Protein tyrosine phosphatase activity regulates endothelial cell-cell interactions, the paracellular pathway, and capillary tube stability

Bradford A. Young, Xiufen Sui, Timothy D. Kiser, Sang Won Hyun, Ping Wang, Serhan Sakarya, Daniel J. Angelini, Kane L. Schaphorst, Jeffrey D. Hasday, Alan S. Cross, Lewis H. Romer, Antonino Passaniti, and Simeon E. Goldblum. Protein tyrosine phosphatase activity regulates endothelial cell-cell interactions, the paracellular pathway, and capillary tube stability. Am J Physiol Lung Cell Mol Physiol 285: L63–L75, 2003. First published March 7, 2003; 10.1152/ajplung.00423.2002.—Protein tyrosine phosphorylation is tightly regulated through the actions of both protein tyrosine kinases and protein tyrosine phosphatases. In this study, we demonstrate that protein tyrosine phosphatase inhibition promotes tyrosine phosphorylation of endothelial cell-cell adherens junction proteins, opens an endothelial paracellular pathway, and increases both trans-endothelial albumin flux and neutrophil migration. Tyrosine phosphatase inhibition with sodium orthovanadate or phenylarsine oxide induced dose- and time- dependent increases in [14C]bovine serum albumin flux across postconfluent bovine pulmonary artery endothelial cell monolayers. These increases in albumin flux were coincident with actin reorganization and intercellular gap formation in both postconfluent monolayers and preformed endothelial cell capillary tubes. Vanadate (25 μM) increased tyrosine phosphorylation of endothelial cell proteins 12-fold within 1 h. Tyrosine phosphorylated proteins were immunolocalized to the intercellular boundaries, and several were identified as the endothelial cell-cell adherens junction proteins, vascular-endothelial cadherin, and β-, γ-, and p120-catenin as well as platelet endothelial cell adhesion molecule-1. Of note, these tyrosine phosphorylation events were not associated with disassembly of the adherens junction complex or its uncoupling from the actin cytoskeleton. The dose and time requirements for vanadate-induced increases in phosphorylation were comparable with those defined for increments in transendothelial [14C]albumin flux and neutrophil migration, and pretreatment with the tyrosine kinase inhibitor herbimycin A protected against these effects. These data suggest that protein tyrosine phosphatases and their substrates, which localize to the endothelial cell-cell boundaries, regulate adherens junctional integrity, the movement of macromolecules and cells through the endothelial paracellular pathway, and capillary tube stability.

THE VASCULAR ENDOTHELIUM PRESENTS a selective barrier that actively regulates paracellular movement of circulating macromolecules and cells into extravascular tissues and compartments (16, 19, 26). In response to a range of stimuli, endothelial cells (EC) undergo cytoskeletal reorganization and profound shape changes that open the paracellular pathway (19). Angiogenesis, the formation of new blood vessels from a preexisting vascular structure, is a tightly orchestrated multistep process that includes similar changes in EC-EC interactions (14, 22). Early in the process, EC must disengage from adjacent EC to migrate toward angiogenic stimuli. With the reestablishment of vessels, EC must again associate with EC to form new capillary tubes. These dynamic changes in EC-EC communication are required for opening of the paracellular pathway and angiogenesis (14, 26).

In the EC, a circumferential band of actin microfilaments (19, 58) is tethered to the EC-EC adherens junction or the zonula adherens (ZA), an intercellular junctional complex that modulates homophilic cell-cell adhesion (18, 44). Vascular endothelial (VE)-cadherin, a membrane-spanning glycoprotein with an ectodomain that dictates homophilic adhesive specificity and a cytoplasmic domain that is indirectly tethered to the actin cytoskeleton, is central to ZA organization (18, 44). Although multiple cadherins can be coexpressed and differentially distributed in EC, VE-cadherin ap-

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pears to be unique in that it is localized to the intercellular junctions (17, 18, 44). At least three cytoplasmic proteins collectively termed the catenins, including α-catenin, β-catenin, γ-catenin, also known as plakoglobin, and possibly p120ctn, participate in anchoring the cytoplasmic domain of cadherins to actin microfilaments. β-, γ-. And p120 catenin each directly binds to cadherin. β- And γ-catenin compete for the same binding site, whereas p120ctn associates with a more membrane-proximal sequence. β- And γ-catenin each bind to α-catenin (1, 51), which couples the cadherin-catenin complex to the actin cytoskeleton (40, 51). This ZA/peripheral actin band forms a continuous belt around the apical portion of the cell, where it is strategically localized to modulate EC-EC interactions and the paracellular pathway (17, 19).

The state of ZA protein tyrosine phosphorylation is central to the regulation of the ZA/cadherin cytoskeletal linkage and homophilic cell-cell adhesion (33, 34, 39) and, as we (31, 60) and others (20, 46, 53) have shown, to angiogenesis and the maintenance of endothelial barrier function. Although the signal transduction pathways that regulate the state of ZA assembly are incompletely understood, ZA proteins can be modified through tyrosine phosphorylation. α-, β-, γ-. And p120catenin, and cadherins themselves each can be phosphorylated on tyrosine residues (1, 20, 33, 54). Multiple stimuli that induce tyrosine phosphorylation of ZA proteins, including src and ras transformation, mitogenic growth factors, proangiogenic agonists, counter-adhesive proteins, and cytokines, profoundly alter their organization. Increased tyrosine phosphorylation of one or more of these proteins, especially β-catenin, reduces cadherin ectodomain homophilic adhesion. In certain cases, increased tyrosine phosphorylation of β-catenin can promote disassembly of the ZA complex, and/or uncoupling of the ZA from the actin cytoskeleton (34, 39).

Protein tyrosine phosphatases (PTPs) are thought to play a crucial role in regulating the state of ZA protein tyrosine phosphorylation and assembly. Increased expression of a number of PTPs parallels increases in cell density (13, 25, 50). As cells achieve confluence, a group of receptor PTPs that participate in strict homophilic adhesion with identical molecules expressed on the surface of neighboring cells is sequestered at the cell-cell interface (10, 15, 23, 28). Here, their catalytic activities are strategically localized and sustained in close proximity to intercellular junctions, including the ZA. Several PTPs directly associate with and/or dephosphorylate ZA proteins (6, 12, 15, 23, 55).

A number of studies support a role for PTPs in the maintenance of EC-EC junctional integrity and endothelial barrier function. In contact-inhibited confluent human umbilical vein EC (HUVEC), membrane-associated PTP activity is increased ~12-fold compared with subconfluent EC (25). One or more PTPs are prominently or almost exclusively expressed in endothelia (9, 24, 55). In mouse development (24) and adult (9) studies, PTP-μ transcription is almost exclusively localized to vascular endothelia where it colocalizes with Flk-1, a receptor PTK that is exclusively expressed in EC. In HUVEC, the SH2 domain-containing PTP, SHP-2, binds to β-catenin and restrains phosphorylation of β-, γ-, and p120 catenins (55). More recently, VE-PTP, the murine homolog of human PTP-β, was shown to associate with VE-cadherin through its ectodomain and to regulate VE-cadherin phosphorylation and barrier-enhancing activity in transfected Chinese hamster ovary cells, independently of its PTP domain (48). We have found that, for some mediators of tyrosine phosphorylation-dependent increments in paracellular permeability, concurrent PTP inhibition is required for a reliable, reproducible phosphotyrosine signal (31, 61). We also found that coadministration of two structurally and functionally dissimilar PTP inhibitors, vanadate and phenylarsine oxide (PAO), at levels that do not in themselves alter barrier function, enhanced transendothelial albumin flux in response to multiple agonists. In one study, nonselective PTP inhibition decreased electrical resistance across bovine brain EC monolayers coincident with tyrosine phosphorylation of proteins associated with intercellular junctions (53). In a more recent study, diperoxovanadate, a potent PTK activator and PTP inhibitor, decreased transendothelial electrical resistance and increased tyrosine phosphorylation of ZA proteins in bovine pulmonary artery EC (27). Finally, several mediators of increased vascular permeability, including thrombin, TNF-α, and IL-1, reportedly modulate PTP activity (2, 29, 32, 55).

Irrespective of the receptor or signaling pathway activated, a final common pathway for increased endothelial paracellular permeability appears to exist, in which decreased PTP catalytic function permits increased tyrosine phosphorylation of ZA proteins and decreased VE-cadherin ectodomain-mediated, homophilic adhesion. In the current study, we asked whether nonselective PTP inhibition that circumvents agonist activation of one or more EC receptors might disrupt EC-EC junctional integrity in postconfluent EC monolayers or preformed EC tubes and whether these effects were associated with tyrosine phosphorylation of one or more ZA proteins, and/or disassembly of the VE-cadherin-catenin complex.

METHODS

**EC tissue culture.** Bovine pulmonary artery EC (American Tissue Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 20% heat-inactivated, fetal bovine serum, l-glutamine (4 mM), nonessential amino acids, and vitamins, in the presence of penicillin G (50 U/ml) and streptomycin (50 μg/ml) (Sigma, St. Louis, MO) (7, 31). Only EC from passages 2–7 were used for these studies.

**Assay of transendothelial albumin flux.** Transendothelial [14C]bovine serum albumin (BSA) flux was assayed as previously described (7, 31). Briefly, gelatin-impregnated polycarbonate filters (13-mm diameter, 0.4-μm pore size) (Nucleopore, Pleasanton, CA) mounted in polystyrene chemotactic chambers (ADAPS, Dedham, MA) were inserted into wells of 24-well plates. EC (2 × 10⁵) were seeded in each upper compartment and were cultured for 72 h. We established the
baseline barrier function of each monolayer by introducing an equivalent concentration of permeability tracer $[^{14}C]$BSA (1.1 pmol, i.e., 4,800–6,200 dpm/0.5 ml) to each upper compartment for 1 h at 37°C, after which 0.5 ml from the lower compartment was mixed with 4.5 ml of Optifluor Scintillation fluid (Packard Instruments, Downers Grove, IL) and counted in a liquid scintillation counter (Packard). Only those monolayers retaining >95% of the tracer were studied. The monolayers were exposed for 5 min–6 h to the nonselective PTP inhibitors sodium orthovanadate (vanadate, Sigma) or PAO (Sigma) or to medium alone. PTP inhibition was also studied in the presence of the PTK inhibitor herbimycin A (1.0 μM, Sigma), which was introduced 16 h before and sustained throughout the vanadate treatment.

**Assay of EC injury.** To determine whether vanadate- or PAO-induced barrier dysfunction could be ascribed to EC injury, a $[^{51}Cr]$ release assay was employed as previously described (31). Briefly, EC were labeled with $[^{51}Cr]$sodium chromate (Amersham, Arlington, IL), and the labeled monolayers were incubated with vanadate, PAO, or medium alone. The supernatants were centrifuged and counted. Other experiments, EC cultured on bronectin-coated glass coverslips, after which they were stained monolayers directly on polycarbonate filters as previously described (31). Lysates of EC treated with vanadate or media alone

**EC tube formation assay.** Each well of a six-well plate was coated with 0.2 ml of Matrigel (8 mg/ml; BD Biosciences, Bedford, MA) as previously described (60). EC were seeded at 5×10⁵ cells/well into the Matrigel-coated wells and cultured to confluence on a six-well plate. The Matrigel-coated wells were incubated with vanadate, PAO, or medium alone. The supernatants were centrifuged and counted. Other experiments, EC cultured on bronectin-coated glass coverslips, after which they were stained monolayers directly on polycarbonate filters as previously described (31).

**Identification of phosphotyrosine-containing proteins.** To maintain EC monolayers under experimental conditions identical to those of our barrier function assay, we stained monolayers directly on polycarbonate filters as previously described (7). Briefly, EC cultured to confluence on these filters were exposed to vanadate, PAO, or medium alone. In some experiments, monolayers were fixed, rendered permeable, and stained with fluorescein-phalloidin (1.65 × 10⁻⁷ M; Molecular Probes, Eugene, OR). The filters and their attached monolayers were photographed through a Zeiss Axioskop 20 microscope equipped for epifluorescence. In other experiments, EC cultured to confluence on these filters were exposed to vanadate, PAO, or medium alone. In some experiments, monolayers were fixed, rendered permeable, and stained with fluorescein-phalloidin (1.65 × 10⁻⁷ M; Molecular Probes, Eugene, OR). The filters and their attached monolayers were photographed through a Zeiss Axioskop 20 microscope equipped for epifluorescence. In other experiments, EC cultured to confluence on these filters were exposed to vanadate, PAO, or medium alone.

**Immunoblotting for EC phosphotyrosine.** An immunoprecipitation strategy was employed to identify substrates for tyrosine phosphorylation as previously described (31). Lysates of EC treated with vanadate or medium alone were preincubated with either anti-murine or anti-goat IgG cross-linked to agarose (Sigma) for 1 h at 4°C and then incubated overnight at 4°C with specific murine monoclonal antibodies raised against β-, γ-, or p120-catenin (Transduction Laboritories, Lexington, KY), or a goat polyclonal antibody raised against VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA). The resultant immune complexes were immobilized by incubation with IgG cross-linked to agarose for 2 h at 4°C, centrifuged, washed, boiled for 5 min in sample buffer, and again centrifuged. For studies with platelet-endothelial cell adhesion molecule (PECAM)-1, a rabbit polyclonal antibody raised against bovine PECAM-1 (generously provided by Dr. S. M. Albeda, University of Pennsylvania Medical School, Philadelphia, PA) and protein G cross-linked to agarose (Sigma) were used. The supernatants were then processed for immunoblotting with antiphosphotyrosine (4G10) antibody as described above. To control for discrepancies in immunoprecipitation and/or loading efficiencies, we stripped and reprobed blots with the immunoprecipitating antibody. The blots were subsequently incubated with HRP-conjugated anti-mouse IgG (Transduction Laboratories) or HRP-conjugated anti-goat IgG (Santa Cruz) and developed with ECL. Blots were scanned by laser densitometry, and the phosphotyrosine-containing bands were normalized to the immunoprecipitated protein of interest.

**Coinmunoprecipitation assays.** EC were lysed with ice-cold lysis buffer containing 50 mM Tris·HCl (pH 7.4), 1% nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mg/m1 phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, 1 mM sodium fluoride, 10 mM disodium pyrophosphate, 500 μM paraaminothiophenol, and 1 mM PMAO (all purchased from Sigma).

The EC lysates were assayed for protein concentration with a Bio-Rad DC Protein assay kit (Bio-Rad, Richmond, CA). Samples were resolved by electrophoresis on an 8–16% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The blots were probed with a biotinylated antiphosphotyrosine monoclonal antibody (0.8 μg/ml, 4G10; Upstate Biotechnology), incubated with horseradish peroxidase (HRP)-conjugated streptavidin (0.5 μg/ml, Upstate Biotechnology), and developed with enhanced chemiluminescence (ECL, Amerham). To confirm equivalent protein loading and transfer, we stripped blots with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris·HCl, pH 6.7, reprobed them with 0.5 μg/ml murine anti-physarum β-tubulin IgG2b (Boehringer-Mannheim, Indianapolis, IN) (31, 61), and developed them as described above. Blots were scanned by laser densitometry (Molecular Dynamics, Sunnyvale, CA), and phosphotyrosine signal was normalized to β-tubulin.

**PTPs REGULATE ENDOTHELIAL CELL-CELL INTERACTIONS**

These samples were photographed through a Nikon TE-200 microscope with ×60 plan-apo objective with a numerical aperture of 1.4. Images were captured with a quadruple dichroic mirror and separate excitation and emission filters for fluorescein and near-infrared fluorescence (Chroma, Portland, ME) mounted in Orbit filter wheels (Manchester, UK), and a Roper HQ charge-coupled device camera, all run with Openlab software (Improvement, Lexington, MA).
Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, 100 mg/ml type I DNase, 1 mM vanadate, 1 mM sodium fluoride, 10 mM disodium pyrophosphate, 500 μM paranitrophenol, and 1 mM PAO, passed through a 25-gauge needle several times, and processed as described above. Ly-sates of EC treated with vanadate or media alone were preincubated with antibody-coated IgG cross-linked to agarose (Sigma) for 1 h at 4°C and then incubated overnight at 4°C with a goat polyclonal antibody raised against VE-cadherin (Santa Cruz). The resultant immune complexes were processed as described above and immunoblotted with specific murine monoclonal antibodies raised against β-, γ-, p120-catenin (Transduction Laboratories) or actin (Amer- sham). The blots were subsequently incubated with HRP-conjugated anti-mouse IgG (Transduction Laboratories) and developed with ECL. To control for efficiency of immunopre-cipitation and protein loading and transfer, we stripped and reprobed with the anti-VE-cadherin antibody. The blots were scanned by laser densitometry, and the catenin of interest was normalized to VE-cadherin.

GST-VE-cadherin binding assays. GST fusion protein con-taining the cytoplasmic domain of human VE-cadherin was preadsorbed to glutathione Sepharose 4B beads (Pharmacia) and then incubated with EC lysates. The VE-cadherin-bind- ing EC proteins bound to the beads were extensively washed, boiled in sample buffer, resolved by SDS-PAGE, transferred to PVDF, and probed with antibodies raised against either β- or γ-catenin. Simultaneous GST/bead controls also were per-formed.

Human neutrophil preparation. Whole peripheral blood from healthy human volunteers was collected into acid citrate dextran (Sigma) solution, and neutrophils were isolated by dextran erythrocyte sedimentation and density gradient centrifugation through Fibavl-Hypaque (Sigma) as previously described (30). Neutrophils were resuspended at 10⁶ cells/ml in Hanks’ balanced salt solution without divalent cations (HBSS) (Life Technologies, Gaithersburg, MD) and incu-bated with 5 μM calcein AM (Molecular Probes) for 40 min with gentle agitation in the dark (3). Neutrophils were washed three times with HBSS – after which their purity was >95% by differential counts of Wright-stained smears and viability >98% by trypan blue dye exclusion.

Assay for transendothelial neutrophil migration. To estab-lish the functional integrity for each monolayer, we per-formed transendothelial [14C]BSA flux across EC cultured to postconfluence on gelatin-impregnated polycarbonate filters (13-mm diameter, 3-μM pore size) as described in Assay of transendothelial albumin flux. Only EC monolayers retaining >97% of the [14C]BSA tracer were studied. The monolay-ers were then exposed to vanadate-enriched medium or to medium alone in the presence or absence of the PTK inhibitor herbimycin A (1.0 μM, Sigma), which was introduced 16 h before and throughout incubation with vanadate or medium alone. The treated monolayers were inserted into wells contai-nating IL-8 (3 μM) or media alone. Calcein AM-labeled neutrophils (5 × 10⁵ cells/well) were introduced into the upper compartments of assay chambers and incubated for 2 h at 37°C. Each lower compartment was sampled and fluoro-metrically assayed. After migration through EC monolayers cultured on filters, >99% of fluorescence remained neutrophil-associated (data not shown). A standard curve was es-tablished for each experiment from which neutrophil num-bers could be interpolated from fluorescence units, and trans-endothelial migration of neutrophils was expressed as a percentage of total cells that migrated.

Statistical methods. Analysis of variance was used to com-pare the mean responses among experimental and control groups for all experiments. Dunnett’s and Scheffe’s F-test were used to determine between which groups significant differences existed. A P value of <0.05 was considered sig-nificant.

RESULTS

Dose- and time-dependent effects of PTP inhibition on transendothelial [14C]BSA flux. To assess whether PTP inhibition alone could increase transendothelial [14C]BSA flux, we tested each of the nonselective PTP inhibitors, vanadate and PAO, in the barrier function assay (Fig. 1). The means (± SE) pretreatment trans-en-dothelial [14C]BSA flux across monolayers used for the vanadate and PAO studies were 0.009 ± 0.003 pmol/h (n = 167) and 0.009 ± 0.002 pmol/h (n = 175), respectively. The mean (± SE) [14C]BSA transfer across naked filters without EC monolayers was 0.210 ± 0.006 pmol/h (n = 5). Vanadate (Fig. 1A) and PAO (Fig. 1B) each increased transendothelial [14C]BSA flux in a concentration-dependent manner. The lowest concentration of vanadate and PAO that increased [14C]BSA flux compared with the media control was 0.5 and 0.25 μM, respectively. The maximum mean (± SE) [14C]BSA flux seen with vanadate (25 μM) and PAO (1.0 μM) over the 6-h study period was 0.074 ± 0.001 pmol/h (n = 10) and 0.112 ± 0.003 pmol/h (n = 8), respectively. The effect of PTP inhibi-tion on endothelial barrier function with vanadate and PAO was also time dependent (Fig. 1, C and D, respec-tively). Transendothelial [14C]BSA flux was assayed after exposure to fixed concentrations of vanadate (25 μM) or PAO (0.5 μM) vs. media alone over a time period from 0.08 h (5 min) to 6 h. A 0.5-μM PAO concentration was employed because it induced comparable increments in [14C]BSA flux (0.073 ± 0.002 pmol/h, n = 12) as did 25 μM vanadate. PTP inhibition with either vanadate (Fig. 1C) or PAO (Fig. 1D) in-creased [14C]BSA flux compared with the simultaneous
media control by 0.5–1 h. \[^{14}C\]BSA flux across media control monolayers did not change throughout the 6-h study period. These combined data demonstrate that two structurally and functionally dissimilar PTP inhibitors each can increase \[^{14}C\]BSA flux across postconfluent EC monolayers.

**Effect of PTP inhibition on EC injury.** A \(^{51}\)Cr release assay was used to determine whether exposures to the same concentrations and exposure times for the PTP inhibitors, vanadate and PAO, that induce endothelial barrier dysfunction also might induce EC injury. The \(^{51}\)Cr release assay detects defects in the plasma membrane that permit passage of molecules ≤1,000 Da. EC monolayers preloaded with \(^{51}\)Cr were exposed for 6 h to vanadate (25 \(\mu M\)), PAO (0.5 \(\mu M\)), or media alone. Mean (± SE) \(^{51}\)Cr release from either vanadate- or PAO-exposed EC (13.07 ± 0.35%, \(n = 10\)), and 12.89 ± 0.47%, \(n = 10\), respectively) was not significantly different from release from the simultaneous media controls (12.32 ± 0.51%, \(n = 10\)). These studies indicate that changes in barrier function in response to PTP inhibition cannot be ascribed to EC injury.

PTP inhibition promotes intercellular gap formation in postconfluent EC monolayers and preformed EC tubes. We questioned whether the vanadate- and PAO-induced loss of barrier function could be explained through opening of the paracellular pathway. Accordingly, EC monolayers exposed for 6 h to media alone (Fig. 2A), 0.5 \(\mu M\) PAO (Fig. 2B), or 25 \(\mu M\) vanadate (Fig. 2C) were stained with fluorescein-phalloidin, an F-actin-specific reagent. By fluorescence microscopy, EC monolayers incubated with media alone exhibited continuous transcytoplasmic actin filaments and cell-to-cell apposition without intercellular gaps (Fig. 2A). Exposure of EC to 0.5 \(\mu M\) PAO (Fig. 2B) or 25 \(\mu M\) vanadate (Fig. 2C) induced disruptions within the F-actin lattice exclusively at the EC-EC interface and circumferential redistribution of F-actin staining to the cell periphery. In other experiments, EC were cultured in Matrigel-coated wells. EC attached within 0.5 h and formed interconnected tubular networks between 6 and 16 h. PTP inhibition with either PAO (Fig. 2E) or vanadate (Fig. 2F) disrupted EC-EC interactions, whereas EC-matrix attachments were sustained (Fig. 2D–F). These data indicate that PTP inhibition with either vanadate or PAO promotes actin reorganization and intercellular gap formation in postconfluent EC.
monolayers as well as in preformed EC tubular structures.

PTP inhibition increases tyrosine phosphorylation of EC proteins. PTP inhibition with pervanadate (vanadate complexed with H₂O₂) has previously been demonstrated to induce rapid and dramatic increases in the tyrosine phosphorylation of proteins localized to the intercellular boundaries (4, 53). We therefore asked whether vanadate-induced intercellular gap formation and increases in transendothelial [¹⁴C]BSA flux could be correlated to increases in protein tyrosine phosphorylation. Accordingly, EC exposed for 1 h to increasing concentrations of vanadate (0.5–25 μM) were processed for phosphotyrosine immunoblotting (Fig. 3). Vanadate at concentrations as low as 0.5 μM increased the phosphotyrosine signal compared with the effect seen with media alone. This same vanadate concentration was the lowest concentration that induced increments in [¹⁴C]BSA flux (Fig. 1A). PTP inhibition with vanadate (25 μM) induced tyrosine phosphorylation of a number of EC proteins including several that migrated with an apparent Mr of 240,000, 220,000, 185,000, 165,000, 135,000, 110,000, 95,000, and 66,000. Over the range of 2.5–25 μM vanadate, this increase in phosphotyrosine signal was concentration dependent. At a fixed concentration of vanadate (25 μM), exposure times as brief as 5 min were associated with increases in phosphotyrosine signal compared with the simultaneous media control (Fig. 3B). These vanadate-induced increases in tyrosine phosphorylation precede the first demonstrable increases in transendothelial [¹⁴C]BSA flux (see Fig. 1C).

Immunolocalization of phosphotyrosine-containing proteins to the EC-EC boundaries with PTP inhibition. To determine the subcellular localization of phosphotyrosine-containing proteins in vanadate-treated EC, postconfluent monolayers exposed to vanadate or media alone were probed with a FITC-conjugated anti-phosphotyrosine antibody and analyzed by epifluorescence microscopy (Fig. 4). EC incubated with vanadate (25 μM) displayed a fluorescence signal that was predominantly restricted to the intercellular boundaries (Fig. 4, B–D), whereas the cells provided with media alone lacked this pattern of immunofluorescence (Fig. 4A). This phosphotyrosine signal was evident as early as 10 min (Fig. 4B) with dramatic increases observed by 1 h (Fig. 4D). These studies indicate that PTP inhibition with vanadate promotes tyrosine phosphorylation of proteins that are either enriched to, or upon, phosphorylation translocate to the EC-EC junctions. Furthermore, these tyrosine phosphorylation events are temporally proximal to intercellular gap formation (Fig. 2, C and E) and opening of the paracellular pathway (Fig. 1C).

PTP inhibition increases tyrosine phosphorylation of EC-EC junctional proteins. To assess whether PTP inhibition with vanadate increases tyrosine phosphorylation of EC-EC adherens junction (ZA) proteins, EC exposed for 1 h to vanadate (25 μM) or media alone were immunoprecipitated with anti-VE-cadherin, anti-

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Fig. 2. Effects of PTP inhibition on intercellular gap formation. A–C: postconfluent endothelial cell (EC) monolayers cultured on filters were exposed for 6 h to media alone (A), 0.5 μM PAO (B), or 25 μM vanadate (C). The monolayers were fixed, rendered permeable, stained with fluorescein-phalloidin, and examined by epifluorescence microscopy. Arrows indicate intercellular gaps. Magnification, ×750. D and E: EC were cultured for 16 h on Matrigel to allow for tube formation, after which they were incubated for 6 h in the presence of PAO (0.25 μM, E), vanadate (25 μM, F), or media alone (D). Arrows indicate intercellular gaps. Magnification, ×320.
PTPs regulate endothelial cell-cell interactions.

**Fig. 3.** Effect of PTP inhibition with vanadate (Van) on protein tyrosine phosphorylation in EC. A: concentration-dependent effect. EC were exposed for 1 h to media alone or increasing concentrations of vanadate (0.5–25 μM). Gel mobility of phosphotyrosine-containing bands is indicated by arrows at right. B: time-dependent effect. EC were exposed to a fixed concentration of vanadate (25 μM, +) or media alone (−) for increasing exposure times. Cell lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, and the blots were probed with antiphosphotyrosine (4G10) IgG. To confirm equivalent protein loading and transfer, we stripped and reprobed each blot with anti-β-tubulin antibody. Molecular masses in kDa are indicated at left. Each blot is representative of 3 experiments.

**Fig. 4.** Immunolocalization of phosphotyrosine-containing proteins in vanadate-treated EC. EC on filters were exposed for 10 min, 30 min, or 1 h to vanadate (25 μM) or media alone, fixed, incubated with FITC-conjugated antiphosphotyrosine antibody, and analyzed by epifluorescence microscopy. A: medium control, 1 h; B: vanadate, 10 min; C: vanadate, 30 min; D: vanadate, 1 h. Arrowheads indicate phosphotyrosine signal at intercellular boundaries. Magnification, ×700.

**Fig. 5.** Identification of tyrosine phosphorylated proteins in EC exposed to vanadate. EC were incubated for 1 h with vanadate (25 μM, +) or media alone (−), lysed, and immunoprecipitated with antibodies raised against vascular endothelial cadherin (VE-cad), β-catenin (β-cat), γ-catenin (γ-cat), or p120ctn (A), or platelet-endothelial adhesion molecule (PECAM-1, B). The immunoprecipitates were resolved by SDS-PAGE and transferred onto PVDF, and the blots were incubated with biotinylated antiphosphotyrosine (4G10) antibody followed by horseradish peroxidase (HRP)-conjugated streptavidin, developed with enhanced chemiluminescence (ECL) and subjected to laser densitometry. For normalization of phosphotyrosine signal to the immunoprecipitated protein of interest, blots were stripped and reprobed with the immunoprecipitating antibodies, i.e., anti-VE-cadherin, anti-β-catenin, anti-γ-catenin, anti-p120ctn, or anti-PECAM-1. IP, immunoprecipitate; IB, immunoblot; IB*, immunoblot after stripping. Each blot is representative of 3 experiments.

β-catenin, anti-γ-catenin, or anti-p120ctn antibodies, and the immunoprecipitates were processed for phosphotyrosine immunoblotting (Fig. 5A). PTP inhibition with vanadate increased tyrosine phosphorylation of VE-cadherin ≥5-fold, β-catenin ≥4-fold, γ-catenin ≥4-fold, and p120ctn ≥5-fold compared with media controls. These data indicate that vanadate, at a concentration and exposure time that opens the paracellular pathway (see Figs. 1, A and C, and 2C), increases tyrosine phosphorylation of components of the ZA multiprotein complex. In similar experiments, vanadate also increased tyrosine phosphorylation of PECAM-1 ≥4-fold (Fig. 5B). Whether tyrosine phosphorylation of one or more of these junctional proteins regulates
EC-EC adhesion in the context of paracellular pathway function and/or angiogenesis is unclear.

**Effect of PTP inhibition on the state of ZA assembly.** Increased tyrosine phosphorylation of ZA proteins can disrupt the intercellular cadherin-catenin linkage and/or uncouple the ZA from the actin cytoskeleton (6, 34, 39). Thus we examined whether vanadate-induced tyrosine phosphorylation of ZA protein components altered the association of VE-cadherin with β-, γ-, or p120-catenin, or with the actin cytoskeleton. Immunoprecipitation of VE-cadherin from lysates of EC treated for 1 h with vanadate or media alone coimmunoprecipitated both catenins and actin, and no differences in coimmunoprecipitation of either the catenins or actin could be demonstrated (Fig. 6A). Immunoprecipitation with an irrelevant antibody did not precipitate VE-cadherin, β-, γ-, and p120-catenin, or actin (data not shown). To confirm our findings that the state of tyrosine phosphorylation of either β- or γ-catenin does not influence its ability to bind VE-cadherin, lysates from EC exposed for 1 h to vanadate (25 μM) or media alone were incubated with a GST fusion protein containing the cytoplasmic domain of human VE-cadherin coupled to glutathione Sepharose beads or a GST bead control (Fig. 6B). The VE-cadherin-binding proteins bound to the beads were extensively washed and processed for immunoblotting with either anti-β-catenin or anti-γ-catenin antibodies. In the presence or absence of PTP inhibition, the binding of either β- or γ-catenin to the cytoplasmic domain of VE-cadherin was equivalent (Fig. 6B). These combined data (Fig. 6, A and B) suggest that in EC, tyrosine phosphorylation of ZA protein components does not dictate disassembly of the ZA multiprotein complex or uncoupling of the ZA from the actin cytoskeleton but is sufficient to promote intercellular gap formation and opening of the paracellular pathway.

**Effect of PTP inhibition on colocalization of VE-cadherin with either β- or γ-catenin.** To extend our in vitro findings to an intact cell system, we applied double-label colocalization fluorescence microscopy. EC monolayers were incubated for 1–6 h with vanadate (25–100 μM) or media alone, after which they were probed with antibodies against VE-cadherin and either β- or γ-catenins and processed for epifluorescence microscopy (Fig. 7). In the media control monolayers, discrete and continuously linear staining for VE-cadherin (Fig. 7, A and C) and either β-catenin (Fig. 7A) or γ-catenin (Fig. 7C) was evident. These fluorescent signals were almost exclusively restricted to intercellular boundaries and revealed a high degree of VE-cadherin-catenin colocalization (Fig. 7, A and C). In some areas, these same control cells also exhibited short, discontinuous regions of EC-EC contact also displaying a high degree of cadherin-catenin colocalization (Fig. 7, A and C). PTP inhibition with increasing concentrations of vanadate for increasing exposure times induced both dose- and time-dependent changes, the most subtle of which were seen after a 1-h exposure to 25 μM vanadate, where no changes in colocalization of VE-cadherin with either β- or γ-catenin could be detected (data not shown). The most dramatic changes were evident after a 6-h exposure to 100 μM vanadate (Fig. 7, B and D). In these EC, PTP inhibition was associated with a marked increase in the arrays of multiple short segments of cadherin/catenin staining that retained a remarkable degree of VE-cadherin/catenin colocalization (Fig. 7, B and D). PTP inhibition with increasing concentrations of vanadate for increasing exposure times induced both dose- and time-dependent changes, the most subtle of which were seen after a 1-h exposure to 25 μM vanadate, where no changes in colocalization of VE-cadherin with either β- or γ-catenin could be detected (data not shown). The most dramatic changes were evident after a 6-h exposure to 100 μM vanadate (Fig. 7, B and D). In these EC, PTP inhibition was associated with a marked increase in the arrays of multiple short segments of cadherin/catenin staining that retained a remarkable degree of VE-cadherin/catenin colocalization (Fig. 7, B and D). PTP inhibition with increasing concentrations of vanadate for increasing exposure times induced both dose- and time-dependent changes, the most subtle of which were seen after a 1-h exposure to 25 μM vanadate, where no changes in colocalization of VE-cadherin with either β- or γ-catenin could be detected (data not shown). The most dramatic changes were evident after a 6-h exposure to 100 μM vanadate (Fig. 7, B and D). In these EC, PTP inhibition was associated with a marked increase in the arrays of multiple short segments of cadherin/catenin staining that retained a remarkable degree of VE-cadherin/catenin colocalization (Fig. 7, B and D).
onist-induced protein tyrosine phosphorylation, intercellular gap formation, and opening of an endothelial paracellular pathway (7, 31, 61). To determine whether PTK-dependent phosphorylation of EC proteins was operative during barrier dysfunction in response to PTP inhibition, we employed the PTK inhibitor herbimycin A (Fig. 8A). The mean (± SE) pretreatment baseline barrier function was 0.010 ± 0.001 pmol/h (n = 72), and there were no significant differences among the experimental groups. [14C]BSA flux across EC monolayers treated with herbimycin A (1.0 μM) alone was not different from that of the media controls. A 6-h vanadate (25 μM) exposure increased transendothelial [14C]BSA flux and pretreatment of EC monolayers with herbimycin A protected against this increment by >80%. To confirm that this concentration of herbimycin A that protected against loss of barrier function also diminished tyrosine phosphorylation of EC proteins, we processed EC exposed to vanadate with or without herbimycin A for phosphotyrosine immunoblotting (Fig. 8B). PTK inhibition with herbimycin A diminished vanadate-induced tyrosine phosphorylation of EC proteins. Thus the PTK inhibitor that protected against opening of the paracellular pathway gained entry into EC and blocked vanadate-induced protein tyrosine phosphorylation. The ability of prior PTK inhibition with herbimycin A to block vanadate-induced increments in albumin flux demonstrates that ongoing, constitutive protein tyrosine phosphorylation is required for opening of the paracellular pathway in response to PTP inhibition. These data indicate that both PTKs and PTPs actively regulate the endothelial paracellular pathway.

**PTP inhibition promotes transendothelial migration of neutrophils.** To determine whether this same tyrosine phosphorylation-responsive paracellular pathway might be accessed by migrating neutrophils, we employed an assay for transendothelial migration of neutrophils. PTP inhibition with vanadate enhanced transendothelial migration of neutrophils ~3.5-fold compared with the simultaneous media controls (Fig. 8C). Prior PTK inhibition with herbimycin A protected against the vanadate effect by >80%. These data suggest that transendothelial paracellular migration of neutrophils is also regulated through the action of both EC PTKs and PTPs.

**DISCUSSION**

In this study, we demonstrate that nonselective PTP inhibition promotes dose- and time-dependent increases in protein tyrosine phosphorylation that precede intercellular gap formation in postconfluent EC monolayers and preformed EC capillary tubes. The phosphotyrosine-containing proteins were immunolocalized to the intercellular boundaries, and several of these substrates were identified as the ZA component VE-cadherin, and β-, γ-, and p120-catenins, as well as the adhesion molecule PECAM-1. Increased tyrosine phosphorylation of ZA proteins was not associated with uncoupling of VE-cadherin from either the catenins or the actin cytoskeleton. Vanadate treatment increased both transendothelial [14C]BSA flux and neutrophil migration, and each was diminished by prior PTK inhibition. These combined data suggest that PTPs regulate the endothelial paracellular pathway through their ability to restrain tyrosine phos-
Tyrosine phosphorylation of ZA proteins reduces EC-EC junctional integrity (57) and decreases both cell-cell junctional permeability (53) and transcellular electrical resistance (53). With these combined data in mind, we asked whether PTPs might participate in the regulation of endothelial barrier function.

In our studies, nonselective PTP inhibition with either of two structurally and functionally dissimilar agents, vanadate and PAO, increased transendothelial albumin flux in a dose- and time-dependent manner (Fig. 1). F-actin fluorescence microscopy of postconfluent EC monolayers subjected to PTP inhibition demonstrated intercellular gap formation with circumferential redistribution of F-actin (Fig. 2, A–C). Vanadate-sensitive PTPs that target cytoskeletal proteins (59) could participate in the actin reorganization seen in EC after PTP inhibition. Similarly, PTP inhibition induced intercellular gap formation in preformed EC tubes (Fig. 2, D–F). These data indicate that nonselective PTP inhibition decreases endothelial barrier function at the level of the paracellular pathway.

To increase our understanding of PTP(s) that may regulate the paracellular pathway, we pursued their substrates. After PTP inhibition, phosphotyrosine immunoblotting demonstrated phosphotyrosine-containing bands, several of which migrated with gel mobilities compatible with one or more EC-EC junctional proteins. The dose and time requirements for increased tyrosine phosphorylation in response to PTP inhibition were compatible with those necessary for loss of barrier function. Phosphotyrosine fluorescence microscopy immunolocalized these phosphotyrosine-containing proteins almost exclusively to EC-EC boundaries. This suggested that PTPs dephosphorylate proteins associated with intercellular junctions and/or proteins that upon tyrosine phosphorylation translocate to the cell periphery. The receptor PTPs that sequester at the cell-cell interface through ectodomain homophilic adhesion (10, 15, 23, 28) might contribute to this phos-
photorysine signal preferentially displayed within intercellular boundaries in response to PTP inhibition (Fig. 4). When we immuno-screened for ZA protein substrates, the tyrosine phosphorylation states of VE-cadherin and β-, γ-, and p120 catenins each were increased. It is conceivable that one or more of the reported ZA-associated PTPs, especially two expressed in EC, PTP-1 and SHP-2 (9, 55), are involved. In another study, PTP inhibition in bovine brain EC increased tyrosine phosphorylation of the tight junctional proteins zona occludens-1 and -2 (53). It is possible that ZA-associated PTPs bind to and/or dephosphorylate substrates within other junctional complexes that reside within the same subcellular compartment. For example, SHP-2, which is recruited to tyrosine phosphorylated PECAM-1, also restrains tyrosine phosphorylation of ZA proteins (55).

The mechanism(s) by which the tyrosine phosphorylation state of one or more ZA proteins can, through in-to-out signaling, regulate VE-cadherin ectodomain-mediated homophilic adhesion is poorly understood. Because ZA disassembly and disruption of the ZA-actin cytoskeletal linkage is known to reduce cell-cell homophilic adhesion (34, 39), we asked whether PTP inhibition might open the endothelial paracellular pathway through uncoupling of VE-cadherin from its catenin binding partners and the actin cytoskeleton. PTP inhibition with vanadate (25 μM) for 1 h, which is associated with dramatic increases in both ZA protein tyrosine phosphorylation (Fig. 5A) and paracellular permeability (Fig. 1C), did not decrease VE-cadherin colocalization with either β- or γ-catenin (data not shown). Similarly, this same PTP inhibition did not, under moderately stringent conditions, diminish coimmunoprecipitation of β-, γ-, or p120 catenin or actin with VE-cadherin (Fig. 6A). An in vitro binding assay, no decrease in binding of either tyrosine-phosphorylated β- or γ-catenin to immobilized nonphosphorylated VE-cadherin could be detected (Fig. 6B), suggesting that the tyrosine phosphorylation state of VE-cadherin is not critical to VE-cadherin-catenin binding. PTP inhibition with vanadate only at higher concentrations for prolonged exposure times was associated with diminished VE-cadherin/catenin staining at intercellular boundaries (Fig. 7). In these monolayers, VE-cadherin and β-/γ-catenins could be localized to short, discrete, linear segments (Fig. 7, B and D). Even after this more rigorous PTP inhibition (i.e., vanadate 100 μM, 6 h), VE-cadherin colocalization with either β-/γ-catenin was remarkably sustained. It is conceivable that these discrete, discontinuous segments of VE-cadherin-catenin colocalization represent an intermediate state for ZA organization that allows for increased flexibility as EC populations transition from a quiescent, contact-inhibited barrier to migratory EC responding to intimal injury or EC clusters organizing into tubes during angiogenesis. These combined data demonstrate that tyrosine hyperphosphorylation of VE-cadherin or any of its associated catenins does not in itself dictate VE-cadherin-catenin disassembly.

In postconfluent EC, membrane-spanning VE-cadherin molecules are trapped at EC-EC junctions through ectodomain homophilic adhesion. Opening of the paracellular pathway theoretically permits disengagement of homophilically bound VE-cadherin ectodomains and lateral mobility in the lipid bilayer with redistribution across the plasma membrane. It is conceivable that as VE-cadherin and either β- or γ-catenin leave the intercellular junctions, they do so together as a VE-cadherin-catenin complex. This would permit more dynamic and efficient ZA disassembly/reassembly in response to rapidly changing physiological demands. The EC response to PTP inhibition was not uniform across the entire EC monolayer. Our VE-cadherin coimmunoprecipitation and GST-VE-cadherin in vitro binding assays reflect only the average of the cellular events occurring throughout the entire monolayer and might not detect subtle changes in protein-protein interactions restricted to an EC subpopulation and/or to a subcellular compartment. Other studies with non-EC systems have generated conflicting results. In several studies, tyrosine phosphorylation of ZA proteins, especially β-catenin, is associated with disruption of the cadherin-actin cytoskeletal linkage and ZA disassembly (34, 39). In other studies, including our own findings, tyrosine phosphorylation of ZA proteins did not appear to promote ZA disassembly or disruption of the cadherin-actin cytoskeleton linkage (8, 20). It is well known that γ-catenin couples VE-cadherin not only to the actin cytoskeleton, but to the intermediate filament network as well (41, 56). In a recent study, histamine, an established mediator of microvascular paracellular permeability, decreased VE-cadherin coimmunoprecipitation of the intermediate filament protein vimentin (52). Therefore, under certain conditions of increased paracellular permeability, VE-cadherin disengages from the intermediate filament network, whereas VE-cadherin-actin association is sustained. The current study indicates that dramatic changes in the state of ZA assembly and its linkage to the actin cytoskeleton are not prerequisites for tyrosine phosphorylation-dependent opening of the endothelial paracellular pathway.

In our studies, EC were subjected to PTP inhibition in the presence of serum and whatever mitogenic growth factors it contains. We therefore asked whether a basal level of ongoing protein tyrosine phosphorylation was required for the paracellular pathway to open in response to PTP inhibition. Prior PTK inhibition with a broad-spectrum PTK inhibitor blocked the PTP effect. Therefore, constitutively tyrosine-phosphorylated proteins may be a prerequisite for EC responsiveness to PTP inhibition. Our studies confirm the importance of both PTKs and PTPs in endothelial paracellular pathway regulation. PTPs appear to restrain PTK-driven protein tyrosine phosphorylation, whereas PTK-driven tyrosine phosphorylation appears to prime and maintain EC in a tonic state of lowered threshold for PTP inhibition.

The same tyrosine phosphorylation-responsive endothelial paracellular pathway also could be accessed by
neutrophils. An EC–EC junctional protein that participates in transendothelial polymorphonuclear neutrophils (PMN) migration, PECAM-1 (or CD31), is a substrate for tyrosine phosphorylation (21, 45, 49). Vanneste et al. (63) and Krumenauer et al. (27) demonstrated that PTPs are operative during the EC response to PTP inhibitors. In this study, we used pharmacological agents that inhibit PTP activity. In this study, we demonstrate that PTP inhibition alone promotes tyrosine phosphorylation of ZA proteins coincident with increases in the movement of macromolecules and neutrophils across the endothelium barrier. Furthermore, our studies indicate that dramatic changes in the state of ZA assembly and its linkage to the actin cytoskeleton are not prerequisites for opening of the paracellular pathway. It is conceivable and even likely that phosphoproteins other than ZA components are operative during the EC response to PTP inhibition. These findings suggest that PTP-dependent modification of ZA protein components may be a vital mechanism employed to regulate movement of macromolecules and neutrophils across the endothelium as well as stability of EC capillary tubes.

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REFERENCES

PTPs REGULATE ENDOTHELIAL CELL-CELL INTERACTIONS


