PKCα Activation of p120-catenin Serine 879 Phospho-Switch

Disassembles VE-cadherin Junctions and Disrupts Vascular Integrity

Emily Vandenbroucke St Amant, Mohammad Tauseef, Stephen M.Vogel, Xiao-Pei Gao,

Dolly Mehta, Yulia Komarova and Asrar B Malik.

Department of Pharmacology and the Center for Lung and Vascular Biology,

The University of Illinois College of Medicine, Chicago, IL 60612, USA

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Corresponding Author:

Dr. Asrar B. Malik

Department of Pharmacology, University of Illinois College of Medicine

835 S Wolcott Ave., M/C 868 Chicago, IL 60612.

(312) 996-7636 (p) (312) 996-1225 (f)

E-mail: abmalik@uic.edu

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Abstract

Rationale: Adherens junctions (AJs) are the primary intercellular junctions in microvessels responsible for endothelial barrier function. Homophilic adhesion of vascular endothelial (VE) cadherin forms AJs, which are stabilized by binding of p120-catenin (p120). p120 dissociation from VE-cadherin results in loss of VE-cadherin homotypic interaction and AJ disassembly; however, the signaling mechanisms regulating p120 dissociation from VE-cadherin are not understood.

Objective: To address the mechanism of protein kinase C (PKC)-α function in increasing endothelial permeability, we determined the role of PKCα phosphorylation of p120 in mediating disruption of AJ integrity.

Methods and Results: We showed that PKCα phosphorylation of p120 at S879 in response to thrombin or LPS challenge reduced p120 binding affinity for VE-cadherin and mediated AJ disassembly secondary to VE-cadherin internalization. In studies in mouse lung vessels, expression of the phosphodeficient S879A-p120 mutant prevented the increase in vascular permeability induced by activation of the thrombin receptor PAR-1.

Conclusions: PKCα phosphorylation of p120 at S879 is a critical phospho-switch mediating disassociation of p120 from VE-cadherin that results in AJ disassembly. Therefore, blocking PKCα-mediated p120 phosphorylation represents a novel targeted anti-inflammatory strategy to prevent disruption of vascular endothelial barrier function.

Keywords: endothelium, inflammation, intercellular junctions, signal transduction, vascular permeability

Non-standard Abbreviations and Acronyms

AJs: adherens junctions

AP-2: adaptor protein-2

CTD: C-terminal domain

EBA: Evans blue albumin

E-cadherin: Epithelial cadherin

LPS: lipopolysaccharide

HLMVEC: human lung microvascular endothelial cell

HPAE: human pulmonary aortic endothelial cells

JMD: juxtamembrane domain

MM control: mismatch control

N-cadherin: neuronal cadherin

NTD: N-terminal domain

p120: p120-catenin

PAR-1: protease activated receptor-1

PKCα: protein kinase C-α

S879: serine 879

S879A: serine 879 mutated to alanine

S879D: serine 879 mutated to aspartic acid

SPR: surface plasmon resonance

VE-cadherin: vascular endothelial cadherin

WT: wild-type

Introduction

The endothelium lining blood vessels regulates passage of protein, solutes and liquid as well as inflammatory cells[1]. Endothelial cells form adherens junctions (AJs) that allows the endothelium to function as a semi-permeable barrier[2]. AJs are formed through vascular endothelial (VE) -cadherin clustering and homophilic binding of VE-cadherin on adjacent cells in a Ca^{2+}-dependent manner, thereby effectively restricting passage of plasma proteins of radii greater that 3 nm[1]. Endothelial cells express VE- and neuronal (N)-cadherins[3]. N-cadherin induces interactions of endothelial cells with smooth muscle cells and pericytes[4], whereas VE-cadherin functions as the primary adhesion molecule regulating barrier function at the level of AJs[5, 6]. The C-terminus (CTD) of cadherins anchors AJs to the actin cytoskeleton through binding of β- and α-catenins[7]. Pro-inflammatory mediators, such as thrombin, signal disassembly of AJs and increase vascular permeability in endothelial cells[8] via activation of their respective G protein-coupled receptors. Thrombin ligation of protease activated receptor (PAR)-1 and leads to protein-rich tissue edema and migration of inflammatory cells into tissue[8]. Other mediators such as LPS can increase vascular permeability through activation of their receptors in endothelial cells, specifically TLR4 and CD14[9].

VE-cadherin stability at AJs is critically dependent on binding of p120-catenin (p120)[10, 11]. Unlike other catenins, p120 binds VE-cadherin at its highly conserved juxta-membrane domain (JMD) covering amino acids 736 to 781[12]. p120, originally described as a Src substrate[13], is a ubiquitously expressed member of a subset of the armadillo repeat motif (ARM) proteins that include δ-catenin[14], ARVCF (Armadillo Repeat gene deleted in Velo-Cranial-Facial syndrome)[15], p0071[16], and plakophilins[17]. Knockout of p120 is embryonically lethal[18] due to impaired microvessel formation. Conditional p120 knockout mice show that p120 is essential for regulating cadherin expression during vascular development[18]as well as assembly of AJs[19, 20]. Loss of p120 binding at the JMD results in internalization of VE-cadherin and other cadherins and their peri-membranous localization[10, 21-24], and subsequent disassembly of AJs[11, 24]. Reduced p120 expression also reduces expression of VE-cadherin secondary to VE-cadherin internalization[10]. Internalization is regulated by binding of p120 and clathrin adaptor protein (AP)-2, a component of clathrin-coated pits (CCPs), to VE-cadherin[22]. p120 binding masks the AP-2 binding site on cadherins[22, 25], and p120 dissociation enables VE-cadherin binding to AP-2, which recruits VE-cadherin into CCPs resulting in VE-cadherin endocytosis[21, 22]. While p120 helps to stabilize AJs, the mechanisms of p120 dissociation from VE-cadherin, the crucial step in VE-cadherin internalization, and its role in intact vessels have not been addressed.

Studies examining p120 function have focused on post-translational modifications of its N-terminal regulatory domain (NTD) that contains multiple phosphorylation sites[26]. Tyrosine phosphorylation of p120 NTD controls p120 binding to RhoA[27], yet it is insufficient to increase endothelial permeability[28]. Furthermore, p120 maintains VE-cadherin surface expression and endothelial permeability independent of the presence of NTD[29]. Here we focused on the CTD of p120, the domain containing two phosphorylation sites: threonine 916 and serine 879 (S879)[30]. Threonine 916 does not appear to affect VE-cadherin interaction with p120[31]. However, S879 is of particular interest since it is known to be phosphorylated by PKCα during inflammation[30, 32]. PKCα is activated by increased intracellular Ca^{2+} and binding to DAG[33]. We have shown in an important observation that PKCα has a key role in signaling increased endothelial permeability[34, 35]. PKCα is also known to phosphorylate S879 on p120 in response to inflammatory mediators and growth factors in endothelial cells, epithelial cells, and

fibroblasts[36]; however, the functional significance of PKCα-mediated phosphorylation with respect to p120 interaction with VE-cadherin and AJ integrity remains unclear. Thus, we studied PKCα phosphorylation of p120 and its role in mediating stability at AJs. We demonstrated that S879 phosphorylation of p120 by PKCα in response to thrombin or LPS challenge decreased p120 affinity for VE-cadherin and induced binding of VE-cadherin to AP-2 and increased endothelial permeability.

Methods

Materials. Endothelial cells and media were obtained from Lonza (Basel, Switzerland). Human α-thrombin was obtained from Enzyme Research (South Bend, IN) and LPS was from Sigma (St. Louis, MO). PAR-1 agonist peptide (TFLLRNPNDK-NH_2) was synthesized[37]. PKCα siRNA (UAAGGAACCACAAGCAGUAUU) and MM control (UAAGGA**G**CCAC**G**AGC**G**GUAUU) siRNA were purchased from Dharmacon (Lafayette, CO). Anti-p120α, anti-VE-cadherin, and anti-GAPDH were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and anti-PKCα, anti-p-PKCα, and anti-Na-K ATPase from Cell Signaling (Beverly, MA). pS879-p120 was purchased from BD Biosciences (San Jose, CA) and anti-GFP from GeneTex (San Antonio, TX). AlexaFluor 488 and 594 secondary antibodies and ProLong were from Invitrogen (Grand Island, NY). S879A-p120 and WT-p120 cDNA were gifts from Dr. Albert Reynolds (Vanderbilt University) and S879D-p120 was generated using the QuikChange site-directed mutagenesis kit from Stratagene (Santa Clara, CA).

Mice. *prkca*$^{-/-}$ mice (gift from Dr. Jeffrey Molkentin, University of Cincinnati) and wild-type mice were of C57/Bl6 genetic background (obtained from Jackson Laboratory, Bar Harbor, ME). Experiments were made using 8-10 wk males in accordance with institutional IUCAC approval.

Mouse lung preparation and vascular permeability measurement. Mice were anesthetized with 3% halothane. Lung vascular filtration coefficient ($K_{f,c}$ in units of ml x min^{-1} x cm H_2O) was measured[38] to quantify vascular permeability.

Liposome delivery of cDNA and transvascular albumin flux assay. Cationic liposomes (100 µl) were prepared[39], mixed with 50 µg cDNA, and injected via a retro-orbital i.v. injection. After 48 hours, Evans blue-albumin (EBA; 20 mg/kg) was injected i.v. with the PAR-1 peptide (TFLLRNPNDK-NH_2, 1 mg/kg) 30 minutes before euthanasia. Lung transvascular EBA uptake was calculated[39].

Cell culture and transfection. Human lung microvascular endothelial cells (HLMVECs) and human pulmonary artery endothelial cells (HPAEs) were cultured in T-75 flasks on 0.1% gelatin in EBM-2 or EGM-2, respectively, supplemented with 10% FBS. HLMVECs were transfected with indicated mutants or siRNA at 80% confluence by nucleofection, using the AMAXA kit (Lonza), and used 72 hr post-transfection. HPAEs were transfected at 70% confluency using Turbofect (Fermentas, Glen Burnie, MD), and used 48 hr post-transfection.

Transendothelial electrical resistance (TER) measurement. TER was measured using ECIS software (Applied Biophysics, Troy, NJ)[40]. Data were normalized to baseline resistance.

Immunofluorescence, live cell imaging and image analysis. Cells were grown to confluence on glass coverslips, serum-starved, and treated with thrombin (4 U/ml). Cells were fixed and visualized as described[40]. For time-lapse imaging, cells were serum starved in phenol-free EBM media (Lonza) and maintained at 37°C with a stage heater (Tempcontrol-37, Zeiss, Thornwood,

NJ). Images were acquired every 10 s using the Nikon Eclipse TE-2000S microscope equipped with UltraView confocal head (PerkinElmer Life Sciences), ORCA-ER-1394 camera (Hamamatsu, Bridgewater, NJ), AR/KR 3-line laser (λ=488, 568 and 647 nm) Plan Apo 100x 1.4NA objective and Volocity 5 software (Improvision, Waltham, MA). Thrombin (4 U/ml) or PBS was applied to cells after the second exposure. As we observed no significant photobleaching, we did not correct for photobleaching. Inter-endothelial gap area and average fluorescence intensity were quantified using MetaMorph software (Molecular Devices, Sunnyville, CA)[40]. Time-lapse videos were quantified by kymograph analysis and GFP intensity was expressed as fluorescence decay.

Immunoprecipitation. Cells were lysed with modified ODG buffer and proteins were immunoprecipitated using the indicated antibodies and A/G agarose beads[40].

Protein binding assay using surface plasmon resonance (SPR). Proteins were generated from cDNA using *in vitro* protein expression kit (Pierce, Rockford, IL). SPR biosensor binding experiments were made at 25°C, using a CM-5 sensor chips (GE Healthcare, Burr Ridge, IL) and 150 mM NaCl (pH 7.4), 10mM HEPES, 3 mM EDTA and 0.005% Surfactant P20 running buffer. VE-cadherin immobilization was made at pH 5.0 followed by regeneration at pH 2.5. p120-catenin was allowed to flow over VE-cadherin at rate of 10 µg/min for 180 s for binding analysis. Measurements were made using Biacore T100 (GE Healthcare).

Statistical analysis. Student's *t* test and ANOVA with Bonferroni post-hoc tests were used to determine significance. $P < 0.05$ denoted significant difference.

Results

PKCα activation increases endothelial permeability secondary to AJ disassembly

To determine the role of PKCα in increasing vascular permeability, PAR-1 agonist peptide, which activates PAR-1 in endothelial cells as well as PKCα[41], was added to perfusate of isolated-perfused mouse lungs. To measure vascular permeability, we determined $K_{f,c}$, a measure of vessel wall permeability to fluid[38], in wild type (WT) and *prkca*$^{-/-}$ mice. Baseline $K_{f,c}$ was not different between *prkca*$^{-/-}$ and WT (Fig. 1A). Activation of PAR-1 increased $K_{f,c}$ of WT lungs to 1.9 $\pm$ 0.06 ml/m/cm H_2O/g dry lung weight whereas $K_{f,c}$ of *prkca*$^{-/-}$ lungs increased only to 0.69 $\pm$ 0.04 (Fig. 1A). We observed a 50% decrease in total PKC activity in *prkca*$^{-/-}$ mouse lungs (Online Fig. IA) and no alterations in expression of other PKC isoforms (Online Fig. IB).

To determine the role of PKCα in mediating alterations in endothelial cell junctions, we transfected human lung microvascular endothelial cells (HLMVECs) with PKCα siRNA or mismatch control siRNA (MM control). PKCα siRNA-treated cells showed 90% depletion of PKCα (Fig. 1B). PKCα-depleted HLMVECs were stimulated with thrombin, and TER changes were taken as a measure of alterations in function of AJs. Baseline TER values were similar between PKCα siRNA- and MM control-treated cells, however, thrombin stimulation produced a 35% decrease in TER in PKCα-depleted cells whereas TER decreased 70% from baseline in MM control cells (Fig. 1C). We next determined whether PKCα activity is required to disrupt AJs. PKCα-depleted cells were stimulated with thrombin and immunostained for VE-cadherin and PKCα (Fig. 1D). Thrombin induced AJ gap formation that was apparent within 15 m in control cells. At 30 m, gaps covered 11% of the monolayer (1E). In contrast, gaps were barely visible in PKCα-depleted monolayers at 15 m and 3% of monolayer contained gaps at 30 m

(Fig. 1E). Control cells lost 66% of VE-cadherin from AJs, whereas 22% of VE-cadherin was lost in PKCα-depleted cells (Fig. 1F). These findings indicate the critical role of PKCα activation in mediating increased permeability secondary to dispersal of VE-cadherin from AJs.

PKCα phosphorylation at S870 of p120 induces disassociation of p120 from VE-cadherin

We surmised that PKCα may function to disrupt AJs by signaling dissociation of p120 from VE-cadherin. Hence, we immunoprecipitated p120 from HLMVEC lysates and immunoblotted with phospho-active PKCα (p-PKCα) or VE-cadherin to determine whether PKCα in its active state interferes with the stable interaction of p120 with VE-cadherin. p120 association with p-PKCα was evident within 5 m after thrombin stimulation, with maximum association occurring at 15 m (Fig. 2A-B). p120 basally interacted with VE-cadherin but this decreased by 73% after 15 m of thrombin stimulation (Fig. 2B). Because p120 was localized at AJs through binding to VE-cadherin, we addressed whether thrombin stimulation altered p120 localization at the membrane. Western blotting of cytosolic and membrane fractions showed that p120 basally associated with the membrane fraction but translocated to the cytosolic fraction after thrombin stimulation (Fig. 2C).

We next addressed whether p120 uncoupling from VE-cadherin was the result of PKCα phosphorylation of p120. We observed that PKCα induced p120 phosphorylation at S879 with the maximal response occurring between 5 and 15 m after thrombin stimulation (Fig. 2D). To determine whether PKCα phosphorylation of p120 induced p120 dissociation from VE-cadherin, we analyzed VE-cadherin content of the p120 immunocomplex. Immunoblots showed that only 7% of p120 dissociated from VE-cadherin in PKCα-depleted cells whereas 47% of p120 was dissociated in control cells (Fig. 2E). We also observed that control cells showed 67% reduction in p120 staining at AJs whereas PKCα-depleted cells lost only 38% of the staining (Fig. 2F-G) indicating that PKCα phosphorylation of p120 at S879 is a crucial determinant of uncoupling of p120 from VE-cadherin.

Reduced p120 affinity for VE-cadherin mediates uncoupling of p120

We next determined the contribution of PKCα phosphorylation of S879 in reducing p120 binding affinity to VE-cadherin. Cells co-immunostained with anti-VE-cadherin antibody along with anti-p120 antibody (Fig. 3A, top panel) or anti-S879 p120 antibody were visualized (p-S879 p120, bottom panel) and localization of total p120 and phosphorylated p120 relative to VE-cadherin was determined (Fig. 3B). Fluorescence intensities showed increased pS879-p120 at AJs after 15 m after thrombin stimulation; however, phosphorylation of S879 on p120 also drastically modified p120 localization within cells. The increase in the cytosolic pool of pS879-p120 was correlated with increased phosphorylation of p120 on S879 (Fig. 3C).

Serine 879 resides within the CTD of p120 adjacent to the ARM repeat where p120 binds cadherins[25, 30]. We mutated serine 879 to alanine to create a phosphodefective p120 mutant (S879A-p120) or to aspartic acid to create a phosphomimetic mutant (S879D-p120). Both were cloned along with wild-type (WT) p120 cDNA into vectors containing a GFP tag and expressed in HPAE cells. VE-cadherin was immunoprecipitated and precipitates were immunoblotted with anti-GFP antibody to identify differences in VE-cadherin affinity for p120 mutants. The S879A-p120 mutant showed basal association with VE-cadherin and significantly increased association with VE-cadherin after thrombin stimulation (Fig. 3D). The S879A-p120 mutant, which cannot be phosphorylated by PKCα, demonstrated greater VE-cadherin binding.

The S879D-p120 mutant interacted weakly with VE-cadherin under basal conditions and the interaction decreased further after thrombin stimulation (Fig. 3D). Equal expression of exogenous p120 was evident by the immunoblots of GFP-p120 in cell lysates with GAPDH serving as loading control (not shown). The finding that phosphodefective p120 remained bound to VE-cadherin after thrombin stimulation (Fig. 3D) further supports the role of S879 phosphorylation as an essential phospho-switch disrupting p120 interaction with VE-cadherin.

To address whether S879 phosphorylation of p120 reduced its binding to VE-cadherin, we used SPR to determine alterations in binding affinity of p120 for VE-cadherin (Fig. 3E). We generated WT-p120, the phosphomimetic p120 mutant and VE-cadherin proteins with a cell-free protein expression kit. We immobilized VE-cadherin onto a CM-5 sensor chip which was exposed to increasing concentrations of WT-p120 or S879D-p120 to determine binding of p120 to VE-cadherin. Phosphomimetic p120 displayed an order of magnitude less affinity to VE-cadherin ($K_d = 3.3 \times 10^{-9}$) a compared to the WT protein ($K_d = 4.2 \times 10^{-10}$).

To determine effects of phosphorylation on p120 localization at AJs, we performed time-lapse imaging of GFP-tagged p120 mutants expressed in HPAE cells after stimulation with thrombin (Fig. 3F). Average fluorescence intensity of GFP at AJs over time was determined by kymograph analysis (Fig. 3G). We showed that p120 mutants localized similarly at AJs (Fig. 3F). The level of S879A-p120 remained unchanged at AJs after 12 m of thrombin (Fig. 3G). However, S879D-p120 decreased to 25% of the initial value at AJs (Fig. 3F). Dissociation of WT-p120 was evident after 5 m of thrombin stimulation, and continued to decrease to 40% of baseline at 12 m (Fig. 3G). These findings further support the key role of S879 phosphorylation as a determinant of p120 association with VE-cadherin at AJs.

S879 phosphorylation of p120 induces AP-2 binding to VE-cadherin and its uncoupling from AJs

We next addressed whether S879 phosphorylation of p120 induced dissociation of VE-cadherin from AJs. Cells transfected with GFP-tagged p120 phospho-mutants were treated with thrombin and immunostained for VE-cadherin. The level of VE-cadherin accumulation at AJ, as a measure of AJ stability, as well as the gap area, a measure of increased permeability, was calculated in response to thrombin. Cells expressing S879A-p120 had junctional gaps covering 7% of total area and lost only 21% of the VE-cadherin from junctions after thrombin stimulation (Fig. 4A-B). S879D-p120-expressing cells had gaps covering 15% of total area and lost 73% of the VE-cadherin from AJs. Thus, S879 phosphorylation promotes the disassociation of VE-cadherin from AJs.

Clathrin adaptor AP-2 binds the same residues on cadherins as p120[22, 25], and p120 dissociation enables AP-2 binding to VE-cadherin, which in turn can activate clathrin-mediated VE-cadherin internalization[22]. Consistent with the observed inhibition of p120 phosphorylation, depletion of PKCα decreased AP-2 interaction with VE-cadherin after thrombin stimulation (Fig. 4C). These findings were recapitulated with the p120 mutants. Expression of S879D-p120 increased the interaction between VE-cadherin and AP-2 after thrombin stimulation whereas expression of either WT or S879A-p120 prevented this increase (Fig. 4D).

p120-S879 phosphorylation increases vascular permeability

To determine whether p120 S879 phosphorylation mediates permeability of vessels, we quantified changes in TER of monolayers transfected with WT, S879A-p120, and S879D-p120 cDNA (Fig. 5A-B). Cells expressing S879A-p120 showed a modest (16%) decrease in TER in response to thrombin, whereas TER decreased 30% and 44% from baseline in cells expressing WT-p120 and S879D-p120, respectively. These data indicate the protective effect of the S879A-p120 mutant in decreasing the junctional permeability response.

To address the barrier-protective effect of S879A-p120 mutant *in vivo*, we expressed p120 mutants in mouse lung vascular endothelial cells using liposomes[39], followed by determination of vascular permeability after stimulation with PAR-1 agonist peptide (Fig. 5C-E). Western blotting confirmed equal expression of WT-p120 and S879A-p120 mutants (Fig. 5C). Lung vascular permeability in mice expressing GFP, WT-p120, and S879A-p120 was measured 30 m after infusion of PAR-1 agonist peptide. An empty GFP vector was used as control. Extravasation of EBA, a measure of albumin transvascular permeability, increased to 30 mg/m dry lung in mice expressing S879A-p120, whereas mice expressing WT-p120 had a significantly greater response (Fig. 5D). Measurements of lung wet:dry ratio (tissue edema) also showed that expression of S879A-p120 significantly reduced lung edema formation in mice receiving PAR-1 agonist peptide compared to expression of WT-p120 (Fig. 5E).

PKCα contributes to LPS-induced increase lung vascular permeability

To address the role of S879-p120 phosphorylation in increasing vascular permeability in another model, we challenged *prkca*$^{-/-}$ and WT mice with LPS (10 mg/kg) Immunoblots of lysates from WT mouse lungs showed p120 was phosphorylated on S879 within 1 h of LPS treatment (Fig. 6A). The lack of S879 phosphorylation in *prkca*$^{-/-}$ mice after LPS treatment demonstrated that S879 phosphorylation was PKCα-dependent. At 6 h post-LPS, $K_{f,c}$ of WT mouse lungs increased significantly whereas in *prkca*$^{-/-}$ mouse lungs $K_{f,c}$ response was markedly reduced (Fig. 6B).

Discussion

We addressed the mechanisms of action of the second messenger PKCα as a crucial signal mediating endothelial barrier disruption. These studies were based on our previous demonstration of the key role of PKCα in signaling increased vascular permeability[34, 41]. We showed that PKCα phosphorylation of p120 at S879 is an essential phospho-switch that reduces p120 binding affinity for VE-cadherin, thereby causing disassembly of AJs and increasing endothelial permeability. Our findings also demonstrated that p120 dissociation from VE-cadherin enables binding of clathrin-adaptor protein AP-2 to VE-cadherin. These findings support the concept that the PKCα-mediated p120 phosphorylation induces AJ disassembly subsequent to VE-cadherin internalization. Using lungs, we further demonstrated that PKCα deletion (*prkca*$^{-/-}$ mice) or expression of a p120 mutant resistant to PKCα phosphorylation in lung vessel endothelia reduced the increase in vascular permeability induced by PAR-1 activation, demonstrating the *in vivo* relevance of our findings.

PKCα in its active state induced by thrombin stimulation was shown to bind and phosphorylate p120, resulting in dissociation of p120 from VE-cadherin. In the absence of PKCα activation, p120 remained bound to VE-cadherin. The findings that PKCα activity is

required for disassembly of the p120/VE-cadherin complex is consistent with the postulated role of post-translational modification of p120 in promoting AJ disassembly during inflammation[35]. Phosphorylation of tyrosine 658 and 731 on VE-cadherin reduced binding with p120[42]; however, phosphorylation at both sites downstream of Src activation was not sufficient to disrupt the endothelial barrier [28]. Tyrosine phosphorylation on NTD of p120 during inflammation, which affects p120 binding and activity RhoA GTPase, also did not affect endothelial barrier function[27, 29]. Here we focused on CTD of p120 which contains two phosphorylation sites, at threonine 916 and serine 879 (S879)[30]. Our results identified the obligatory role of PKCα phosphorylation at S879 in mediating disruption of AJ barrier and increasing endothelial permeability.

The strength of cadherin binding to p120 is weak compared to cadherin binding to β-catenin[25, 43], suggesting the malleability of p120/cadherin interaction during inflammation. We observed that the phospho-mimicking p120 mutant (S879D) had significantly reduced binding affinity to VE-cadherin compared to WT-p120 supporting the role of S879 phosphorylation as a key regulator of p120 binding to VE-cadherin. The basal VE-cadherin/p120 K_D value was less than values for E-cadherin and p120[38], which might be due to intrinsic differences between the two cadherins and endothelial vs. epithelial barrier restrictiveness. They may also reflect methodological differences (e.g., we used full-length construct while others have used fragments of p120[24]) to assess interactions. Upon stimulation with thrombin, both WT and S879D-p120 dissociated from VE-cadherin whereas expression of phospho-defective p120 mutant (S879A-p120) demonstrated increased association due to its greater VE-cadherin binding potential. These results collectively show that PKCα-induced p120 phosphorylation functions by reducing p120 binding affinity to VE-cadherin, which appears to be a key factor regulating the plasticity of AJs.

The plastic nature of the interactions between VE-cadherin and p120 can also be inferred from the crystal structure of the p120/E-cadherin complex[25]. The stronger static interaction encompasses a greater fraction of p120 molecule binding to the JMD whereas the dynamic interaction appears to be subject to regulation[25] by modifications induced by phosphorylation. Moreover, the dynamic binding site masks the AP-2 binding site on VE-cadherin[25]. Because PKCα-induced p120 phosphorylation not only interfered with p120 binding to VE-cadherin but also allowed AP-2 binding in a *pari passu* manner at the same site, we suggest that phosphorylation of S879 alters the dynamic interaction between p120 and VE-cadherin.

VE-cadherin has a different amino acid sequence in JMD core from E-cadherin[25]. VE-cadherin lacks the canonical di-leucine motif to which clathrin adaptors such as AP-2 bind to induce endocytosis[25], however, we showed the VE-cadherin JMD functions like E-cadherin because of its ability to bind AP-2. Thus, once p120 dissociated from VE-cadherin, VE-cadherin is extricated from AJs through endocytosis after binding AP-2. It remains to be seen if S879 phosphorylation of p120 also induces dissociation from other cadherins as it does for VE-cadherin.

We showed using TER measurements that AJ integrity is restored w 2 hours of disruption after PAR-1 activation; thus, the process at least with thrombin stimulation is fully reversible. We did not address how p120 interaction with VE-cadherin is re-established after PKCα-induced p120 phosphorylation. One possibility is that internalized VE-cadherin re-cycles to AJs on dephosphorylation of p120 (undefined phosphatase) and its return to AJs. In support of this idea, p120 level at junctions was restored by 60 min after thrombin stimulation.

To connect the relevance of PKCα-induced p120 phosphorylation at S879 to endothelial permeability *in vivo*, we expressed WT-p120 and phosphodefective p120 (S879A-p120) constructs described above in mouse lung vessel endothelia. Expression of S879A-p120 significantly reduced the vascular permeability response induced by PAR-1 activation. Inhibition of S879 phosphorylation also increased VE-cadherin retention at AJs and maintained permeability at a low level even after stimulation with thrombin.

To extend these studies to another model of increased vascular permeability, we used LPS to address the role that PKCα-mediated S879-p120 phosphorylation in increasing permeability. Using *prkca*$^{-/-}$ and WT mice challenged with LPS, we observed that S879 was phosphorylated in response to LPS. We also found that deletion of PKCα significantly reduced LPS-induced increase in lung vascular permeability. The model (Fig. 6C) describes the role of PKCα-mediated phosphorylation of p120 on S879 in disrupting p120 binding to VE-cadherin, which in turn results in VE-cadherin internalization and increases endothelial permeability. These results raise the intriguing prospect that manipulation of S879 phosphorylation on p120 is a potentially important anti-inflammatory target.

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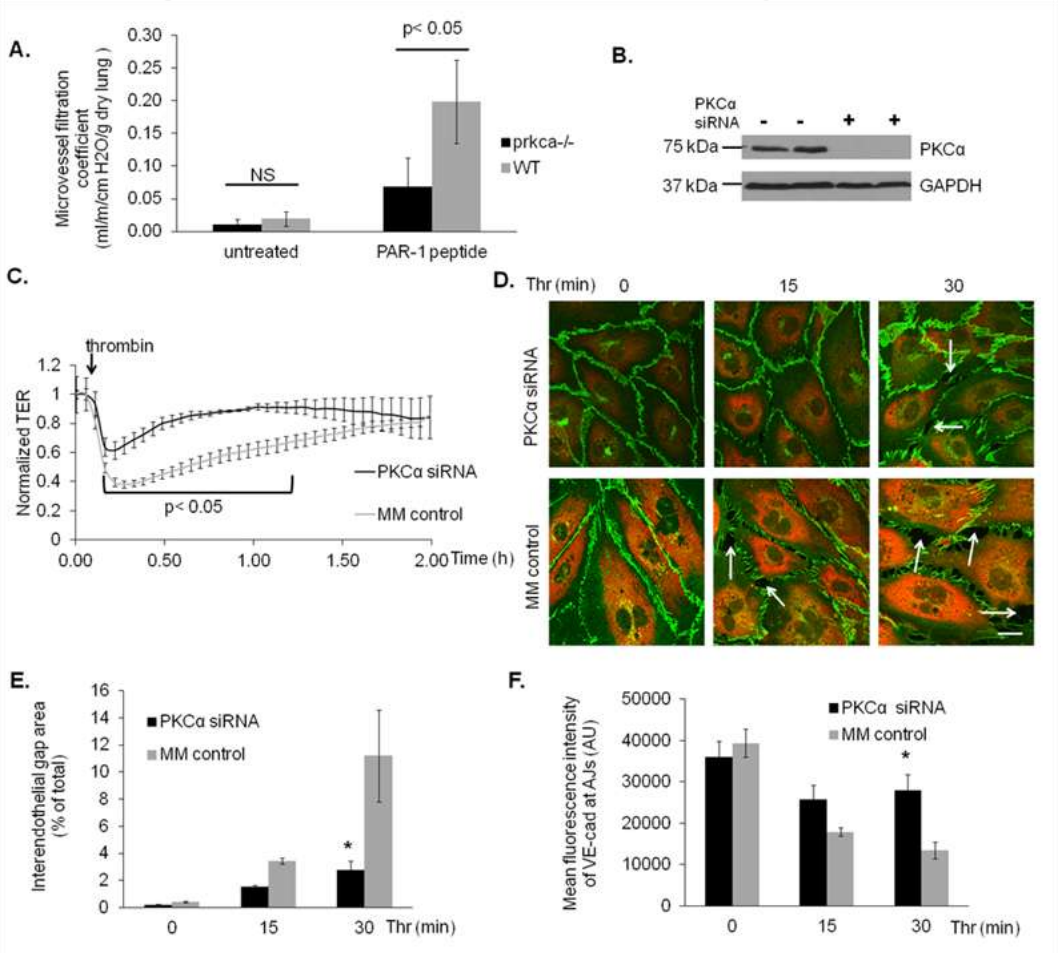
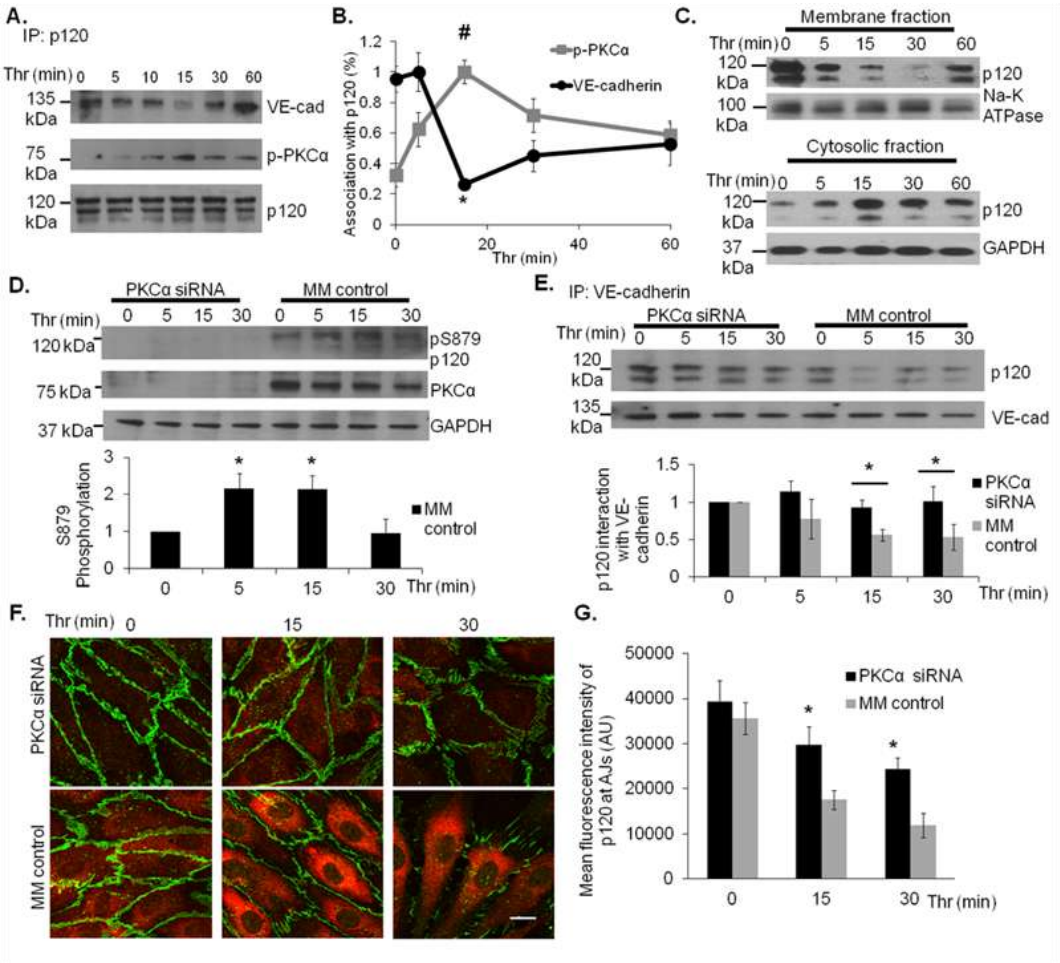
Figure Legends

Figure 1. PKCα regulates paracellular endothelial permeability.



(A) PKCα contributes to mechanism of increase vascular permeability induced by PAR-1 agonist peptide. Lungs from PKCα knockout (*prkca*$^{-/-}$) and wild-type (WT) mice were perfused with PAR-1 agonist peptide (1 mg/kg) or left untreated (control group). Graph shows lung microvessel filtration coefficient ($K_{f,c}$) as mean ± SEM (n=4). NS=not significant. * $p<0.05$ denotes significance from WT after PAR-1 peptide stimulation. (B) Immunoblots showing PKCα expression in HLMVECs 72 h after treatment with PKCα siRNA (+) or MM control siRNA (-). GAPDH is shown as loading control. (C) Deletion of PKCα reduces thrombin-induced decrease in TER. Representative traces of TER in HLMVECs transfected with PKCα siRNA or MM control siRNA. Arrow shows addition of α-thrombin (Thr; 4 U/ml). Results are expressed as mean ± SEM (n=3); * $p<0.05$ denotes difference from MM control. (D-F) PKCα induces interendothelial gap formation. (D) Immunofluorescent staining of HLMVECs transfected with PKCα or MM control siRNA for VE-cadherin (green) and PKCα (red) post-thrombin stimulation (4 U/ml). Arrows indicate interendothelial gaps. Scale bar, 10 μm. * denotes difference ($p<0.05$) between MM control and PKCα. (E) Bar graph shows mean ± SEM of interendothelial gap area expressed as percent of total area in cells expressing PKCα siRNA or MM control siRNA. (n=5). (F) Bar graph shows mean ± SEM of fluorescence intensity of VE-cadherin at AJs in cells expressing PKCα siRNA or MM control siRNA (n=5). * denotes diffeence ($p<0.05$) between MM control and PKCα.

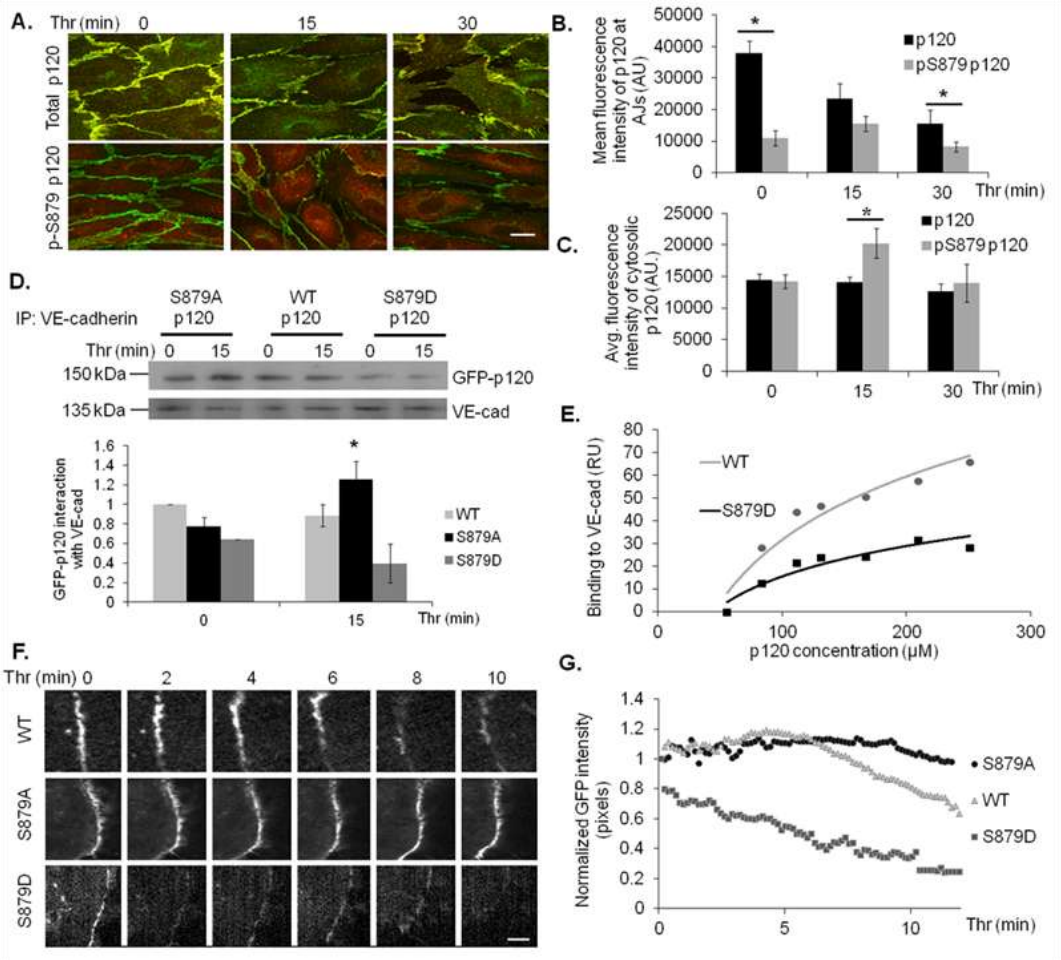
Figure 2. PKCα regulates the association between p120-catenin and VE-cadherin.



(A-B) Thrombin alters p120 association with VE-cadherin and PKCα. p120 was immunoprecipitated from HLMVEC lysates at indicated times after thrombin stimulation (4 U/ml), and precipitates were immunoblotted for phospho-PKCα (p-PKCα; p-Ser657), VE-cadherin (VE-cad), and p120. (B) densitometric analysis of interactions of VE-cadherin and PKCα with p120. Data represent mean ± SEM (n=3). * and # denote difference from baseline ($p<0.05$). (V) Thrombin induces redistribution of p120 in endothelial cells. Lysates from thrombin-stimulated HLMVECs were separated into cytosolic and membrane fractions followed by Western blotting with anti-p120 antibody. Immunoblots with anti-Na-K ATPase or anti-GAPDH antibodies were used as loading controls. (D) PKCα knockdown prevents p120 phosphorylation on S879. Western blots show changes in p120 phosphorylation on S879 following thrombin stimulation in HLMVECs transfected with PKCα or MM control siRNA. Immunoblot with anti-PKCα shows equal protein depletion of PKCα with GAPDH as a loading control (n=3). Bar graph shows densitometric analysis, * denotes difference from MM control 0 timepoint. (E) PKCα depletion prevents p120 dissociation from VE-cadherin. VE-cadherin was immunoprecipitated from PKCα siRNA or MM control-transfected HLMVECs lysates after thrombin stimulation. Precipitates were immunoblotted with anti- p120 and anti-VE-cadherin antibodies (n=3). Bar graph shows densitometic analysis; * denotes difference from MM control ($p<0.05$). (F-G) p120 is retained at

junctions in cells depleted of PKCα after thrombin treatment. (F) siRNA-treated HLMVECs were immunostained for p120 (green) and PKCα (red). Representative images shown at indicated times after thrombin stimulation. Scale bar, 10 μm. (G) Bar graph showing mean ± SEM of fluorescence intensity of p120 at the junctions (n=5). * denotes significance from MM control ($p<0.05$).

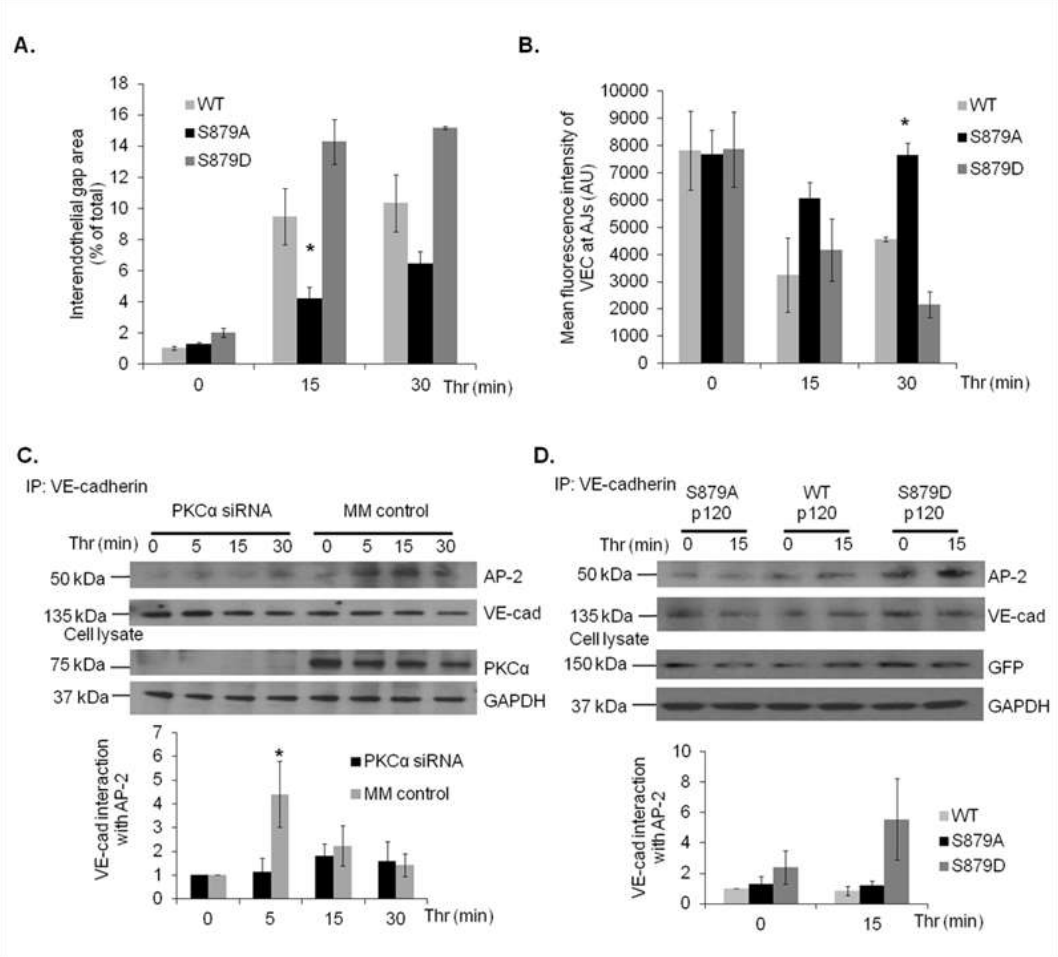
Figure 3. S879 phosphorylation of p120-catenin reduces p120 affinity for VE-cadherin.



(A-C) p120 phosphorylated on S879 resides primarily in cytosol. (A) HLMVECs were co-immunostained for VE-cadherin (green) and p120 (top panel; red) or pS879 p120 (bottom panel; red). Representative images show total p120 or pS879-p120 localization at indicated times after thrombin stimulation (4 U/ml). Scale bar, 10 μm. (B-C) Graph of p120 accumulation at AJs (B) or in cytosol (C) shown as mean fluorescence intensity ± SEM (n=5). * denotes difference from total p120 ($p<0.05$). (D) S879A-p120 maintains binding to VE-cadherin after thrombin stimulation. VE-cadherin was immunoprecipitated from HPAE lysates expressing WT p120, S879A-p120, or S879D-p120. Immunoblots show association of VE-cadherin with GFP-tagged p120 (GFP) mutants following thrombin stimulation (4 U/ml; n=3). Bar graph shows densitometric analysis; * denotes difference from WT-p120 ($p<0.05$). (E) S879D-p120 has decreased binding affinity to VE-cadherin. Increasing concentrations of WT-p120 or S879D-

p120 protein were exposed to immobilized VE-cadherin, and bound p120 was measured with SPR. Results are expressed as best-fit concentration-response curve. (F-G) S879A-p120 remains at junction while S879D-p120 dissociates on thrmobin stimulation. (F) Cells transfected with GFP-p120 phospho-mutants were imaged before and after thrombin stimulation (4 U/ml). Representative time-lapse images show p120 localization at AJs at indicated time points after thrombin. Scale bar, 2 µm. (G) Fluorescence intensity of GFP at AJs was quantified and expressed as percent change in average fluorescence intensity over time (n=25 cells).

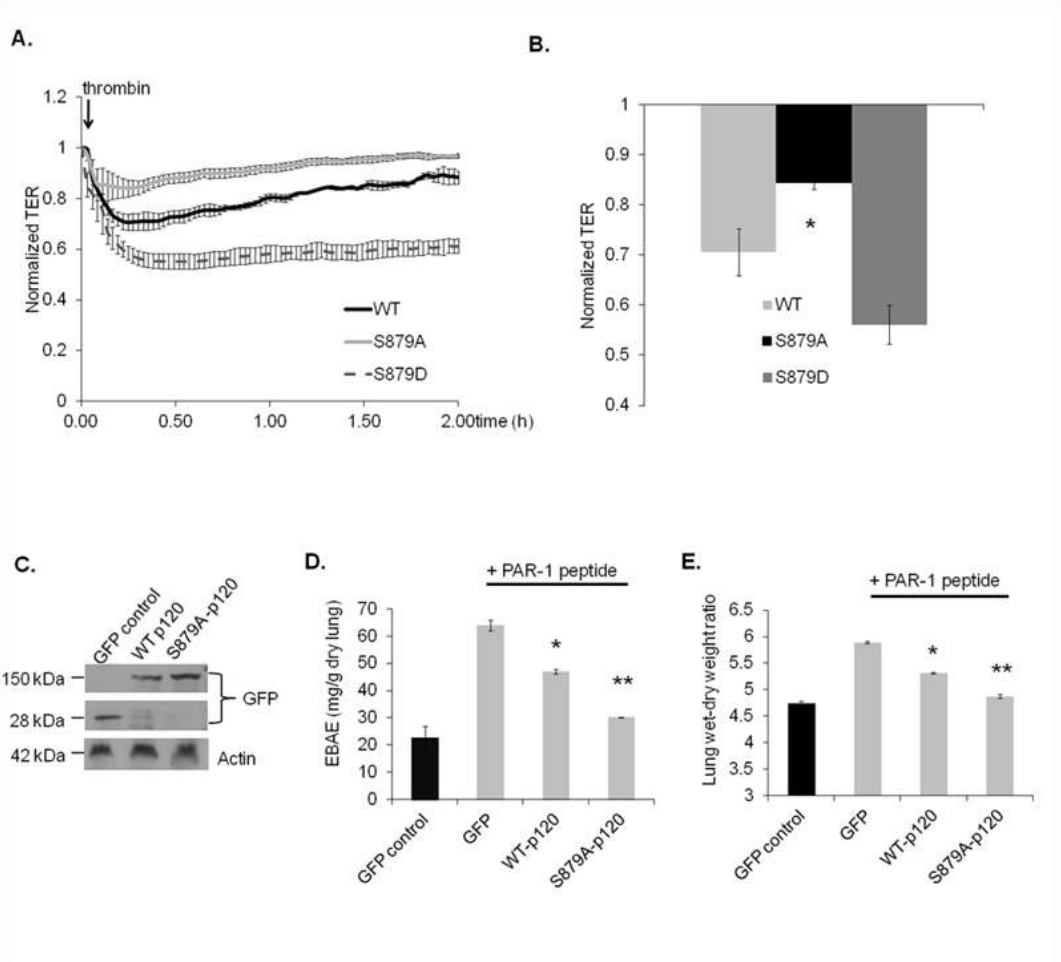
Figure 4. p120 S879 phosphorylation regulates VE-cadherin localization at AJs.



(A-B) VE-cadherin remains at junctions in cells expressing S879A-p120 after thrombin stimulation. (A) Graph of mean ± SEM of interendothelial gap area expressed as percent of total area (n=5). (B) Graph of average fluorescence intensity of VE-cadherin at AJs. Results are expressed as mean ± SEM (n=5). * denotes difference from WT ($p<0.05$). (C) VE-cadherin interaction with AP-2 is decreased in cells depleted of PKCα. VE-cadherin was immunoprecipitated in HLMVECs treated with PKCα or MM control siRNA. Immunoblots show AP-2 interaction with VE-cadherin (n=3). Immunoblots from cell lysates show equal knockdown of PKCα. Bar graph shows densitometric analysis; * denotes significance from MM control ($p<0.05$). (D) VE-cadherin interaction with AP-2 is increased in cells expressing S879D-p120.

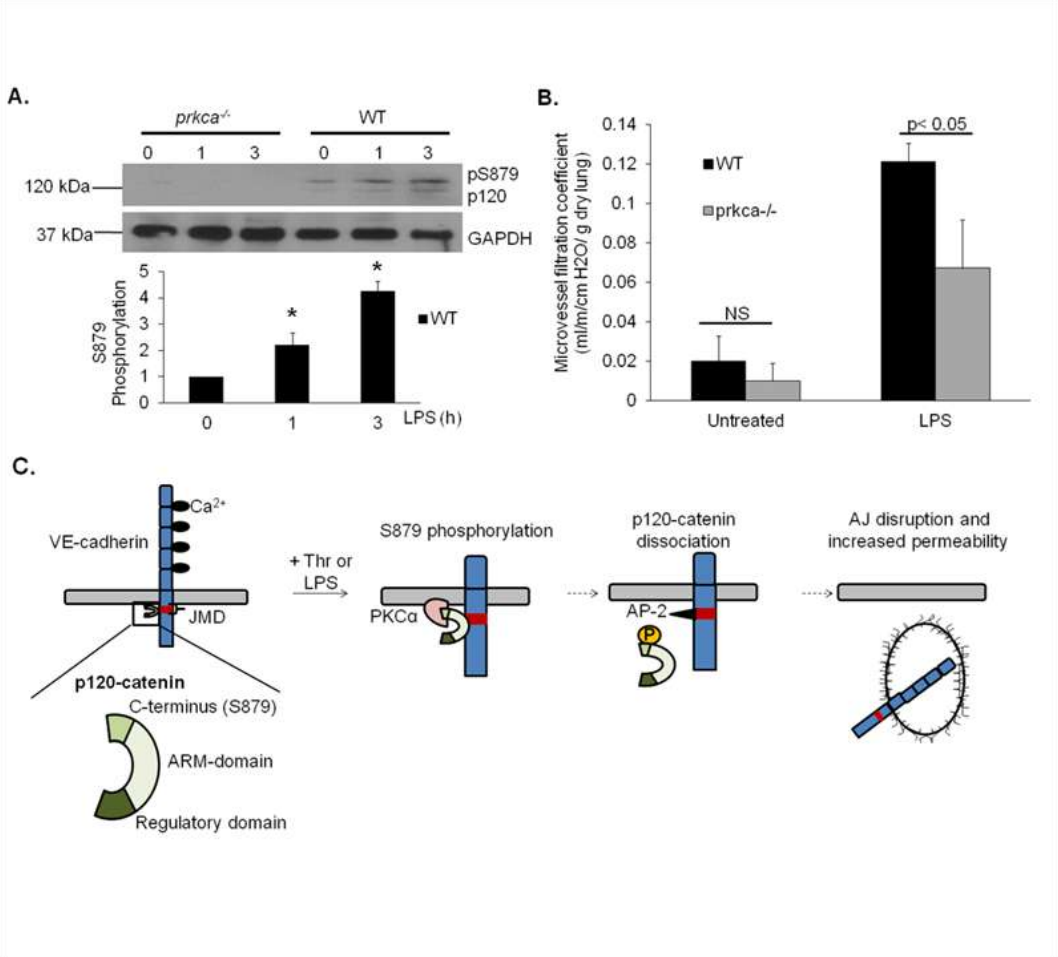
VE-cadherin was immunoprecipitated in HPAE cells transfected with indicated p120 phospho-mutants; precipitates were immunoblotted for AP-2 and VE-cadherin (n=3). Immunoblots from whole cell lysates show equal expression of p120 phospho-mutants. Bar graph shows densitometric analysis; * denotes significance from WT-p120 ($p<0.05$).

Figure 5. S879 phosphorylation of p120-catenin increases endothelial permeability.



(A-B) Expression of S879A-p120 attenuates thrombin-induced decrease in transendothelial resistance (TER). (a) Graph shows mean ± SEM of changes in TER in HPAE cells expressing WT-p120, S879A-p120, or S879D-p120 mutants; arrow indicates thrombin addition (4 U/ml; n=3). (B) Mean ± SEM of maximum decrease in TER after thrombin stimulation (n=3). * denotes significance ($p<0.05$) from WT-p120. (C-E) Expression of S879A-p120 reduces PAR-1-induced increased in lung vascular permeability. GFP-WT-p120, GFP-S879A-p120, or empty vector (GFP) were transduced in WT mouse lung endothelia using liposomes. (C) Immunoblots show expression of GFP (28 kDa), GFP-tagged WT-p120 and S879A-p120 (150 kDa) in whole lungs. Actin is shown as a loading control. (D-E) Evans blue was simultaneously injected i.v. with PAR-1 agonist peptide (1 mg/kg). At 30 minutes thereafter, we determined (D) EBA extravasation and (E) lung wet:dry ratio. Data represent mean ± SEM (n=4). * denotes difference from GFP and ** denotes difference from WT-p120 ($p<0.05$).

Figure 6. PKCα-mediated S879-p120 phosphorylation contributes to LPS-induced increase in lung vascular permeability.



(A) LPS induces S879-p120 phosphorylation in a PKCα-dependent manner. Western blot analysis was performed on mouse lung homogenates after mice were challenged with LPS (10 mg/kg) for the indicated times. Immunoblots show phosphorylation of S879 on p120 after LPS treatment and deletion of PKCα (n=3). Bar graph shows densitometric analysis; * denotes significance from WT 0 timepoint ($p<0.05$). (B) Deletion of PKCα significantly reduced the LPS-induced increase in lung vascular permeability. Lungs from PKCα knockout (*prkca*$^{-/-}$) and wild-type (WT) mice were perfused with LPS (40 mg/kg) and compared to untreated control group (from previous data; Fig 1A). Bar graph shows lung vascular filtration coefficient ($K_{f,c}$) as mean $\pm$ SEM (n=3). NS=not significant. * $p<0.05$ denotes significance from WT after LPS treatment. (C) Model of effects of PKCα-mediated S879 phosphorylation of p120 on stability of VE-cadherin adhesion and AJ assembly. p120 binds the JMD (aa 736-781) of VE-cadherin but after stimulation with thrombin or LPS, PKCα is activated and translocated to the AJ where it phosphorylates S879 on p120. Phosphorylated p120 dissociates from VE-cadherin leaving VE-cadherin susceptible to binding by AP-2 and recruitment into clathrin-coated pits. VE-cadherin undergoes endocytosis and AJs are disassembled.