PECAM-1 Affects GSK-3 β -Mediated β -Catenin Phosphorylation and Degradation

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Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) regulates a variety of endothelial and immune cell biological responses. PECAM-1-null mice exhibit prolonged and increased permeability after inflammatory insults. We observed that in PECAM-1-null endothelial cells (ECs), β -catenin remained tyrosine phosphorylated, coinciding with a sustained increase in permeability. Src homology 2 domain containing phosphatase 2 (SHP-2) association with β -catenin was diminished in PECAM-1-null ECs, suggesting that lack of PECAM-1 inhibits the ability of this adherens junction component to become dephosphorylated, promoting a sustained increase in permeability. β -Catenin/Glycogen synthase kinase 3 (GSK-3 β) association and β -catenin serine phosphorylation levels were increased and β -catenin expression levels were reduced in PECAM-1-null ECs. Glycogen synthase kinase 3 (GSK-3 β) serine phosphorylation (inactivation) was blunted in PECAM-1-null ECs after histamine treatment or shear stress. Our data suggest that PECAM-1 serves as a critical dynamic regulator of endothelial barrier permeability. On stimulation by a vasoactive substance or shear stress, PECAM-1 became tyrosine phosphorylated, enabling recruitment of SHP-2 and tyrosine-phosphorylated β -catenin to its cytoplasmic domain, facilitating dephosphorylation of β -catenin, and allowing reconstitution of adherens junctions. In addition, PECAM-1 modulated the levels of β -catenin by regulating the activity of GSK-3 β , which in turn affected the serine phosphorylation of β -catenin and its proteosomal degradation, affecting the ability of the cell to reform adherens junctions in

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Platelet endothelial cell adhesion molecule-1 (PECAM-1), a cell adhesion molecule, is a 130-kd glycosylated single-pass transmembrane protein with an ectodomain, a transmembrane region, and a cytoplasmic tail.¹ Although the ectodomain is involved in adhesion,² the cytoplasmic domain serves as a scaffold for a variety of proteins and thus mediates biological events.³ There are tyrosine and serine residues in the cytoplasmic tail that become differentially phosphorylated, determining the repertoire of proteins that associate with PECAM-1 and allowing formation of dynamic molecular complexes.⁴

PECAM-1 has been demonstrated to associate with β -catenin.^{5,6} Furthermore, PECAM-1 alters the quantity, activation state, phosphorylation state, and localization of β -catenin.^{6–8} We have demonstrated diminished expression of β -catenin in endothelial cells (ECs) that lack PECAM-1 expression.⁷ Moreover, there is decreased transcriptionally active β -catenin and a lack of its nuclear localization in ECs that lack PECAM-1 expression, which correlates with a slower proliferative rate of these cells at baseline.⁷

When PECAM-1 was transfected into SW480 cells (colon adenocarcinoma cells that do not express endogenous PECAM-1), Ilan et al^{6,9} demonstrated membrane sequestration of β - and γ -catenin, suggesting that PECAM-1 functions as a modulator of several cytoskeletal components. Additionally, the association between PECAM-1 and β -catenin is altered during *in vitro* angiogenesis.⁹ Thus, PECAM-1/ β -catenin association is dynamic and thought to facilitate the development of ad-

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herens junctions by serving, in part, as a reservoir for β -catenin.⁶

Numerous other factors have been demonstrated to be contributory to the regulation of endothelial barrier permeability. Interestingly, Garcia et al demonstrated the role of GSK-3 β as an initial responder and effector of permeability maintenance.³⁹ Specifically, hepatocyte growth factor induced phosphorylation of GSK-3 β , enabling tightening of adherens junctions.¹⁰ GSK-3 β is a pluripotent enzyme¹¹ and is also critically involved in the Wnt pathway,¹² where it regulates cytosolic β -catenin levels.¹³ In the absence of Wnt signals, GSK-3 β is unphosphorylated and "active," whereby it serine phosphorylates β -catenin, targeting it for degradation by the ubiquitin-proteasome system.¹⁴ On stimulation by a Wnt signal, GSK-3 β becomes serine phosphorylated, thus inactivating it and allowing β -catenin accumulation and translocation either to the nucleus to initiate transcription or to the membrane to serve in adherens junctions regulation.¹⁵

In a mouse model of experimental autoimmune encephalomyelitis (EAE; a mouse model of multiple sclerosis) and after intradermal histamine injection, Graesser et al¹⁰ recently demonstrated that there is a more severe and earlier onset of disease in PECAM-1 knockout (KO) mice compared with wild-type (WT) animals. This was attributed to a "leakier" blood-brain barrier, facilitating plasma and cellular transit into the brain, thus inducing disease in the EAE model and edema after histamine injection. Recently, Carrithers et al¹⁶ have demonstrated that lipopolysaccharide (LPS) induces increased mortality in PECAM-1 KO mice attributable to defective permeability regulation on loss of PECAM-1. Thus, maintenance of endothelial barrier permeability appears to be a key feature of PECAM-1. In this report, we demonstrate that PECAM-1 functions as a key modulator of vascular integrity by influencing β -catenin degradation and β -catenin localization^{4,6,9,17} and expression.⁷

Materials and Methods

Antibodies

Western blots of human and mouse ECs were performed using PECAM-1 C-20 and M-20 (Santa Cruz Biotechnologies, Santa Cruz, CA), β -catenin monoclonal antibody (BD Pharmingen, San Diego, CA), antiphosphotyrosine antibody (PY-99; Santa Cruz Biotechnologies), and anti-histamine H1 receptor (H-300; Santa Cruz Biotechnologies). Anti-GSK-3 β (9332), anti-phospho-serine9-GSK-3 β (9336), anti- β -catenin (9562), anti-Ser33, Ser37, Thr41- β -catenin (9561), anti-phospho Akt (Ser473) (9271), anti-Akt (9272), and anti-phospho-(Tyr) p85-binding motif (4292) were purchased from Cell Signaling Technology (Beverly, MA). In addition, Western blotting for mouse PECAM-1 was performed using "Sleet-4" antibody (a rabbit polyclonal antibody generated by Pinter et al⁸).

Secondary antibodies were horseradish peroxidase conjugates and were obtained from Santa Cruz Biotechnologies or Cell Signaling Technologies. Radioimmuno-

precipitation lysis buffer was from Upstate Biotechnologies (Lake Placid, NY), complete protease inhibitor cocktail was from Roche Pharmaceuticals (Indianapolis, IN), phosphatase inhibitor cocktails I and II were from Calbiochem (San Diego, CA), and leupeptin and aprotinin were from Sigma (St. Louis, MO). Chemiluminescent developing reagents were purchased from Santa Cruz Biotechnologies, Perkin Elmer (Norwalk, CT) (Western Lightning), and Pierce Biotechnology (Rockford, IL) (Western Dura).

Tissue Culture

The wild-type line bEnd.WT and the PECAM-1-null line bEnd.PECAM-1.2, derived from WT and PECAM-1 KO mouse brain endothelium, respectively, were immortalized by retroviral transduction of primary endothelial cell culture with the polyoma virus middle T oncogene. They were the generous gift of Dr. Britta Engelhardt (Theodor Kocher Institute, University of Bern, Switzerland).^{10,18} Similarly, the lung microvascular endothelial cell line luEnd.PECAM-1.1 (PECAM-1 KO) was established by retroviral transduction of primary lung endothelial cells derived from the PECAM-1 knockout mouse with the polyoma virus middle T oncogene.^{10,18,19} These lung microvascular-derived PECAM-1 KO cells were retrovirally transduced with full-length murine PECAM-1 cDNA to generate a PECAM-1 reconstituted (RC) cell line.^{10,18,19} Cells were cultured in medium consisting of Dulbecco's modified Eagle medium, 10% fetal bovine serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1% nonessential amino acids, pyruvate, 10⁻⁵ mol/L 2-mercaptoethanol, and antibiotics. Selection of PECAM-1 expression was maintained with 1 μ g/ml puromycin. Lung microvascular-derived PECAM-1 KO endothelial cells were not transfected with control plasmid and not selected with puromycin. Before and during experiments, the PECAM-1 RC cells were taken off puromycin selection. All of these endothelial cell lines have been shown to exhibit essentially identical expression of ICAM-1, ICAM-2, VCAM-1, and VE-cadherin by several investigators, including us, and have been used to investigate a variety of endothelial cell behaviors.^{10,18-23} These cell lines were used for experiments from passages 8 to 25.

Human umbilical vein endothelial cells (HUVECs) were purchased from the Boyer Center for Molecular Medicine cell culture core (Yale Medical School) and cultured on gelatin in medium consisting of M199 medium, 20% fetal bovine serum, 50 mg/ml endothelial cell growth supplement, 50 mg/ml heparin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, and antibiotics.

PECAM-1 Knockdown

Antisense oligonucleotide 5'-TCCTTCCAGGG ATGT-GATC-3' for human PECAM-1 and control scrambled oligonucleotide 5'-TTCTACCTCGCGCGATTAC-3' were gifts of F. Bennett and T. Condon (Isis Pharmaceuticals, Carlsbad, CA). HUVEC cultures at 70 to 80% confluence were transfected using Lipofectin according to manufacturer's instructions.

Permeability Assay

KO and RC ECs were grown to confluence on collagen IV inserts placed into Biocoat 24-well plates (BD Pharmingen). Medium was replaced with colorless Hank's balanced salt solution after cells were confluent. Histamine was added to the upper chamber. At various time points after adding histamine, 20 μ l of Evans blue dye was added to the upper chamber for 2 minutes. Inserts were removed, and liquid was collected from the lower chamber and read at 650 nm on the spectrophotometer as described previously.¹⁰

Immunofluorescence

To measure the integrity of the EC monolayers before and after histamine treatment (3.2×10^{-5} mol/L; Sigma), confluent cultures of RC and KO ECs plated on type I collagen-coated 60-mm Falcon bacteriological plastic petri dishes were fixed in Streck's Tissue Fixative (Streck Laboratories, La Vista, NE) at various times after histamine stimulation, permeabilized with 0.5% Triton X-100 in Tris-buffered saline, and stained with anti- β -catenin antibody (Santa Cruz Biotechnologies) followed by incubation with tetramethylrhodamine- or fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). After coverslipping with Antifade Mounting Medium (Molecular Probes, Eugene, OR), cells were photographed using an Olympus X71 Research microscope (Olympus, Nashua, NH) and SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Proteasome Inhibition

Lactacystin (Peptides International, Louisville, KY) was used at a concentration of 10 μ mol/L to inhibit proteasomal activity. Briefly, lactacystin was added to the cell cultures 6 hours before histamine treatment. Inhibition of proteasomal degradation of β -catenin was assessed by Western blotting using a phospho-serine (Ser33, Ser37, and Thr41) β -catenin antibody. Polyubiquitinated β -catenin appears as higher molecular weight bands on the blot.

Shear Stress

Shear stress was applied to confluent cultures with an orbital shaker (Lab-Line, Melrose Park, IL).^{17–19} Although this technique does not result in uniform application of laminar shear stress across the entire monolayer, the majority of the cells are exposed to near maximal shear stress ($\tau_{\max}$), which can be calculated as

$$\tau_{\max} = a\sqrt{\rho\eta(2\pi f)^3}$$

where a is the radius of orbital rotation (1.4 cm), ρ is the density of the culture medium (1.0 g/ml), η is the viscosity of the medium (0.0075 poise measured by viscometer), and f is the frequency of rotation (rotations/second). A shaking frequency of 270 rpm results in a shear stress of 14 dynes/cm², which is a normal level in arteries.²⁴

Figure 1. Endothelial cells devoid of PECAM-1 (KO) and endothelial cells reconstituted with PECAM-1 (RC) express similar levels of the H1 histamine receptor. **Top panel:** Representative Western blots of lysates derived from lung microvascular endothelial cells isolated from CD31 KO mice (KO) and similar CD31 KO endothelial cells transfected with and stably expressing full-length wild-type PECAM-1 (RC) express similar levels of H1 histamine receptor. **Bottom panel:** β -Actin was used to normalize the protein loads.

Western Blotting and Immunoprecipitation

Confluent monolayers were washed twice in ice-cold phosphate-buffered saline and lysed in 1 $\times$ RIPA buffer (Upstate Biotechnology) supplemented with Complete protease inhibitor cocktail (Boehringer Mannheim) and 1 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 mmol/L sodium orthovanadate. Cells were lysed on ice for 20 minutes and centrifuged for 20 minutes at 12,000 rpm at 4°C, and protein concentrations of supernatants were assessed using the BCA Protein assay kit (Pierce).

Five hundred micrograms of protein was immunoprecipitated with specific antibody in 1 ml of immunoprecipitation buffer (50 mmol/L Tris [pH 7.4], 5 mmol/L EDTA [pH 7.4], 150 mmol/L NaCl, and 0.5% NP-40), with specific antibody and with 20 μ l of protein A-G agarose (Santa Cruz Biotechnologies) overnight at 4°C. Immunoprecipitations were washed with immunoprecipitation buffer, solubilized in 2 $\times$ sample buffer, boiled at 100°C for 10 minutes, and loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels. Gels were transferred using semidry transfer systems (Bio-Rad Transblot System; Bio-Rad, Hercules, CA), and Western blotting was performed as described in detail previously.⁷ Blots were stripped in buffer containing 2% SDS, blocked in milk, and reprobed with primary antibody. Blots were scanned with an Arcus II scanner, or images were captured on a Bio-Rad Chemidoc. Densitometry was performed using QuantityOne Software (Bio-Rad). All experiments were performed at least three times from independent lysates. Figures demonstrate representative experiments.

Results

Histamine 1 Receptor Expression Is Not Modulated on Loss of PECAM-1

Sheibani and colleagues²¹ have demonstrated that on loss of PECAM-1 expression, there is a decline in flt-1 receptor levels, whereas flk-1 receptor is equally expressed. This implies that PECAM-1 may serve a regulatory role in cytokine receptor expression. Because we elected to use histamine as the vasoactive agent in these studies, an analysis of histamine receptor expression in our KO and RC ECs was warranted. Four histamine receptors have been identified, namely H1 to H4. H1 is expressed on ECs and is primarily involved in permeability regulation and mediation of vasospasm.²⁵ We wanted

Figure 2. Histamine induces prolonged increase in permeability in endothelial cells lacking PECAM-1. **A:** A graph illustrating the quantitation of the changes in the permeability of reconstituted (RC; **open boxes**) and PECAM-1-deficient (KO; **dark shaded boxes**) microvascular endothelial cell confluent monolayers after exposure to histamine. Permeability of the monolayers to Evans blue dye was measured by collecting medium from the lower wells of the transwell chambers and measuring the absorbance at 650 nm. $n = 5$; $*P < 0.05$. **B:** A graph illustrating the quantitation of the changes in tyrosine-phosphorylated β -catenin expression in reconstituted (RC; **open boxes**) and PECAM-1-deficient (KO; **dark shaded boxes**) microvascular endothelial cell confluent monolayers after exposure to histamine. Samples were immunoprecipitated with anti- β -catenin antibodies, followed by anti-phosphotyrosine (PY) antibody immunoblotting, stripping, and anti- β -catenin immunoblotting. Phosphotyrosine band intensities were normalized to β -catenin band intensities. **Vertical bars** represent SD. $n = 4$; $*P < 0.05$. **C:** Immunofluorescence micrographs illustrating changes in the cell periphery β -catenin staining in reconstituted (RC; **A, C, and E**) and PECAM-1-deficient (KO; **B, D, and F**) microvascular endothelial cell cultures before (**A and B**) and after exposure to histamine at 5-minute (**C and D**) and 15-minute (**E and F**) exposure times. Confluent cultures were labeled with anti- β -catenin. At the zero time point, both WT and KO cultures exhibited uniform, continuous peripheral staining outlining individual cells. Five minutes after histamine treatment, both WT and KO endothelial monolayers exhibited discontinuous peripheral β -catenin staining, consistent with disruption of monolayer integrity. At 15 minutes, the WT cultures exhibited re-formation of continuous peripheral β -catenin staining, whereas the KO cultures demonstrated a persistence of discontinuous, peripheral β -catenin staining, consistent with sustained disruption of monolayer integrity. Scale bar = 25 μ m; **arrows** indicate discontinuities in the peripheral β -catenin staining.

to investigate whether the differences in endothelial permeability in histamine-treated ECs expressing PECAM-1 or not were attributable to modulation in H1 receptor expression. Western blotting for H1 receptor was performed in KO and RC endothelial cells and then normalized with actin, which was used as a loading control. As depicted in Figure 1, there was no appreciable difference in H1 expression in ECs that express or lack PECAM-1. This suggests that the differences in permeability observed on histamine treatment are attributable to other regulatory functions of PECAM-1 downstream of the H1 receptor.

ECs Lacking PECAM-1 Have Sustained Increase in Permeability after Histamine Treatment, Which Correlates with Prolonged β -Catenin Tyrosine Phosphorylation

Because the morphological findings observed in this study were consistent with changes in vascular integrity

(which we have noted in PECAM-1-null animals in a model of central nervous system inflammation¹⁰), we chose to further dissect the molecular mechanisms by which PECAM-1 affects vascular permeability. An *in vitro* permeability assay using transwells was developed. Histamine was chosen as the stimulator because it is a cytokine that predominantly affects permeability acutely as opposed to other endothelial functions such as motility, proliferation, apoptosis, or tube formation.

To determine the effects of PECAM-1/ β -catenin interactions on endothelial monolayer permeability, we treated endothelial cells with histamine for 0, 0.5, 1, 2, 5, 10, 15, 30, and 60 minutes and assessed the permeability of the monolayers to Evans blue dye, which was measured by collecting medium from the lower wells and measuring the absorbance at 650 nm. Although both cell types begin with comparable permeability characteristics, KO cell monolayers exhibited prolonged permeability to the dye lasting 20 minutes, whereas the RC cell monolayers were impermeable to the dye by 5 minutes, as shown in Figure 2A.

Because β -catenin is known to associate with VE-cadherin, an adherens junction component,⁶ and β -catenin tyrosine phosphorylation is associated with increased permeability, we assessed the chronology and quantity of tyrosine-phosphorylated β -catenin in these cells after histamine treatment. Cell lysates from KO and RC monolayers treated with histamine for 0, 5, 15, 30, and 60 minutes were immunoprecipitated with β -catenin antibody and Western blotted with an anti-phosphotyrosine antibody. Figure 2B demonstrates a persistence of tyrosine-phosphorylated β -catenin in KO cells, whereas the RC cells demonstrate earlier dephosphorylation of tyrosine-phosphorylated β -catenin, correlating with the observed differences in permeability.

Furthermore, as illustrated in Figure 2C, immunofluorescence with a β -catenin antibody demonstrates that 5 minutes of treatment with histamine results in the formation of intercellular "gaps" in both cell types, which remain persistent in the KO cells even at the 15-minute time point, consistent with sustained increase in paracellular permeability and with the persistence of tyrosine-phosphorylated β -catenin in KO cells. Recovery of the KO monolayer, as evidenced by a lack of observable intercellular gaps, is not observed until 60 minutes after stimulation (data not shown).

This experiment suggests that there is no defect in permeability at baseline in ECs that express or lack PECAM-1. On stimulation by a vasoactive agent, ECs lacking PECAM-1 retain increased vascular permeability for longer periods associated with increased levels of tyrosine-phosphorylated β -catenin and with visible persistent intercellular gaps. This suggests that the lack of PECAM-1 affects the dephosphorylation of β -catenin, thereby inhibiting reconstitution of adherens junctions.

Histamine Treatment Induces Tyrosine Phosphorylation of PECAM-1 and Thus Facilitates SHP-2- β -Catenin Complex Formation

SHP-2, a cytosolic protein tyrosine phosphatase, has been demonstrated to be recruited to PECAM-1 on tyrosine phosphorylation of PECAM-1. PECAM-1 RC cells untreated or treated with histamine for 15 minutes were harvested, and their lysates were immunoprecipitated using an antiphosphotyrosine antibody. Immunoprecipitates were then Western blotted with β -catenin antibody. Figure 3A demonstrates that histamine results in tyrosine phosphorylation of PECAM-1 within 15 minutes of treatment. Western blotting of RC and KO lysates was performed to demonstrate that equal amounts of SHP-2 are expressed in both cell types (as demonstrated in Figure 3B). Furthermore, lysates of RC and KO cells treated with histamine were immunoprecipitated with SHP-2 antibody and then Western blotted with an antibody directed against β -catenin. A fourfold induction in β -catenin-SHP-2 association was observed in RC cells after histamine treatment, whereas no such change is observed in KO cells (Figure 3, C and D).

Figure 3. Histamine induces tyrosine phosphorylation of PECAM-1, and PECAM-1 promotes interactions between β -catenin and SHP-2. **A:** Confluent RC monolayers were treated with histamine for 10 minutes, and lysates were immunoprecipitated with anti-phosphotyrosine antibody followed by Western blotting for PECAM-1. An increase in tyrosine-phosphorylated PECAM-1 is noted 10 minutes after histamine treatment. **B:** Lysates of confluent RC and KO monolayers were normalized for protein, run on SDS-polyacrylamide gel electrophoresis, and immunoblotted for SHP-2, revealing essentially equal levels of expression of this phosphatase. **C:** Confluent RC and KO monolayers were treated with histamine for 15 minutes, and lysates were immunoprecipitated with anti-SHP-2 antibody followed by Western blotting for β -catenin. Very little β -catenin is observed to be associated with SHP-2 prior histamine treatment in the RC cell cultures. However, after histamine treatment, a significant increase in β -catenin/SHP-2 association was observed after histamine treatment. In contrast, very little β -catenin is observed to be associated with SHP-2 before and after histamine treatment in the KO cell cultures. Expression of SHP-2 was similar in both RC and KO cultures. **D:** Averages of four independent experiments assessing β -catenin/SHP-2 association in RC and KO cultures before and after histamine treatment illustrating significant ($P < 0.05$) increases in β -catenin/SHP-2 association in RC cultures after histamine treatment, whereas no significant changes ($P > 0.05$) in the modest β -catenin/SHP-2 association were noted in the KO cultures. **Vertical lines** represent SD.

Figure 4. Histamine treatment elicits increased β -catenin/PECAM-1 association. Representative Western blots of RC cultures treated with histamine for 0, 5, 15, and 60 minutes. The RC endothelial cells exhibited increased β -catenin/PECAM-1 association when assayed by immunoprecipitation with anti-PECAM-1 followed by Western blotting with anti- β -catenin (**top panel**) and PECAM-1 (**bottom panel**).

Additionally, we treated RC with histamine for 0, 5, 15, and 60 minutes. Lysates were immunoprecipitated with PECAM-1 antibody and then Western blotted for β -catenin. As demonstrated in Figure 4, top panel, treatment with histamine induces recruitment of β -catenin to PECAM-1 at 15 minutes and remains sustained for 1 hour. Stripping and reprobing the blot with PECAM-1 antibody shows that approximately equal amounts of PECAM-1 were immunoprecipitated from all samples (Figure 4, bottom panel).

These studies illustrate that PECAM-1 is tyrosine phosphorylated on histamine and facilitates the formation of a complex comprised of tyrosine-phosphory-

lated β -catenin and SHP-2, which then leads to the de-phosphorylation of β -catenin and timely adherens junction reassembly.

PECAM-1 Abrogates β -Catenin-GSK-3 β Association, Diminishing β -Catenin Serine Phosphorylation and Degradation

We have previously demonstrated that lack of PECAM-1 expression diminishes cellular β -catenin expression levels.⁷ This suggests that less β -catenin would be available in KO cells to participate in adherens junction assembly. Garcia et al³⁹ have demonstrated that hepatocyte growth factor phosphorylates GSK-3 β at serine residue 9, inactivating it^{26,27} and allowing stabilization of β -catenin, allowing for optimal adherens junction dynamics and gene expression.¹⁰ Here, we demonstrate that although the expression levels of GSK-3 β are similar in RC and KO ECs (Figure 5A, bottom panel), the fraction of serine-phosphorylated GSK-3 β in RC is greater compared with KO cells (Figure 5A, top panel). This suggests that GSK-3 β , at baseline, is less active in WT cells, whereas it is more active in KO cells. Concordantly, we find increased levels of serine-phosphorylated β -catenin in KO cells

Figure 5. PECAM-1 blunts GSK-3 β activity, abrogates β -catenin-GSK-3 β association, and abrogates β -catenin serine phosphorylation, diminishing β -catenin degradation. **A:** Representative Western blot of RC and KO endothelial cell cultures labeled with anti-pSer9-GSK-3 β (pGSK-3 β) (**top panel**) and anti-GSK-3 β illustrating an increased pSer9-GSK-3 β fraction of pGSK-3 β in the RC cultures. **B:** Representative Western blot of RC and KO endothelial cell cultures labeled with anti-pS- β -catenin (anti-Ser33, Ser37, and Thr41- β -catenin), illustrating increased pS- β -catenin in the KO cultures. **C:** Representative Western blot of RC and KO endothelial cell cultures labeled with anti- β -catenin (**top panel**) and anti-GSK-3 β after an immunoprecipitation with anti-GSK-3 β , illustrating increased GSK-3 β -associated β -catenin in the KO cultures. **D:** Quantitation of Western blots of brain WT (**open boxes**) and KO (**gray filled boxes**) endothelial cell cultures, before and after histamine treatment (0, 5, and 10 minutes), labeled with anti-pS-GSK-3 β and anti-GSK-3 β and normalized to total GSK-3 β . Note the increase in the WT pS-GSK-3 β fraction at the 5-minute time point. $n = 3$. **E:** Quantitation of Western blots of lung RC (**open boxes**) and KO (**gray filled boxes**) endothelial cell cultures, before and after histamine treatment (0, 5, and 10 minutes), labeled with anti-pS-GSK-3 β and anti-GSK-3 β and normalized to total GSK-3 β . Note the increase in the WT pS-GSK-3 β fraction at the 10-minute time point. $n = 3$. **F:** Representative Western blots of lung CD31 KO lysates with and without a 6-hour pretreatment with lactacystin (10 μ mol/L) followed by a 10-minute treatment with vehicle or before a 10-minute histamine treatment (100 μ mol/L). The blot was labeled with anti-phospho-serine β -catenin (anti-Ser33, Ser37, and Thr41- β -catenin), illustrating a robust increase in pS- β -catenin in the cells pretreated with lactacystin followed by histamine treatment at the 10-minute time point (**top panel**). When stripped and re-blotted with anti- β -catenin, the blot also revealed a robust increase in β -catenin in the cells pretreated with lactacystin followed by histamine treatment at the 10-minute time point (**bottom panel**). The samples were normalized to protein load. **G:** Representative Western blots of HUVEC lysates before and after histamine treatment (0, 5, and 15 minutes) labeled with anti-GSK-3 β (**top panel**), anti-pSer9-GSK-3 β GSK-3 β (**second panel**), illustrating a transient increase in pSer9-GSK-3 β after histamine treatment at the 5-minute time point. The blots were normalized to Erk-2 (**third panel**), and the pS-GSK-3 β /GSK-3 β ratios were calculated (**bottom panel**).

Figure 6. Shear stress increases GSK-3 β inactivation and abrogates β -catenin serine phosphorylation in PECAM-1-expressing endothelial cells but not in PECAM-1-null cells. **A:** Representative Western blots of lung-derived CD31 KO (top two panels) and CD31RC (bottom two panels) cultures labeled with anti-serine⁹ phosphorylated GSK-3 β (pGSK-3 β) and anti-GSK-3 β (Tot GSK) before and after 0, 5, 10, 30, and 60 minutes of shear stress. The bottom panel is a quantitation of the percent change in serine-phosphorylated GSK-3 β in the CD31KO (squares) and CD31RC (circles) cultures before and after various time periods of shear stress. Vertical lines represent SEM. $n = 4$. **B:** Representative Western blots of lung-derived CD31 KO (top two panels) and CD31RC (bottom two panels) cultures labeled with anti-serine-phosphorylated β -catenin (anti-Ser33, Ser37, and Thr41- β -catenin) (p β cat) and anti- β -catenin (Tot β cat) before and after 0, 5, 10, 30, and 60 minutes of shear stress. The bottom panel is a quantitation of the percent change in serine/threonine phosphorylated β -catenin in the CD31KO (squares) and CD31RC (circles) cultures before and after various time periods of shear stress. Vertical lines represent SEM. $n = 4$.

compared with RC cells (Figure 5B). This also suggests that KO cells may have a higher fraction of β -catenin (serine phosphorylated) that is targeted for degradation. Thus, we immunoprecipitated lysates of KO and RC cells with GSK-3 β antibody and then Western blotted for β -catenin. Figure 5C, top panel, demonstrates that there is stronger association between (serine-phosphorylated) β -catenin and GSK-3 β in KO compared with RC endothelial cells. These findings obtained from microvascular endothelial cells derived from the pulmonary parenchyma were confirmed using WT and KO microvascular endothelial cells derived from the brain.¹⁰ Histamine treatment evoked an increase in Ser9-phosphorylated (and thus inactivated) GSK-3 β in WT brain-derived and PECAM-1-reconstituted PECAM-1-deficient lung-derived lung microvascular endothelial cells (albeit with different kinetics) but not in brain- or lung-derived PECAM-1-null cells (Figure 5, D and E). These data are consistent with the notion that a lack of PECAM-1 results in a greater fraction of active (non-Ser9-phosphorylated) GSK-3 β , facilitating β -catenin association with GSK-3 β ; subsequent serine and threonine phosphorylation of β -catenin at residues Ser33, Ser37, and Thr41; and targeting β -catenin for degradation as evidenced by the increased lower molecular forms observed on Western blotting (Figures 5B and 6B).

To confirm our hypothesis that the serine-phosphorylated β -catenin expressed in lung-derived PECAM-1 KO endothelial cells is targeted for proteosomal degradation, we used the use of the proteasome inhibitor lactacystin. As illustrated in Figure 5F, pretreatment with lactacystin results in the persistence of increased amounts of phos-

pho-serine- β -catenin and in the persistence of higher molecular weight bands representing polyubiquitinated serine-phosphorylated β -catenin as well as lower molecular weight bands representing degraded β -catenin. Additional experiments, using brain WT and PECAM-1 KO endothelial cells, revealed that in WT cell cultures treated with lactacystin and histamine, there were only minimal nonsignificant changes in β -catenin levels, whereas in brain-derived PECAM-1 KO cell cultures treated with lactacystin and histamine, there were robust increases in β -catenin levels compared with the WT cultures ($n = 3$; data not shown).

We confirmed our findings using primary cultures of HUVECs to confirm whether PECAM-1 affects β -catenin-GSK-3 β association. First, we treated HUVECs with histamine for 15 minutes and then performed immunoprecipitation of lysates with anti-phosphotyrosine antibody and Western blotting for PECAM-1. We noted the same findings as with RC cells (data not shown). Furthermore, we treated HUVECs with histamine for 0, 5, 15, and 60 minutes and noted a threefold increase in serine-phosphorylated GSK-3 β within 5 minutes with return to baseline levels within 15 minutes (Figure 5G). This suggests that in HUVECs, histamine induces an early and intense phosphorylation and inactivation of GSK-3 β to enable more β -catenin to associate with VE-cadherin to form adherens junctions and translocate to the nucleus to modulate gene expression.¹⁰

To ascertain whether the modulation of GSK-3 β serine phosphorylation (Ser9 phosphorylation = inactivation) by PECAM-1 is unique to histamine treatment, we used both our lung-derived PECAM-1-deficient endothelial cells and our PECAM-1-reconstituted PECAM-1-deficient lung-derived endothelial cells in a shear-stress study. Shear stress is a known modulator of endothelial cell permeability, affecting junctional complexes (of which β -catenin is an important component) and cytoskeletal organization.^{28–30} As illustrated in Figure 6A, whereas the PECAM-1-expressing PECAM-1 RC cells respond to shear stress by increasing their serine phosphorylation (at Ser9) and thus inactivation of GSK-3 β , the PECAM-1 KO cells do not. In addition, the levels of serine-phosphorylated β -catenin do not change in the PECAM-1-null lung-derived endothelial cells reconstituted with PECAM-1 (CD31RC) cells but are increased in the PECAM-1 KO cells (Figure 6B), further supporting our hypothesis that the absence or presence of PECAM-1 determines, in part, the dynamics of β -catenin degradation as well as its localization^{4,6,9,17} and expression.⁷

Activity of GSK-3 β Correlates Inversely with Activity Levels (Phosphorylation) of Phosphatidylinositol 3-Kinase (PI3K) and Akt

Because it is known that GSK-3 β activity can be inhibited through a PI3K-dependent pathway involving Akt,^{31,32} we investigated the effects of knocking down PECAM-1 expression (using PECAM-1 antisense oligonucleotides) in HUVECs on PI3K and Akt activities (phosphorylation) and

Figure 7. Levels of pP85 (pPI3K), pAkt, and pS-GSK-3 β correlate directly with PECAM-1 expression levels. **A:** Representative Western blots of HUVECs treated with either a scrambled PECAM-1 oligonucleotide (scrambled CD31) and/or antisense PECAM-1 oligonucleotide (antisense CD31), revealing a significant knockdown of PECAM-1 expression in the antisense CD31-treated cultures, which correlated with significant knockdowns of tyrosine-phosphorylated (active) PI3K and Akt. $n = 3$. **B:** Quantitation of three independent experiments consisting of HUVECs treated with either a scrambled PECAM-1 oligonucleotide (scrambled CD31) and/or antisense PECAM-1 oligonucleotide (antisense CD31), illustrating significant knockdown of phospho-Akt and phospho-PI3K. **C:** Representative Western blots of three independent experiments consisting of lung-derived RC and CD31 KO and brain-derived WT and CD31 KO endothelial cell cultures, illustrating decreased phospho-PI3K and phospho-Akt levels in the CD31 KO cultures compared with the WT and RC cultures.

assessed potential correlations with GSK-3 β activity (Ser9 phosphorylation) (Figure 7, A and B). Lung RC and PECAM-1 KO and brain WT and PECAM-1 KO endothelial cell phospho-Akt and phospho-PI3K levels were also assessed (Figure 7C). As illustrated in Figure 7, reduction in HUVEC PECAM-1 expression elicited a reduction in PI3K and Akt activation (phosphorylation) as does loss of PECAM-1 expression in the lung- and brain-derived endothelial cells harvested from PECAM-1 KO animals. In all endothelial cells tested, knockdown or loss of PECAM-1 expression resulted in decreased pAkt and pPI3K levels, which correlated with decreased GSK-3 β Ser9 phosphorylation (Figures 5 and 6) and thus an increase in GSK-3 β activity.

Discussion

Loss of endothelial permeability barrier function often has devastating consequences in hospitalized patients.³³ EC damage and its effects have been recognized as perpetuating tissue and organ damage after a variety of injuries including ischemia/reperfusion.³⁴ Enhanced permeability due to EC separation occurs in the 24 hours after reperfusion, allowing transit of solutes, fluid, and cells into the interstitial space.³⁵ In normal tissues and organs, reconstitution of the endothelial barrier involves, in part, dephosphorylation of tyrosine-phosphorylated adherens junction proteins, leading to reassembly of adherens junctions, thus closing gaps between ECs and preventing further passage of solutes and cells into the interstitium. Previously, we have demonstrated that in PECAM-1 KO mice, there is significantly more interstitial edema/hemorrhage 24 hours after autoimmune (inflammatory) insult,¹⁰ vasoactive amine stimulation,¹⁰ or endotoxin challenge.¹⁶ Furthermore, in the case of endotoxin challenge, Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) staining of several organs 24 hours after reperfusion, showed extensive necrosis/apoptosis in the PECAM-1 KO mice.¹⁶ This suggests that lack of PECAM-1 promotes tissue/organ injury, in part, by prolonging vascular barrier incompetence, in addition to other possible mecha-

nisms, and clearly implicates PECAM-1 as playing a critical role in the re-establishment/maintenance of barrier permeability after several types of injury.

We used ECs in culture that either express PECAM-1 or not, challenged them with histamine, and measured barrier permeability. We noted that lack of PECAM-1 predisposed ECs to a prolonged increase in barrier permeability. To verify that the varied permeability response in cells lacking PECAM-1 expression was not due to differences in H1 receptor expression, we performed Western blots for H1 receptor in KO and RC cells and normalized the blots with actin. No apparent difference in H1 receptor expression level was noted, suggesting that the lack of PECAM-1 affects histamine-mediated signaling downstream to the H1 receptor. Because β -catenin tyrosine phosphorylation state closely correlates with both endothelial and epithelial barrier competence,³⁶ we performed immunoprecipitations for β -catenin and then Western blotted using an anti-phosphotyrosine antibody to determine the fraction of β -catenin that was tyrosine phosphorylated in these cells. We determined that in ECs lacking PECAM-1 expression, the prolonged increase in permeability correlates closely with sustained elevation in β -catenin tyrosine phosphorylation. This suggests that loss of PECAM-1 results in a reduced ability to dephosphorylate β -catenin in a timely fashion after a cytokine challenge, thereby resulting in increased prolonged permeability. This is in keeping with the increased vascular permeability observed in mice after EAE induction¹⁰ and treatment with LPS,¹⁶ where humoral factors likely precipitate barrier incompetence, which the PECAM-1 KO mice are not able to compensate for in a timely fashion.

The recruitment of SHP-2 to PECAM-1 has been demonstrated by numerous groups including our own.⁴ Moreover, it is clear that phosphorylation of the PECAM-1 ITAM tyrosines are required to enable association with SHP-2. A multitude of factors are known to tyrosine phosphorylate PECAM-1, including osmotic stress, mechanical stretch, and cytokines such as Vascular Endothelial Growth Factor (VEGF) and IL3. Fyn and src have been implicated as kinases that mediate PECAM-1 tyrosine phosphorylation; however, descrip-

Figure 8. Working model illustrating the effects of presence or absence of PECAM-1 on GSK-3 β -mediated β -catenin phosphorylation and degradation. **A:** In PECAM-1-expressing endothelial cells, tyrosine phosphorylated β -catenin is efficiently complexed with SHP-2 in a tripartite complex with immunoreceptor tyrosine-based activation motif tyrosine phosphorylated PECAM-1, dephosphorylated, and made available to participate in reformation of adherens junctional complexes and translocation to the nucleus where it modulates gene expression on complexing with Lef/Tcf. PECAM-1 expression is also associated with increased activity (phosphorylation) of PI3K and Akt, which increase the serine phosphorylation of GSK-3 β , inactivating it, thus reducing the fraction of β -catenin that is serine/threonine phosphorylated and thus targeted for proteosomal degradation. **B:** In the absence/reduction of PECAM-1 expression, tyrosine-phosphorylated β -catenin is inefficiently complexed with and dephosphorylated by SHP-2, reducing its ability to participate in the reformation of adherens junctions (dashed lines). In addition, the absence/reduction of PECAM-1 expression is also associated with a reduction in PI3K and Akt activity (phosphorylation), leading to a persistent high activity of GSK-3 β (dashed line), resulting in an increased serine/threonine phosphorylation of β -catenin and its targeting for proteosomal degradation (heavy solid lines) and reduced nuclear translocation (light solid lines).

tions of other kinases or mechanisms and chronology of PECAM-1 phosphorylation still remain unclear. Here, we show that PECAM-1 is tyrosine phosphorylated after histamine treatment. Previous studies have shown that PECAM-1 tyrosine phosphorylation increases SHP-2 recruitment to PECAM-1. We demonstrate that in EC-expressing PECAM-1, there is a fourfold increase in SHP-2- β -catenin molecular complex formation after histamine treatment, whereas there is practically no change in association between SHP-2 and β -catenin after histamine treatment in ECs that lack PECAM-1 expression. This suggests that PECAM-1 serves as a scaffold, where on PECAM-1 tyrosine phosphorylation, SHP-2 is recruited and β -catenin is actively dephosphorylated. Histamine is known to tyrosine phosphorylate β -catenin, and when tyrosine phosphorylated, β -catenin increases its association with PECAM-1.^{4,6,9,17} This would imply that on vasoactive substance stimulation, PECAM-1 becomes tyrosine phosphorylated and recruits SHP-2 and tyrosine-phosphorylated β -catenin forming a tripartite molecular complex, resulting in dynamic tyrosine dephosphorylation of β -catenin, with the resultant return of β -catenin to the adherens junctions, facilitating the reformation of a functional endothelial barrier.

In addition to histamine treatment, the presence or absence of PECAM-1 affected endothelial cell responses to shear stress (Figure 6). Specifically, PECAM-1-expressing cells exhibited an increase in the fraction of

inactive GSK-3 β and maintenance of β -catenin levels, whereas PECAM-1-null endothelial cells exhibited unchanged constitutive GSK-3 β activity levels and increased levels of serine-phosphorylated β -catenin, leading to proteosomal degradation.

We also examined the effects of VEGF A₁₆₅ on GSK-3 β phosphorylation and found that there was no appreciable change in GSK-3 β phosphorylation in the time frame studied (15 minutes) ($n = 3$; data not shown), suggesting that the permeability changes observed after VEGF administration are due to different mechanisms than those observed after histamine treatment and shear stress. This is not surprising because distinct receptors are engaged/stimulated and distinct signaling cascades are initiated following these diverse stimuli^{25,28–30,37}. In addition it is likely that there are redundant pathways modulating permeability in ECs, depending on the vascular bed and stimuli involved.

The modulation of GSK-3 β activity by PECAM-1 adds another aspect to the mechanisms in which PECAM-1 is involved in the control of β -catenin biology, namely its degradation after its serine phosphorylation by GSK-3 β (Figure 8). Although our studies highlight the role of PECAM-1 as a regulator of permeability by dynamically functioning as a scaffold, it also raises the question of whether overexpression of PECAM-1 might be a mechanism by which to promote a more rapid reconstitution of junctions. Although this hypothesis remains untested, if its feasibility were determined, it would pro-

vide a potential therapeutic option to patients where tissue/organ ischemia and its consequences are anticipated.

In analyzing these data, it would be important to mention that expression of β -catenin is decreased in ECs on loss of PECAM-1.⁷ This suggests that there may be less total β -catenin at the adherens junctions at baseline. Although a reduced level of β -catenin might be adequate for barrier maintenance in the unperturbed state, it may be insufficient for timely reconstitution of the permeability barrier when the PECAM-1 KO ECs are challenged by a vasoactive stimulus.¹⁰ Dejana and colleagues³⁸ recently demonstrated that β -catenin-null animal vessel endothelial cells displayed an elongated phenotype, less complex interendothelial cell junctions, more fenestrae with significantly leakier vessels, and resultant hemorrhage and edema causing embryonic lethality. Interestingly, the PECAM-1 KO ECs are also more elongated, and the vessels of PECAM-1 KO mice, while displaying normal integrity under baseline conditions, exhibit prolonged loss of barrier function when challenged by induction of autoimmunity,¹⁰ a vasoactive amine,¹⁰ or LPS.¹⁶ This suggests that although some of the permeability defects observed in our system might be directly attributable to PECAM-1, the consequences of decreased β -catenin expression in PECAM-1-null ECs also might contribute to the observed phenotype.

Thus, from our previously published data coupled with the data presented here, we hypothesize that PECAM-1 modulates β -catenin via two distinct pathways: 1) its ability to facilitate the tyrosine de-phosphorylation of β -catenin via SHP-2, influencing adherens junction assembly;^{4,6,9} and 2) its ability to modulate the serine phosphorylation of GSK-3 β influencing β -catenin serine phosphorylation levels, thus affecting targeting of β -catenin for proteosomal degradation (Figure 8). Our data highlight the crucial role of PECAM-1 in permeability regulation and studies exploring up-regulation of PECAM-1 in the microvasculature before procedures known to cause tissue/organ injury should be pursued.

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