Role of Protein Tyrosine Phosphatase 1B in Vascular Endothelial Growth Factor Signaling and Cell–Cell Adhesions in Endothelial Cells

Yoshimasa Nakamura, Nikolay Patrushev, Hyoe Inomata, Dolly Mehta, Norifumi Urao, Ha Won Kim, Masooma Razvi, Vidisha Kini, Kalyankar Mahadev, Barry J. Goldstein, Ronald Mckinney, Tohru Fukai, Masuko Ushio-Fukai

Abstract—Vascular endothelial growth factor (VEGF) binding induces phosphorylation of VEGF receptor (VEGFR)2 in tyrosine, which is followed by disruption of VE-cadherin–mediated cell–cell contacts of endothelial cells (ECs), thereby stimulating EC proliferation and migration to promote angiogenesis. Tyrosine phosphorylation events are controlled by the balance of activation of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Little is known about the role of endogenous PTPs in VEGF signaling in ECs. In this study, we found that PTP1B expression and activity are markedly increased in mice hindlimb ischemia model of angiogenesis. In ECs, overexpression of PTP1B, but not catalytically inactive mutant PTP1B-C/S, inhibits VEGF-induced phosphorylation of VEGFR2 and extracellular signal-regulated kinase 1/2, as well as EC proliferation, whereas knockdown of PTP1B by small interfering RNA enhances these responses, suggesting that PTP1B negatively regulates VEGFR2 signaling in ECs. VEGF-induced p38 mitogen-activated protein kinase phosphorylation and EC migration are not affected by PTP1B overexpression or knockdown. In vivo dephosphorylation and cotransfection assays reveal that PTP1B binds to VEGFR2 cytoplasmic domain in vivo and directly dephosphorylates activated VEGFR2 immunoprecipitates from human umbilical vein endothelial cells. Overexpression of PTP1B stabilizes VE-cadherin–mediated cell–cell adhesions by reducing VE-cadherin tyrosine phosphorylation, whereas PTP1B small interfering RNA causes opposite effects with increasing endothelial permeability, as measured by transendothelial electric resistance. In summary, PTP1B negatively regulates VEGFR2 receptor activation via binding to the VEGFR2, as well as stabilizes cell–cell adhesions through reducing tyrosine phosphorylation of VE-cadherin. Induction of PTP1B by hindlimb ischemia may represent an important counterregulatory mechanism that blunts overactivation of VEGF2 during angiogenesis in vivo. (Circ Res. 2008;102:0-0.)

Key Words: protein tyrosine phosphatase 1B ■ vascular endothelial growth factor ■ endothelial cell ■ cell–cell adhesions ■ angiogenesis

Vascular endothelial growth factor (VEGF) induces angiogenesis by stimulating endothelial cell (EC) migration and proliferation primarily through the VEGF type 2 receptor (VEGFR2) (KDR/Flik-1). VEGF binding initiates autophosphorylation of VEGFR2, which, in turn, creates docking sites for Src homology domain 2–containing adaptor molecules. This event is followed by activation of diverse angiogenic enzymes such as extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK), which are involved in EC proliferation and migration, respectively. One of the initial responses of quiescent ECs to induce angiogenesis is the loss of established cell–cell contacts, which is followed by EC migration, proliferation, and formation of capillary tube networks. The molecule primarily responsible for cell–cell adhesions of ECs is the transmembrane homophilic adhesion molecule vascular endothelial (VE)-cadherin, whose tyrosine phosphorylation is important for disruption of cell–cell contacts. Thus, tyrosine phosphorylation of VEGFR2 and VE-cadherin is important initial signaling events by which VEGF stimulates angiogenesis in ECs. Tyrosine phosphorylation events are controlled by the balance of activation of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). PTP inhibitors accelerate neovascularization in rat ischemia hindlimb models; however, little is known about role of endogenous PTPs in VEGF signaling in ECs.
In preliminary studies to identify PTPs involved in angiogenesis in vivo, we identified PTP1B as among the major PTPs whose expression is dramatically induced during angiogenesis in a mouse ischemia hindlimb model. PTP1B is the first PTP isolated in homogenous form, and is ubiquitously expressed enzymes. Studies using PTP1B-deficient mice show that PTP1B is a key negative regulator of insulin and leptin signaling and a therapeutic target for the type 2 diabetes and obesity. PTP1B is also implicated in growth factor–mediated and integrin-mediated signaling. Furthermore, PTP1B binds to E- and N-cadherin and regulates the cell–cell adhesions in neural retina cells and epithelial cells, respectively. However, the specific role of PTP1B in VEGF signaling and VE-cadherin–mediated cell–cell adhesions in ECs is unknown.

We performed the present study to test the hypothesis that PTP1B may play an important role in VEGF signaling linked to angiogenesis. Using overexpression and knockdown strategies, we demonstrate that PTP1B negatively regulates VEGFR2 receptor autophosphorylation via binding to the VEGFR2, which, in turn, inhibits downstream ERK1/2 phosphorylation and EC proliferation. We also found that PTP1B associates with VE-cadherin, thereby reducing its tyrosine phosphorylation, which may contribute to stabilizing cell–cell adhesions. PTP1B expression and activity are markedly increased in a mouse hindlimb ischemia model, which may represent important counterregulatory mechanism that blunts overactivation of VEGFR2 during angiogenesis in vivo.

**Materials and Methods**

Materials, cell culture, immunoprecipitation, immunoblotting, transient transfection of CHO cells, adenovirus transduction, small interfering (si)RNA transfection, PTPase activity assay, in vivo receptor dephosphorylation assays, cell proliferation and migration assays, confocal immunofluorescence microscopy, transendothelial electric resistance (TER) measurement, mouse ischemic hindlimb model, and statistical analyses are described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

**Results**

**PTP1B Overexpression Inhibits, Whereas PTP1B Knockdown Enhances, VEGF-Induced Autophosphorylation of VEGFR2**

To determine the function of PTP1B in VEGF signaling, we examined the effect of PTP1B overexpression on VEGFR2 autophosphorylation in human umbilical vein endothelial cells (HUVECs). Figure 1 shows that transduction of adenovirus expressing wild-type PTP1B (Ad.PTP1B-WT) significantly increased PTP1B protein expression and activity (Figure 1A) and inhibited VEGF-induced VEGFR2 tyrosine phosphorylation without affecting VEGFR2 expression (Figure 1B). In contrast, Ad.PTP1B-C/S (catalytically inactive C215S mutant), which increased PTP1B protein expression at similar extent as PTP1B-WT but had no effect on PTP1B activity (Figure 1A), significantly enhanced VEGF-induced VEGFR2 tyrosine phosphorylation without affecting VEGFR2 expression (Figure 1B). In contrast, Ad.PTP1B-C/S (catalytically inactive C215S mutant), which increased PTP1B protein expression at similar extent as PTP1B-WT but had no effect on PTP1B activity (Figure 1A), significantly enhanced VEGF-induced VEGFR2 tyrosine phosphorylation without affecting VEGFR2 expression (Figure 1B). These results suggest that overexpression of PTP1B inhibits VEGFR2 phosphorylation in a catalytic activity–dependent manner and that PTP1B-C/S protects from dephosphorylation by endogenous PTP1B. In contrast, Figure 2 shows that knockdown of endogenous PTP1B protein and specific activity with siRNA (Figure 2A) enhanced VEGF-induced VEGFR2 tyrosine phosphorylation (Figure 2B). These results suggest that PTP1B functions as negative regulator for VEGFR2 autophosphorylation.

**PTP1B Dephosphorylates and Associates With VEGFR2**

To determine whether VEGFR2 can serve as a substrate for PTP1B, we performed in vivo dephosphorylation assay. Figure 3A shows that recombinant active PTP1B protein dephosphorylated VEGFR2 obtained from VEGFR2 immu-
noprecipitates of VEGF-stimulated HUVEC lysates in a dose-dependent manner. To assess the mechanism by which PTP1B negatively regulates VEGFR2 phosphorylation, we examined whether PTP1B physically interacts with VEGFR2. Figure 3B shows that PTP1B was coimmunoprecipitated with VEGFR2 in HUVECs infected with Ad.PTP1B-WT, but not with Ad.LacZ, in basal state and after VEGF stimulation. These results suggest that PTP1B associates with VEGFR2 in a phospho-tyrosine-independent manner and that endogenous PTP1B interaction with VEGFR2 is unstable to be detected by coprecipitation. To confirm further their interaction in vivo, glutathione S-transferase (GST)-tagged PTP1B-WT or substrate-trapping mutants PTP1B-D/A (D181A) or PTP1B-C/S, and Myc-tagged entire intracellular domain of human VEGFR2 (VEGFR2cyto, residues 790 to 1356) were cotransfected in CHO cells. Figure 3C shows that either PTP1B-WT or PTP1B-D/A or PTP1B-C/S, but not empty vector alone, coprecipitated with Myc-VEGFR2cyto. These results suggest that PTP1B associates with VEGFR2 kinase domain, independent of phosphorylation status of the receptor and phosphatase activity.

PTP1B Overexpression Inhibits, Whereas PTP1B Knockdown Enhances, VEGF-Induced Phosphorylation of ERK1/2 but Not p38 Mitogen-Activated Protein Kinase
To further characterize the effect of PTP1B on VEGF signaling, we examined the effect of overexpression of

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Knockdown of endogenous PTP1B by siRNA enhances VEGFR2 autophosphorylation. HUVECs were transfected with scrambled or PTP1B siRNA. A, PTP1B activity (upper graph) and PTP1B protein expression (lower blot). PTP1B activity was expressed as fold change from control (the ratio in untransfected cells was set to 1). *P<0.05 vs scrambled siRNA. B, Cells were stimulated with VEGF (20 ng/mL), and lysates were immunoprecipitated with anti-VEGFR2 antibody, followed by immunoblotting with anti-pTyr or VEGFR2 antibody. Bottom, Averaged data, expressed as fold change of phosphorylation over basal (means±SE, n=3). *P<0.05 vs scrambled siRNA at each time point.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** PTP1B dephosphorylates and associates with VEGFR2 in vivo. A, In vivo receptor dephosphorylation assay. VEGFR2 immunoprecipitates obtained from HUVECs stimulated with VEGF for 5 minutes were incubated with active recombinant PTP1B protein at 30°C for 10 minutes. Samples were immunoblotted with anti-pTyr or VEGFR2 antibody. B, HUVECs infected with Ad.LacZ (control) or Ad.PTP1B-WT were stimulated with VEGF (20 ng/mL) for 5 minutes. Lysates were immunoprecipitated with anti-VEGFR2 antibody, followed by immunoblotting with anti-PTP1B or VEGFR2 antibody. The bottom blot shows Western blot analysis with anti-PTP1B antibody. C, CHO cells were cotransfected with Myc-tagged VEGFR2 cytoplasmic domain (myc-VEGFR2cyto) and various GST-tagged mutants of PTP1B (GST-PTP1B-WT, D181A, C215A) or empty vector. Lysates immunoprecipitated with VEGFR2 antibody (IP: VEGFR2) or without immunoprecipitation (no IP) were immunoblotted with anti-GST antibody.
PTP1B on phosphorylation of downstream signaling such as ERK1/2 and p38 MAPK, which are involved in endothelial proliferation and migration, respectively. Figure 4A shows that Ad.PTP1B-WT significantly inhibited, whereas Ad.PTP1B-C/S slightly enhanced, VEGF-induced ERK1/2 phosphorylation, without affecting p38 MAPK phosphorylation. Conversely, PTP1B siRNA significantly enhanced VEGF-induced phosphorylation of ERK1/2 without affecting p38 MAPK phosphorylation (Figure 4B). We also found that PTP1B siRNA has no effect on Akt phosphorylation (data not shown). These results suggest that PTP1B is an endogenous negative regulator for the VEGFR2-ERK1/2 pathway. This notion is further supported by the observation that overexpression of PTP1B-WT inhibited, but PTP1B siRNA enhanced, VEGF-stimulated phosphorylation of VEGFR2 at Tyr1175 and phospholipase (PL)Cγ, which are upstream of ERK1/2 (Figure I in the online data supplement).

PTP1B Negatively Regulates VEGF-Stimulated EC Proliferation

Because VEGF-induced ERK1/2 is involved in EC proliferation, we examined the effects of overexpression of PTP1B and PTP1B siRNA on this response using MTT assay. Figure 5A shows that Ad.PTP1B-WT, but not LacZ or PTP1B-C/S,
significantly inhibited VEGF-stimulated EC proliferation. Neither PTP1B nor PTP1B siRNA treatment affected basal proliferation. Conversely, PTP1B siRNA significantly enhanced VEGF-stimulated EC proliferation without affecting basal response (Figure 5B). Similar responses are obtained by cell number measurement (data not shown). In contrast, both PTP1B overexpression and knockdown had no effects on VEGF-mediated migration (supplemental Figure II) and cell survival (data not shown), which are dependent on p38 MAPK and Akt, respectively.

**PTP1B Stabilizes VE-Cadherin–Mediated Cell–Cell Adhesion**

Disruption of cell–cell contacts by reducing VE-cadherin from the cell–cell adhesion sites or tyrosine phosphorylation of VE-cadherin is important for initiating angiogenesis. We thus examined the role of PTP1B in localization of VE-cadherin at adherence junctions in confluent monolayer of ECs. Figure 6A shows that Ad. PTP1B-WT increased VE-cadherin staining at sites of cell–cell contact, whereas Ad. PTP1B-C/S markedly reduced it, resulting in small gaps between adjacent cells in basal state. VEGF stimulation reduced VE-cadherin staining at sites of cell–cell contact in LacZ-infected cells, which was prevented by PTP1B-WT and further decreased by PTP1B-C/S. Furthermore, basal and VEGF-induced tyrosine phosphorylation of VE-cadherin was significantly reduced by overexpression of PTP1B-WT, whereas it was enhanced by PTP1B-C/S (Figure 6B and supplemental Figure IIIA). Conversely, PTP1B siRNA markedly reduced VE-cadherin staining at cell–cell contacts and significantly enhanced basal and VEGF-induced tyrosine phosphorylation of VE-cadherin (Figure 7A and 7B). We also determined TER, a measure of intercellular adhesion between ECs, and found that PTP1B-WT significantly increased, whereas PTP1B-C/S and PTP1B siRNA significantly decreased TER (Figures 6C and 7C). Neither Ad. PTP1B-WT nor Ad. PTP1B-C/S, nor PTP1B siRNA, affected protein expression of VE-cadherin (data not shown). These results suggest that PTP1B activity stabilizes VE-cadherin–mediated cell–cell adhesion in ECs. We also found that PTP1B was coimmunoprecipitated with VE-cadherin in unstimulated and VEGF-stimulated confluent HUVECs infected with Ad.LacZ, Ad. PTP1B-WT, or Ad. PTP1B-C/S (supplemental Figure IIIC). Moreover, confocal analysis with HUVECs transiently transfected with GFP-PTP1B-D181A (D/A) revealed that PTP1B colocalized with VE-cadherin at cell–cell contacts, but the majority of PTP1B was found at perinucleus as reported (data not shown). Colocalization of GFP-PTP1B-WT with VE-cadherin was weaker than the D/A mutant, indicating that stabilization of the enzyme–substrate complex is necessary for visualization of PTP1B-VE-cadherin complex. These results suggest that PTP1B associates with VE-cadherin at...
cell–cell junctions and dephosphorylates VE-cadherin in confluent ECs, thereby stabilizing cell–cell adhesions.

Increase of PTP1B Expression and Activity in Mouse Ischemic Hindlimb Model of Angiogenesis

To assess the role of PTP1B in angiogenesis in vivo, we confirmed the expression of PTP1B and examined its activity in a mouse hindlimb ischemia model. Figure 8A shows that hindlimb blood flow recovery after femoral artery ligation was markedly decreased immediately after surgery (day 0) and recovered to the level of that of the nonischemic limb by day 7. Western blot analysis shows that PTP1B protein was robustly increased in the ischemic hindlimb at 7 days after operation compared with nonischemic legs (Figure 8C), which was associated with the increase in PTP1B activity (Figure 8B). Of note, their increase was time-dependent, with a peak at day 7, and there was no significant difference between day 0 and 1 (data not shown). Furthermore, immunofluorescence analysis demonstrated that PTP1B expression was increased at lectin-positive capillary ECs and skeletal myocyte at 3 and 7 days after ischemic injury in a time-dependent manner (supplemental Figure IV). In contrast, expression of Src homology domain 2–containing phosphatase (SHP)-2 was not changed after hindlimb ischemia (Figure 8C). We also found that SHP-2 siRNA had no effect on VEGFR2 autophosphorylation and ERK1/2 phosphorylation in HUVECs (data not shown). Given the functional role of PTP1B in VEGF signaling and cell–cell adhesion in ECs, these data suggest that PTP1B may be involved in the process by which new blood vessels are formed in vivo.

Discussion

Many studies have focused on mechanism of VEGFR2 signal activation as opposed to signal termination, which is much less studied. The present study provides evidence that (1) overexpression of PTP1B inhibits VEGFR2 autophosphorylation, ERK1/2 phosphorylation, and EC proliferation, whereas knockdown of PTP1B using siRNA significantly enhances these responses; (2) PTP1B binds to VEGFR2 in HUVECs overexpressing PTP1B, as well as in CHO cells cotransfected with PTP1B and VEGFR2 cytoplasmic domain; (3) recombinant active PTP1B protein dephosphorylates VEGFR2 immunoprecipitates of VEGF-stimulated HUVECs; (4) overexpression of PTP1B stabilizes endothelial

Figure 7. PTP1B knockdown reduces VE-cadherin–mediated cell–cell adhesion and increases VE-cadherin tyrosine phosphorylation. HUVECs transfected with scrambled or PTP1B siRNA were used for VE-cadherin staining (A), VE-cadherin tyrosine phosphorylation analysis (B), and real-time measurement of TER (C). A, White arrows show decrease in VE-cadherin staining at cell–cell contacts with formation of small gaps. B, Cells stimulated with VEGF (20 ng/mL) were used for measurement of VE-cadherin tyrosine phosphorylation and PTP1B protein expression as described in the legend of Figure 6. Results are representative of 3 independent experiments. C, Values are the means±SE for 8 independent duplicate experiments. *P<0.05 vs scrambled siRNA.

Figure 8. Increase of PTP1B expression and activity in mouse ischemic hindlimb model of angiogenesis. Hindlimb ischemia was induced by the right femoral artery ligation. A, Laser Doppler blood flow analysis. Arrows indicate a low perfusion signal (dark blue) at immediately after operation (day 0) and a high perfusion signal (yellow to red) detected on day 7 in the ischemic hindlimbs. B, PTP1B specific activity in nonischemic and ischemic tissue at 7 days after ischemia. C, Upper blots show PTP1B and SHP2 protein expression in nonischemic and ischemic tissues at 7 days after ischemia. Each lane indicates a different mouse. For B and C, the bar graph shows averaged data, expressed as fold change over basal (the value in nonischemic tissue was set to 1). Values are the means±SE (n=4). *P<0.05 vs hindlimb ischemia.
cell–cell junction by reducing VE-cadherin tyrosine phosphorylation and increasing TER, whereas PTP1B siRNA causes opposite effects; (5) PTP1B physically associates with VE-cadherin in HUVECs; and (6) PTP1B expression and activity are dramatically increased in ischemic hindlimb model.

Previous studies demonstrated that several PTPs, including SHP-1, SHP-2, and HCPTPA, bind to VEGFR2 directly or indirectly. However, their endogenous role in VEGFR2 autophosphorylation under physiological condition remains unclear. SHP-1 is involved in tumor necrosis factor α-mediated prevention of VEGFR2 phosphorylation, whereas SHP-2 inhibits tyrosine phosphorylation of VEGFR2 in ECs when they are cultured only on type I collagen. HCPTPA overexpression attenuates VEGFR2 autophosphorylation, but this has not been studied with a loss-of-function approach. Using overexpression and knockdown approaches, as well as coexpression and cotransfection assays, the present study demonstrates that PTP1B functions as a negative regulator for VEGFR2 autophosphorylation in ECs via binding to the VEGFR2 cytoplasmic kinase domain. Of note, VEGFR2 is coprecipitated with PTP1B in HUVECs overexpressing PTP1B-WT, as well as in CHO cells transfected with PTP1B-WT or substrate-trapping mutants of PTP1B (PTP-D/A and PTP1B-C/S), suggesting that PTP1B binds to VEGFR2 in a phosphorylation-independent manner, as demonstrated by its binding to p130Cas. We could not detect PTP1B association with VEGFR2 in LacZ-infected cells, which may be due to the unstable complex formation of endogenous PTP1B with its substrate. In addition, VEGFR2 dephosphorylation assay demonstrates that ligand-induced, activated VEGFR2 is dephosphorylated by PTP1B. Consistent with our findings, PTP1B has been shown to bind to and dephosphorylate platelet-derived growth factor receptor, indicating that PTP1B is a biologically relevant receptor tyrosine kinase phosphatase. Our study cannot rule out the possibility that PTP1B–VEGFR2 interaction is indirect, perhaps through intermediate proteins, which is required for PTP1B-induced dephosphorylation of VEGFR2.

VEGFR2 autophosphorylation is required for activation of diverse signaling pathways, such as ERK1/2 and p38 MAPK, which are involved in EC proliferation and migration, respectively. The downstream signaling events that derive from the autophosphorylated tyrosines within VEGFR2, as well as dephosphorylation of RTKs by PTPs are highly site-specific. Previous reports show that phosphorylation of Y1175 within VEGFR2 is essential for activation of PLCγ, and subsequent ERK phosphorylation and cell proliferation, whereas pY1214 is involved in VEGF-stimulated p38 MAPK activation and cell migration. The present study demonstrates that PTP1B siRNA enhances, whereas overexpression of PTP1B inhibits, phosphorylation of Y1175 of VEGFR2, PLCγ, ERK, and EC proliferation without affecting phosphorylation of p38 MAPK or Akt, as well as EC migration. Consistent with our result, Milarski et al. reported that PTP1B specifically dephosphorylates the epidermal growth factor receptor pY992, which is the specific binding site for PLCγ. On the other hand, using porcine aortic ECs transfected with chimeric murine VEGFR2, Holmqvist et al. and Dayanir et al. showed that VEGFR2-pY1175/1173 is required for phosphatidylinositol 3-kinase activation, which is involved in EC migration and proliferation, respectively. Moreover, PTP1B inhibitors enhance phosphorylation of Akt and endothelial NO synthase and improve peripheral endothelial dysfunction in a chronic heart failure model of mice. This conflicting evidence may be attributable to differences in experimental conditions (cell culture conditions or transfection system with chimeric murine VEGFR2 or cell types or species). It is possible that PTP1B may preferentially bind to the VEGFR2–PLCγ complex but not to the VEGFR2–phosphatidylinositol 3-kinase complex, which may form appropriate conformation or localize at PTP1B-associated subcellular compartments. Furthermore, PTP1B may affect other VEGFR2 phosphorylation sites in addition to pY1175 of the receptor. Nevertheless, our data clearly demonstrate that PTP1B negatively regulates EC proliferation, at least in part, by selectively inhibiting VEGFR2-pY1175-PLCγ–ERK pathway in intact human ECs. Detailed analysis using mutant Y1175F-VEGFR2, characterization of the interaction of PTP1B and VEGFR2 and their localization, and identification of the dephosphorylation site(s) of VEGFR2 by PTP1B are objectives of future studies.

VE-cadherin–mediated cell–cell adhesion is important for preserving endothelial integrity of ECs. Tyrosine phosphorylation of VE-cadherin is associated with a loss of cell–cell contacts in confluent monolayer of ECs and an increase in endothelial permeability, which is initial events to promote angiogenesis. PTPs activity also regulates the integrity of cell–cell junctions. We found that PTP1B binds to and colocalizes with VE-cadherin at cell–cell contacts in the basal state. Overexpression of PTP1B stabilizes endothelial cell–cell junction by reducing VE-cadherin tyrosine phosphorylation and increasing TER, whereas PTP1B siRNA causes opposite effects. Thus, PTP1B functions to maintain endothelial barrier integrity. Consistent with our results, PTP1B has been shown to localize at cell–cell junctions in confluent rat corneal ECs and, in other systems, to bind directly to E-cadherin or N-cadherin, which are involved in stabilizing intercellular junctions. Given that a fraction of VEGFR2 is localized at VE-cadherin–containing cell–cell contacts, it is conceivable that PTP1B localized at adherens junction may form multiple complex with VEGFR2 and VE-cadherin to downregulate VEGF signaling, thereby limiting angiogenic program. Density-enhanced phosphatase-1 (DEP1)/CD148 indirectly associates with VE-cadherin/VEGFR2 complex, which in turn promotes contact-dependent inhibition of VEGFR2 phosphorylation, ERK activation, and EC proliferation. Thus, PTP1B-mediated inhibition of VEGFR2-ERK-EC proliferation may be, at least in part, through promoting cell–cell contacts, thereby contributing to DEP1–mediated contact-dependent inhibition of VEGFR2 signaling in ECs. Additionally, vascular endothelial PTP (VE-PTP) and SHP-2 bind to VE-cadherin and β-catenin, respectively, which are involved in VE-cadherin–mediated barrier function in confluent ECs. These suggest that PTP1B and other PTPs which localize at cell–cell contacts may collaborate to maintain low levels of tyrosine...
PTPs have a reactive Cys residue in the enzyme active site (eg, Cys215 in human PTP1B), which is deprotonated at neutral pH and is susceptible to the reversible oxidative inactivation by agonist-induced reactive oxygen species (ROS).\textsuperscript{35} We and others demonstrated that ROS are involved in VEGFR2 autophosphorylation,\textsuperscript{36–38} as well as loss of cell–cell adhesions through increasing tyrosine phosphorylation of VE-cadherin and/or other junctional proteins.\textsuperscript{39–41} Whether VEGF-induced ROS are involved in oxidative inactivation of PTP1B in ECs is currently under investigation.

General PTP inhibitors promote VEGFR2 activation, blood flow recovery, or angiogenesis after hindlimb ischemia.\textsuperscript{5,6} suggesting PTPs as potential therapeutic targets to promote neovascularization. Using mouse hindlimb ischemia model, we demonstrate that PTP1B expression is increased at lectin-positive ECs and skeletal myocyte in a time-dependent manner with a peak at 7 days after hindlimb ischemia, which is associated with an increase in PTP1B activity. This staining pattern is consistent with the previous report for the induction of VEGFR2 in ischemic skeletal muscle.\textsuperscript{42} Together with our in vitro data, ischemia-induced upregulation of PTP1B may represent an important compensatory mechanism that blunts overactivation of angiogenic signaling in vivo, at least in part, by inhibiting tyrosine phosphorylation of VEGFR2 and VE-cadherin. Our results are consistent with previous reports that PTP1B inhibits platelet-derived growth factor– or fibroblast growth factor–induced proliferation of cultured vascular smooth muscle,\textsuperscript{43} whereas PTP1B expression and neointima formation are increased in vascular injury model.\textsuperscript{43,44} This was interpreted as a counterregulatory function of PTP1B in neointima formation in response to vascular injury. In contrast, SHP-2 expression was not changed after ischemia, and SHP-2 siRNA had no effects on VEGFR2 autophosphorylation in cultured ECs, suggesting specific involvement of PTP1B in angiogenic responses in vitro and in vivo. Sugano et al\textsuperscript{17} reported that SHP-1 protein is increased in a rat hindlimb ischemia model, which prevents tumor necrosis factor α–induced negative inhibitory effects on VEGF signaling. We also found that SHP-1 protein is increased after ischemia, but the extent of its increase is much less than that of PTP1B. Previous reports show that PTP1B inhibitor enhances VEGF-mediated angiogenesis using a mouse Matrigel model,\textsuperscript{45} which cannot eliminate the nonspecific effects. Because our ischemia hindlimb data implicates but does not conclude that PTP1B may play a role in VEGF-induced angiogenesis in vivo, further investigation will be required using PTP1B\textsuperscript{−/−} mice in future study. Given that PTP1B\textsuperscript{−/−} mice prevent type 2 diabetes and obesity\textsuperscript{46,48} in which ischemia-induced angiogenesis is impaired, inhibiting PTP1B may be an important therapeutic strategy to promote new vessel formation in cardiovascular diseases.

In summary, we demonstrate that PTP1B functions as negative regulator of VEGF signaling by dephosphorylating VEGFR2 via binding to the receptor, as well as by stabilizing adherens junctions via inhibiting tyrosine phosphorylation of VE-cadherin that mediates contact-dependent inhibition of VEGFR2 signaling. These mechanisms may contribute to specific inhibition of ERK-EC proliferation pathway. Inhibition of PTP1B by hindlimb ischemia may represent an essential counterregulatory mechanism that blunts widespread, uncontrolled activities of VEGFR2 and other RTKs during angiogenesis in vivo. These findings should provide novel insight into PTP1B as a potential therapeutic target to promote neovascularization in ischemic cardiovascular diseases.

Sources of Funding
This research was supported by NIH grants RO1 HL077524 (to M.U.-F.), DH 43396 (to B.J.G.), and HL70187 (to T.F.); American Heart Association Grants-in-Aid 0555308B and 0755805Z (to M.U.-F.); a Ministry of Education, Culture, Sports, and Science Grant-in-Aid (to Y.N.); Technology of the Japanese Government grant 17688006 (to Y.N.); and a Japan Heart Foundation/Bayer Yakuhin Research Grant Abroad (to H.I.).

Disclosures
None.

References


20. Liu F, Hill DE, Chernoff J. Direct binding of the proline-rich region of protein tyrosine phosphatase 1B interacts with and is tyrosine phosphorylated by the epidermal growth factor receptor. *Biochem J*. 1997;327:139–145.


Role of Protein Tyrosine Phosphatase 1B in Vascular Endothelial Growth Factor Signaling and Cell–Cell Adhesions in Endothelial Cells
Yoshimasa Nakamura, Nikolay Patrushev, Hyoe Inomata, Dolly Mehta, Norifumi Urao, Ha Won Kim, Masooma Razvi, Vidisha Kini, Kalyankar Mahadev, Barry J. Goldstein, Ronald Mckinney, Tohru Fukai and Masuko Ushio-Fukai

Circ Res. published online May 1, 2008;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2008/05/01/CIRCRESAHA.107.167080.citation

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/05/01/CIRCRESAHA.107.167080.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Online data supplement

Role of Protein Tyrosine Phosphatase 1B in VEGF Signaling and Cell-Cell Adhesions in Endothelial Cells

Yoshimasa Nakamura\textsuperscript{1,5}, Nikolay Patrushev\textsuperscript{3}, Hyoe Inomata\textsuperscript{1}, Dolly Mehta\textsuperscript{1}, Ha Won Kim\textsuperscript{2}, Norifumi Urao\textsuperscript{1}, Masooma Razvi\textsuperscript{1}, Vidisha Kini\textsuperscript{1}, Kalyankar Mahadev\textsuperscript{4}, Barry J. Goldstein\textsuperscript{4}, Ron Mckinney1, Tohru Fukai\textsuperscript{2}, Masuko Ushio-Fukai\textsuperscript{1}

\textsuperscript{1} Department of Pharmacology, and \textsuperscript{2}Departments of Medicine and Pharmacology, University of Illinois at Chicago, Chicago, IL
\textsuperscript{3}Emory University School of Medicine, Atlanta, GA
\textsuperscript{4}Division of Endocrinology, Diabetes and Metabolic Diseases
Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA
\textsuperscript{5} Department of Biofunctional Chemistry, Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan

Running title: Role of PTP1B in VEGF signaling

Address correspondence to:

Masuko Ushio-Fukai, Ph.D
Dept. of Pharmacology
Center for Lung and Vascular Biology
University of Illinois at Chicago
835 S. Wolcott, M/C868,
E403 MSB
Chicago, IL 60612
Phone: 312-996-7665
Fax: 312-996-1225
Email: mfukai@uic.edu
Materials and Methods

Materials- Antibodies to VEGFR2, phosphotyrosine (pY99), VE-cadherin, and phospho-PLCγ (pY783), actin and GAPDH were from Santa Cruz. Antibodies to phospho-VEGFR2 (pY1175), phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-p38 mitogen-activated protein kinase (MAPK), ERK1/2, and p38 MAPK were from Cell Signaling. Anti-PTP1B monoclonal antibody and rabbit polyclonal antibody were from Calbiochem and Upstate, respectively. Human recombinant VEGF165 was from R&D Systems and BRB Preclinical Repository. Oligofectamine, and Opti-MEMI Reduced-Serum Medium were from Invitrogen Corp. CellTiter 96® solution cell proliferation assay was obtained from Promega. Other materials including anti-vinculin antibody were purchased from Sigma.

Cell Culture- Human umbilical vein ECs (HUVECs) were purchased from VEC Technologies, Inc. (Rensselaer, NY) and were grown in endothelial basal medium2 (EBM2, Clonetics) containing 10% fetal bovine serum (FBS) as described. Experiments were performed using cells between passages 3 and 6.

Immunoprecipitation and Immunoblotting- Growth-arrested HUVECs were stimulated with VEGF (10 ng/ml) and cells were lysed in lysis buffer, pH 7.4 (in mM) 50 HEPES, 5 EDTA, 50 NaCl, 1% Triton X-100, protease inhibitors (10 μg/ml aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) and phosphatase inhibitors ((in mmol/L) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate). Cell lysates were used for immunoprecipitation and immunoblotting as described previously.

Transient Transfection of CHO cells- CHO cells were transiently transfected with pcDNA3-myc-VEGFR2cyto (entire intracellular domain of human KDR, residues 790-1356) and pMT2-GST-PTP1B-WT or PTP1B-D181A or PTP1B-C215S which were kindly provided by Dr. Nicholas Tonks using the Polyfect according to manufacturer’s instruction (Qiagene).

Adenovirus Transduction - HUVECs were incubated with 10 multiples of infection (MOI) of either adenovirus expressing wild-type PTP1B (Ad.PTP1B-WT) or PTP1B-C/S (catalytically inactive C215S mutant; Ad.PTP1B-C/S) or Ad.LacZ (control) in 10% FBS
containing culture medium for 24 hr, followed by incubation in 0.5%FBS without virus for 12 hr before experiments, as we described previously 4.

**siRNA Transfection**- RNA oligonucleotides were obtained from Sigma. The sequences of specific siRNA against PTP1B is; 5’-AAATCAACGGAAGAGGTTCT-3’. The scrambled siRNA control is 5’-GAGATGACACGACUGAGATAA-3’. We performed a Blast search and confirmed that the PTP1B and scrambled siRNA sequences have no overlap with other proteins. HUVECs were grown to 60 % confluence in 100 mm dishes and transfected with 30 nM siRNA using Oligofectamine (Invitrogen), as described previously 5. Cells were used for experiments at 48 hr after transfection.

**PTP1B Activity Assay**- HUVEC cells or mice hindlimb muscles were snap-frozen in liquid N₂, and disrupted by scraping into ice-cold, deoxygenated homogenization buffer (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, in 50 mM Hepes, pH 7.5, containing a protease inhibitor mixture (Sigma), 1% (v/v) Triton X-100, and 0.5% (v/v) NP-40, followed by brief sonication. The whole cell lysate was cleared by centrifugation at 15,000 g for 20 min. Specific PTP1B activity was measured by the hydrolysis of p-nitrophenyl phosphate(pNPP; Sigma) in PTP1B immunoprecipitates. Briefly, PTP1B immunoprecipitates from 500 μg of cell lysates were incubated in a final volume of 100 μl at 37 °C for 30 min in reaction buffer containing 10 mM pNPP and 2 mM EDTA in 20 mM MES at pH 6.0. The reaction was stopped by the addition of 200 μl of 5 M NaOH, and the absorption was determined at 410 nm 6.

**In Vivo Receptor Dephosphorylation Assays**- Growth-arrested HUVECs were stimulated with VEGF for 5min, and cell lysates were immunoprecipitated with rabbit anti-VEFGR2 antibody and immobilized on protein A/G agarose for 1.5 hr. Beads were washed and incubated in the presence of recombinant active PTP1B protein (Biomol, 50-200ng) at 30 °C for 10 min in 50 μl of buffer (20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 12 mM MgCl, 1 mM dithiothreitol, 10 μM ATP). The reaction was stopped by adding 2x sample buffer and samples were immunoblotted with anti-phosphotyrosine or VEGFR2 antibodies.
Cell Proliferation Assay- HUVECs (10^5 cells) were seeded in 6-well plates in EBM containing 10% FBS overnight, and incubated in EBM containing 0.5% FBS for 24 hours and then incubated with or without stimulants in EBM containing 0.2% FBS for 48 hours. After culturing, 20 μl of an CellTiter 96® solution was added to each well, and the absorbance was measured at 490 nm according to the manufacturer's instructions after incubation at 37°C for 2-3 h. In some experiments, after trypsinization, the cell number was determined by counting with a hemocytometer.

Modified Boyden Chamber Migration Assay- Migration assays using a Modified Boyden Chamber method were conducted in 24-well transwell chambers as described previously.

Confocal Immunofluorescence Microscopy- HUVECs growing on 0.1% gelatin-coated glass coverslips were stimulated with VEGF or vehicle alone, and fixed with 4% paraformaldehyde in PBS, and permeabilized in 0.05% Triton X-100/PBS for 5 min. After blocking, cells were incubated with rabbit anti-VE-cadherin antibody for 1 hr, incubated in FITC-conjugated goat anti-rabbit IgG for 1 hr. Images were taken using the confocal laser scanning imaging system Zeiss LSM 510. In some experiments, HUVECs were transiently transfected with plasmid encoding GFP-PTP1B-D/A or GFP only which were kindly provided by Dr. Carlos Arregui using Amaxia Nucleofector System according to manufacturer’s instruction, as reported previously. After transfection, cells were stimulated with VEGF, fixed, permeabilized, and were incubated with anti-VE-cadherin antibody, followed by Rhodamine Red X-conjugated goat anti-rabbit IgG.

Transendothelial Electrical Resistance Measurement- The time course of endothelial cell retraction in real time, as a measure of increased endothelial permeability, was recorded as described previously.

Mouse Ischemic Hindlimb Model- Study protocols were approved by the Animal Care and Use Committee of University of Illinois at Chicago. Female C57BL/6J mice (8-9 weeks of age) were obtained from The Jackson Laboratory. The right superficial femoral artery was ligated proximally and distally with 5-0 silk ligatures, and excised. To measure hind limb blood flow we used a laser Doppler blood flow (LDBF) analyzer (Lisca AB,
Sweden) as described previously. At 0, 1, 3 7 days after ischemia, thigh adductor muscle in ischemic hindlimbs were used for immunoblotting and immunohistochemistry as described previously.

**Statistical Analysis** - Results are expressed as mean ± S.E. Statistical significance was assessed by Student's paired two-tailed t-test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the Super ANOVA statistical program (Abacus Concepts, Berkeley, CA). A p value of <0.05 was considered to be statistically significant.
References


Figure legends for Supplemental Figures

Supplemental Figure I. PTP1B overexpression inhibits while PTP1B knockdown enhances VEGF-induced phosphorylation of VEGFR2-Tyr1175, PLCγ1 and ERK1/2. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled (control) or PTP1B siRNAs (B) were stimulated with VEGF (20 ng/ml) for 5 min. Lysates were immunoblotted with anti-phospho-VEGFR2 (pY1175) or phospho-PLCγ1 (pY783) or phospho-ERK1/2 antibodies. Bottom panels show averaged data, expressed as fold change of phosphorylation over basal (means ± S.E., n=3). *P < 0.05 for VEGF-induced changes in cells infected with Ad.PTP or transfected with PTP1B siRNA vs control.

Supplemental Figure II. PTP1B overexpression or knockdown has no effects on VEGF-induced cell migration. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled or PTP1B siRNAs (B) were used for measurement of cell migration with the modified Boyden chamber method. Cells were stimulated with 50 ng/ml VEGF for 6 hours. Bar graph represents averaged data, expressed as cell number counted per 10 fields (X200) and fold change in cell number over that in unstimulated LacZ-infected (A) or scrambled siRNA-transfected (B) cells (control).

Supplemental Figure III. PTP1B negatively regulates tyrosine phosphorylation of VE-cadherin through binding to VE-cadherin. HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S (A) or transiently transfected with scrambled or IQGAP1 siRNAs (B) were stimulated with VEGF (20 ng/ml). Lysates were immunoprecipitated with anti-pTyr antibody, and then immunoblotted with VE-cadherin antibody, and averaged data for tyrosine phosphorylation of VE-cadherin, as expressed by mean±SE for 3 independent experiments are shown. C, HUVECs infected with Ad.LacZ (control), Ad.PTP1B-WT, or Ad.PTP1B-C/S were stimulated with VEGF (20 ng/ml) for 5 min, and lysates were immunoprecipitated with anti-PTP1B antibody, and then immunoblotted with VE-cadherin antibody. The blots are representative of 3 separate
Supplemental Figure IV. Increase of PTP1B expression at lectin-positive ECs and skeletal myocyte in mouse ischemic hindlimb model of angiogenesis. Hindlimb ischemia was induced by the right femoral artery ligation as described in Materials and Methods. Immunostaining of ischemic adductor skeletal muscle with anti-PTP1B antibody (green) or lectin which stains ECs of capillaries (red) at 0, 3 and 7 days after ischemia was visualized with a confocal fluorescence microscope. NC indicates staining without primary antibody (negative control).
Supplemental Figure I

A

<table>
<thead>
<tr>
<th>VEGF</th>
<th>Ad.LacZ</th>
<th>Ad.PTP1B-WT</th>
<th>Ad.PTP1B-C/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

IB

- VEGFR2-pY1175
- p-PLCγ1
- p-ERK1/2
- PTP1B
- Actin

Fold change

VEGF - + Ad.LacZ Ad.PTP1B-WT Ad.PTP1B-C/S

*
Supplemental Figure III

A

B

C

**Figure A**

*VE-cadherin-pY Fold change*

0  5  15
LacZ  PTP1B-WT  PTP1B-C/S

**Figure B**

*VE-cadherin-pY Fold change*

0  5  15
Scrambled siRNA  PTP1B siRNA

**Figure C**

<table>
<thead>
<tr>
<th></th>
<th>LacZ</th>
<th>PTP1B-WT</th>
<th>PTP1B-C/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Western Blot**

*IP: PTP1B  IB: VE-cadherin  IB: PTP1B  IB: Actin*
Supplemental Figure IV

Day 0
Day 3
Day 7