cell monolayers, and to protect against LPS-induced pulmonary edema in intact lungs. This observation strongly supports a role for the Nox-4-mediated oxidation and inhibition of PTP1B in settings of LPS-induced ALI. Furthermore, reduced PTP1B activity and the association with β -catenin may offer a mechanism for the protective role of the oxidation-resistant PTP1B. Unlike the acetaldehyde-induced activation of PTP1B, which results in AJ disruption, the low activity status of the oxidation-resistant PTP1B may mediate the protection of the endothelial barrier from LPS-induced injury (10), possibly by stabilizing AJ structures.

Endogenous PTP1B is cleaved by calpain-2, an intracellular, calcium-dependent protease that functions in the turnover of cellular adhesions and cell migration (12, 48–51). This cleavage produces a catalytically active cytosolic fragment. The hyperactivation of calpain-2 and the increased expression of PTP1B were noted in a number of human cancers (52), as well as in Type 2 diabetes (3, 53). Interestingly, individuals with Type 2 diabetes appear to be protected against sepsis-induced ALI (54–56). We speculate that a component of this observed protection is perhaps mediated by the overexpression of PTP1B and increased cleavage by calpain-2, resulting in an abundance of cytosolic PTP1B that is free from Nox-4-mediated oxidation and inhibition.

Overall, the present study extends previous findings regarding the role of PTP1B in the regulation of intercellular AJs, demonstrating a critical role for PTP1B in the regulation of the pulmonary endothelial barrier. In addition, the present findings provide insights into the mechanism by which PTP1B is post-translationally modified in sepsis-induced ALI. The observation that a cleavage fragment of PTP1B that is resistant to Nox-4-mediated oxidation is able to significantly attenuate pulmonary endothelial permeability, both *in vitro* and *in vivo*, holds potential for future therapies aimed at the restoration of pulmonary vascular function after the formation of pulmonary edema.

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