Phospholipase C-ε signaling mediates endothelial cell inflammation and barrier disruption in acute lung injury

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Bijli KM, Fazal F, Slavin SA, Leonard A, Grose V, Alexander WB, Smrcka AV, Rahman A. Phospholipase C-ε signaling mediates endothelial cell inflammation and barrier disruption in acute lung injury. Am J Physiol Lung Cell Mol Physiol 311: L517–L524, 2016. — Phospholipase C-ε (PLC-ε) is a unique PLC isoform that can be regulated by multiple signaling inputs from both Ras family GTPases and heterotrimeric G proteins and has primary sites of expression in the heart and lung. Whereas the role of PLC-ε in cardiac function and pathology has been documented, its relevance in acute lung injury (ALI) is unclear. We used PLC-ε−/− mice to address the role of PLC-ε in regulating lung vascular inflammation and injury in an aerosolized bacterial LPS inhalation mouse model of ALI. PLC-ε−/− mice showed a marked decrease in LPS-induced proinflammatory mediators (ICAM-1, VCAM-1, TNF-α, IL-1β, IL-6, macrophage inflammatory protein 2, keratinocyte-derived cytokine, monocyte chemoattractant protein 1, and granulocyte-macrophage colony-stimulating factor), lung neutrophil infiltration and microvascular leakage, and loss of VE-cadherin compared with PLC-ε+/+ mice. These data identify PLC-ε as a critical determinant of proinflammatory and leaky phenotype of the lung. To test the possibility that PLC-ε activity in endothelial cells (EC) could contribute to ALI, we determined its role in EC inflammation and barrier disruption. RNAi knockdown of PLC-ε inhibited NF-κB activity in response to diverse proinflammatory stimuli, thrombin, LPS, TNF-α, and the nonreceptor agonist phorbol 13-myristate 12-acetate (phorbol esters) in EC. Depletion of PLC-ε also inhibited thrombin-induced expression of NF-κB target gene, VCAM-1. Importantly, PLC-ε knockdown also protected against thrombin-induced EC barrier disruption by inhibiting the loss of VE-cadherin at adherens junctions and formation of actin stress fibers. These data identify PLC-ε as a novel regulator of EC inflammation and permeability and show a hitherto unknown role of PLC-ε in the pathogenesis of ALI.

endothelial cells; adhesion molecules; transcription factors; signal transduction; lung inflammation

THE ACQUISITION OF PROINFLAMMATORY PHENOTYPE and disruption of vascular endothelial barrier leading to inflammatory cell infiltration and protein-rich edema formation are prominent pathogenic features of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (3, 31, 50). The major mechanisms of inflammatory cell extravasation and increased vascular permeability in the inflamed lung and other tissues include activation of the transcription factor NF-κB, a master regulator of inflammation, and disassembly of VE-cadherin homodimers, the main constituent of inter-EC adherens junctions (AJs), respectively (7, 10, 38). Activation of NF-κB is initiated by IKK-β-mediated phosphorylation of Ser32 and Ser36 of Ik-Bo, an inhibitory protein that associates with NF-κB to retain it as an inactive complex in the cytoplasm (22, 38). Phosphorylation of Ik-Bo marks it for ubiquitination and proteasome-mediated degradation, causing the release of NF-κB. The released NF-κB [predominantly RelA/p65 homodimer in endothelial cells (EC) (35)] undergoes rapid nuclear translocation and binding to the promoter of proinflammatory genes. Phosphorylation of RelA/p65 at Ser336 serves to confer transcriptional competency to NF-κB bound to the promoter (22, 38). Activated NF-κB renders the otherwise “antiadhesive” lung microvasculature into a “proadhesive” one via activation of proinflammatory genes such as adhesion molecules (ICAM-1, VCAM-1, E-selectin), cytokines (TNF-α, IL-1β, IL-6), and chemokines [IL-8, monocyte chemoattractant protein (MCP-1)] (13, 33, 34, 39, 53, 54).

Disassembly of AJs is primarily regulated by loss of VE-cadherin from AJs, which is further aided by the contractile forces generated by actin-myosin interaction (actin stress fiber formation) (10, 11, 31). Thus VE-cadherin disassembly and actin-myosin interaction are important determinants of AJ disruption and increased endothelial permeability caused by proinflammatory mediators (10, 11, 30, 31). The coordinated and concerted actions of these events (induction of proinflammatory genes via NF-κB activation and disruption of endothelial AJs via VE-cadherin disassembly) serve to facilitate adhesion and transendothelial migration of inflammatory cells, particularly neutrophils (polymorphonuclear leukocytes, PMN) (29, 39, 43), and to increase endothelial permeability (6, 11, 32, 44) associated with ALI/ARDS (3, 5, 14, 18, 20, 45, 50).

Phosphoinositide-specific phospholipase C-ε (PLC-ε) is the most recently described member of the PLC family that hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (8, 26). The IP3 and DAG in turn lead to generation of Ca2+ and activation of protein kinase C (PKC), respectively (8, 26). PLC-ε contains Ras association (RA) domains that confer regulation by Ras family members and a guanine nucleotide exchange factor (GEF) domain that regulates activity of the small GTPase, Rap (8, 26). PLC-ε is directly regulated by small GTPases including Rho, Rac, Ras, and Rap downstream of G protein-coupled receptors (GPCRs), including thrombin and lysophosphatidic acid receptors, as well as receptor tyrosine kinases (RTK) such as insulin growth factor receptor (8, 12, 26, 41). PLC-ε is implicated to play a role in the development and function of heart as well as in skin
inflammation and tumor promotion (24, 28, 49). In cardiac myocytes, PLC-ε hydrolyzes phosphatidylinositol 4-phosphate as a substrate instead of PI(4,5)P2 at the Golgi apparatus to generate local DAG (55). Other work indicates that PLC-β isoforms respond to acute receptor signals, whereas PLC-ε is responsible for longer-duration signaling (25). These data indicate that PLC-ε may play an important role in chronic receptor signaling and DAG generation at intracellular compartments. Studies using PLC-ε−/− mice have identified novel PLC-ε-dependent pathways for adrenergic regulation of cardiac contraction and GPCR- and RTK-dependent regulation of cardiac hypertrophy (49, 55). Lung is another primary site of PLC-ε expression; however, its role in the lung, particularly in the context of ALI, has not been studied. In this study, we provide evidence that PLC-ε is an important component of lung vascular inflammation and injury and that this action of PLC-ε relies, at least in part, on its ability to promote EC inflammation and barrier disruption.

MATERIALS AND METHODS

Reagents. Human thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). LPS of Escherichia coli (E. coli) origin, diethylaminoethyl (DEAE)-dextran, and an anti-PLC-ε antibody were from Sigma–Aldrich Chemical (St. Louis, MO). Antibodies to VCAM-1, ICAM-1, RelA/p65, IκB-α, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to VE-cadherin were obtained from Abcam (Cambridge, MA) and BD Biosciences (San Jose, CA). Antibodies to phospho-(Ser12) and phospho-(Ser78)-IκBα and phospho-(Ser73)-RelA/p65 were obtained from Cell Signaling Technology (Beverly, MA). Plasmid maxi kit was from Qiagen (Valencia, CA). All other materials were from Fisher Scientific (Pittsburg, PA) or VWR Scientific Products (Gaithersburg, MD).

Mouse model of ALI. PLC-ε−/− (knockout, KO) mice were generated as described previously (49). Age-matched C57BL/6 PLCE-ε+/+ (wild-type, WT) mice were used as controls (Jackson Laboratory, Bar Harbor, ME). WT and KO mice were exposed to aerosol of LPS (0.5 mg/ml) for 30 min as described (40, 52). After 18 h, bronchoalveolar lavage (BAL) fluids and lungs from these mice were collected and analyzed for the various markers of lung inflammation and injury as described (5, 14, 16). All mice care and treatment procedures were approved by the University of Rochester Committee on Animal Resources and performed in adherence to the National Institute of Health guidelines.

EC. Human pulmonary artery ECs (HPAEC) were obtained from Lonza (Walkersville, MD) and cultured as described (4) in gelatin-coated flasks using endothelial basal medium 2 (EBM2) with bullet kit additives (BioWhittaker, Walkersville, MD). Experiments were performed in HPAEC < passage 6.

Measurement of lung inflammation and injury. Lung homogenates were prepared in radioimmune precipitation (RIPA) buffer [50.00 mM Tris-HCl, pH 7.4, 150.00 mM NaCl, 0.25 mM EDTA, pH 8.0, 1.00% deoxycholic acid, 1.00% Triton X-100, 5.00 mM NaF, 1.00 mM EGTA, 1.00 mM DTT, and 1.00% PMSF]. After 0.5 h at 4°C, lysates were centrifuged to collect the supernatants containing the cytoplasmic proteins. The pelleted nuclei were resuspended in 50 µl of buffer A (10.00 mM HEPES, pH 7.9, 10.00 mM KCl, 0.10 mM EDTA, 1.00 mM magnesium chloride, 10.00% glycerol, 0.5–1.0% Triton X-100, 1.00 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail). Equal amounts of protein from the cell lysates as determined by the BCA assay (Pierce) were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes for Western blotting as described (15). Representative blots presented in RESULTS come from the same membrane that may have more samples in various groups.

Nuclear extract preparation and assessment of NF-κB DNA binding. Nuclear extracts were prepared and analyzed for NF-κB DNA binding activity as described (1). After appropriate treatments, cells were washed twice with ice-cold PBS and resuspended in 400 µl of buffer A (10.00 mM HEPES, pH 7.9, 10.00 mM KCl, 0.10 mM EDTA, 1.00 mM magnesium chloride, 10.00% glycerol, 0.5–1.0% Triton X-100, 1.00 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail). Equal amounts of protein from the cell lysates as determined by the BCA assay (Pierce) were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes for Western blotting as described (15). Representative blots presented in RESULTS come from the same membrane that may have more samples in various groups.

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of PMN in the lung was determined by monitoring the differential cell counts in BAL fluids and myeloperoxidase activity in the lung tissues as described (5, 14, 52). Total protein levels in BAL fluids were determined using bicinchoninic acid (BCA) kit (Pierce, Rockford, IL).

RNAi knockdown. Predesigned short interfering RNA (siRNA) specific for human PLC-ε (si-PLC-ε) and a nontargeting siRNA control (si-Con) were obtained from Dharmacon (Lafayette, CO). HPAEC were transfected with si-PLC-ε or si-Con using DharmaFect1 siRNA transfection reagent (Dharmacon) essentially as described (4). Briefly, 50–100 nM siRNA and DharmaFect1 were mixed together and then added to cells that were 50–60% confluent. At 24–36 h after transfection, cells were challenged with thrombin or other agonists and then lysed after the indicated time periods for various analyses.

Immunoblot analysis. Immunoblotting was performed as described previously (15). In brief, cells after appropriate treatments were lysed in RIPA buffer or phosphorylation lysis buffer [50.00 mM HEPES, 150.00 mM NaCl, 200.00 µM sodium orthovanadate, 10.00 mM sodium pyrophosphate, 100.00 mM sodium fluoride, 1.00 mM EDTA, 1.50 mM magnesium chloride, 10.00% glycerol, 0.5–1.0% Triton X-100, 1.00 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail]. Equal amounts of protein from the cell lysates as determined by the BCA assay (Pierce) were subjected to SDS-PAGE and then transferred onto nitrocellulose membranes for Western blotting as described (15). Representative blots presented in RESULTS come from the same membrane that may have more samples in various groups.

Reporter gene constructs and luciferase assay. The reporter plasmid pNF-κB-LUC (Stratagene, La Jolla, CA) containing five copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene was used to determine the transcriptional activity of NF-κB. The pTKRLUC plasmid (Promega, Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter was used to normalize the transfection efficiencies. Reporter gene transfections and luciferase assays were performed essentially as described (36). Briefly, DEAE-dextran (50 µg/ml) in serum-free EB2M was mixed with 5 μg pNF-κB-LUC and 0.125 μg pTKRLUC. The resulting mixture was applied onto cells that were 60–70% confluent. After 1 h, cells were exposed to 10% DMSO in serum-free EB2M for 4 min and then washed twice with PBS and allowed to grow in EB2M-10% FBS. The confluent monolayers were treated with appropriate agonists and then lysed in passive reporter lysis buffer (Promega). The cell extracts were assayed for firefly and Renilla luciferase activities using dual luciferase reporter assay system (Promega), and the data were expressed as a ratio of firefly to Renilla luciferase activity. In experiments evaluating the effect of PLC-ε knockdown on NF-κB transcriptional activity, cells were first transfected with PLC-ε siRNA and 24 h later cells were again transfected with pNF-κB-LUC and pTKRLUC using DEAE-dextran as described (4).

Immunofluorescence. Confluent HPAEC monolayers grown on coverslips were subjected to immunofluorescence staining as described (15). To localize F-acitin filaments, cells were incubated with Alexa Fluor 488-phalloidin. VE-cadherin antibody (BD Biosciences)
was used to visualize AJ. DNA was stained using Hoechst dye to visualize nuclei. Images were obtained using a Nikon fluorescence microscope (Nikon Instech, Tokyo, Japan).

Measurement of endothelial permeability by transendothelial electrical resistance. The endothelial barrier integrity was measured by monitoring transendothelial electrical resistance (TER) across confluent monolayers using the highly sensitive electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as described (32). Briefly, cells were grown to confluence on gelatin-coated gold microelectrodes in complete culture medium (EBM2 containing 10% FBS and bullet kit additives). After 24 h, culture medium was replaced with EBM2 containing 1% FBS, and 2 h later thrombin was added and the TER measured over a period of 4 h. Resistance was normalized to the initial voltage and expressed as a fraction of the normalized resistance value.

Statistical analysis. Data presented are means ± SE and were analyzed by using standard one-way ANOVA. The significance between the groups was determined using Tukey’s test (Prism 5.0; GraphPad Software, San Diego, CA). A P value <0.05 between two groups was considered statistically significant.

RESULTS

Role of PLC-ε in lung vascular inflammation and injury. We used PLC-ε+/− (KO) mice to address the role of PLC-ε in regulating lung inflammatory injury in an aerosolized bacterial LPS inhalation mouse model of ALI (40, 52). LPS inhalation induced the levels of proinflammatory mediators (ICAM-1, VCAM-1, TNF-α, MCP-1, KC, MIP-2, and GM-CSF) in the lungs of PLC-ε+/+ (WT) mice, but these responses were significantly inhibited in KO mice (Fig. 1A, a−g). Similarly, the levels of proinflammatory mediators (IL-1β, IL-6, MCP-1, and KC) in the BAL fluids of KO mice were also attenuated compared with WT mice after LPS challenge (Fig. 1B, a−d). Consistent with this, KO mice showed a marked decrease in LPS-induced lung PMN recruitment and microvascular leakage compared with WT mice (Fig. 2, A−C). Because the levels of VE-cadherin, a major regulator of endothelial barrier integrity (10, 47), are decreased in inflamed lungs (18, 20), we determined whether deficiency of PLC-ε influences VE-cadherin levels in mice challenged with LPS. Results showed that KO mice were protected against LPS-induced decrease in VE-cadherin levels (Fig. 3). These data identify PLC-ε as a critical determinant of proinflammatory and leaky phenotype of the lung.

PLC-ε regulates EC inflammation via NF-κB activation. To test the possibility that PLC-ε activity in EC could contribute

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**Fig. 1.** Effect of phospholipase C-ε (PLC-ε) deficiency on LPS-induced proinflammatory mediators in the lung. Age-matched C57BL/6L wild-type (WT) and PLC-ε−− (knockout, KO) mice were aerosolized with saline alone or saline containing Escherichia coli LPS as described in MATERIALS AND METHODS. At 18 h after LPS inhalation, lungs and bronchoalveolar lavage (BAL) fluids from these mice were collected, and levels of proinflammatory mediators were measured. A: lung homogenates were analyzed for ICAM-1 and VCAM-1 (a and b) levels by ELISA and TNF-α, monocyte chemoattractant protein 1 (MCP-1), keratinocyte-derived cytokine (KC), macrophage inflammatory protein 2 (MIP2), and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels (c−g) by multiplex immune assay system. B: BAL fluids were analyzed for IL-1β, IL-6, MCP-1, and KC levels (a−d) by ELISA. Data are means ± SE (n = 3−5 for each condition). *P <0.05 vs. saline WT; #P vs. LPS WT. nd, not detected.
to the pathogenesis of ALI, we determined its role in mediating EC inflammation. Cells were transfected with siRNA targeting PLC-ε (si-PLC-ε) or control si-RNA (si-Con), and VCAM-1 level was measured as a marker of EC inflammation. Transfection of EC with si-PLC-ε resulted in significant depletion of PLC-ε (Fig. 4A) and inhibition of VCAM-1 expression in response to thrombin (Fig. 4B), an important edemagenic and proinflammatory agonist whose concentration is elevated in plasma and lavage fluids of patients with ALI/ARDS (17, 23) and known to induce PLC-ε activity (9). Because VCAM-1 is primarily regulated by NF-κB (38), we determined the possibility that PLC-ε mediates EC inflammation by activating NF-κB. To this end, cells were transfected with si-PLC-ε or si-Con in combination with pNF-κB-Luc, and luciferase activity was measured as an indicator of NF-κB activity. We observed that depletion of PLC-ε prevented thrombin-induced NF-κB activity (Fig. 4C), consistent with its effect on VCAM-1 expression (Fig. 4B). We also examined whether PLC-ε depletion affects NF-κB activity following stimulation of EC with other proinflammatory stimuli. We found that PLC-ε depletion was effective in preventing NF-κB activity in response to stimuli as diverse as LPS, TNF-α, and the non-receptor agonist phorbol 13-myristate 12-acetate (phorbol esters) (Fig. 4, D–F). These data indicate that PLC-ε is an important regulator of NF-κB activity in the endothelium.

We analyzed NF-κB activation pathway to determine the mechanism by which PLC-ε regulates NF-κB activity. We first evaluated the effect of PLC-ε depletion on Ik-Bo degradation, a prerequisite for the release of NF-κB (predominantly RelA/p65 homodimer in EC) (15, 35) for its translocation to the nucleus, where its binds to the promoters of target genes to activate their transcription (7, 22, 38). We found that thrombin induced Ik-Bo degradation in cells transfected with si-PLC-ε, but this response was markedly reduced in cells transfected with si-PLC-ε (Fig. 5A). Consistent with this, depletion of PLC-ε was also effective in inhibiting thrombin-induced nuclear DNA binding of RelA/p65 (Fig. 5B). We next assessed the role of PLC-ε in mediating phosphorylation of Ser^536 within the transactivation domain 1 of RelA/p65, a critical event conferring transcriptional competency to the bound NF-κB (22, 38). Results showed that thrombin-induced Ser^536 phosphorylation of RelA/p65 was attenuated in cells depleted of PLC-ε (Fig. 5C). Considered together, these data show that PLC-ε controls NF-κB activation and thereby VCAM-1 expression by a dual mechanism involving Ik-Bo degradation-dependent nuclear DNA binding and phosphorylation-dependent increase in transcriptional capacity of the bound RelA/p65.

PLC-ε mediates EC barrier disruption by promoting VE-cadherin disassembly and actin stress fiber formation. We also determined whether PLC-ε is required for EC barrier disruption. Real-time measurement of TER showed that thrombin induced the characteristic time-dependent change in TER (32). The maximal decrease occurred around 0.5 h after thrombin challenge, which gradually recovered to baseline TER by 4 h (Fig. 6A). Depletion of PLC-ε had no effect on baseline TER but protected against thrombin-induced decrease in TER (Fig. 6A). These data identify PLC-ε as a novel regulator of EC barrier function.

We next determined whether the barrier-disruptive action of PLC-ε derives from its ability to mediate the loss of VE-cadherin at AJs and formation of actin stress fibers. Visualization of VE-cadherin junctions showed that thrombin induced a decrease in immunostaining of VE-cadherin at AJs and formation of actin stress fibers. Visualiza-
ε-depleted EC (Fig. 6C). Together, these data identify a novel role of PLC-ε in regulating EC barrier disruption via its ability to induce VE-cadherin disassembly and actin cytoskeleton rearrangement.

**DISCUSSION**

In this study, we have identified a novel role of PLC-ε in mediating EC inflammation and barrier disruption and in causing lung inflammatory injury in mice. Our in vivo experiments using PLC-ε KO mice establish PLC-ε as a key mediator of lung vascular inflammation and injury. Our in vitro data using cultured EC reveal that PLC-ε is an important regulator of EC inflammation and barrier disruption. We show that PLC-ε mediates EC inflammation by promoting NF-κB signaling via 1k-Bax degradation-dependent DNA binding and phosphorylation-dependent increase in transcriptional activity of the bound RelA/p65. We also demonstrate that PLC-ε promotes endothelial barrier disruption by inducing actin stress fiber formation and loss of VE-cadherin at AJs. Thus the pathogenic role of PLC-ε in ALI derives, at least in part, from its ability to induce EC inflammation and permeability.

The vascular endothelium represents a critical interface that serves to regulate vascular homeostasis by virtue of regulating leukocyte trafficking, controlling vessel wall permeability, and maintaining blood fluidity. The extravasation of PMN and loss of endothelial barrier function are key early events in the pathogenesis of ALI and other inflammatory diseases (3, 31, 50, 51). Our results indicate that PLC-ε serves to promote lung vascular inflammation and leakage associated with ALI. Genetic ablation of PLC-ε in the lungs of mice exposed to LPS. Moreover, PLC-ε−/− mice showed a significant protection against the loss of VE-cadherin in the lungs of mice exposed to LPS. Similarly, we also observed that PLC-ε deficiency was associated with reduced lung PMN sequestration and migration in mice exposed to LPS. Consistent with this, the levels of adhesion molecules, cytokines, and chemokines, which act in concert to aid the adhesion of PMN to the endothelium and subsequently extravasation of adherent PMN into the surrounding tissues, were reduced in the lungs of PLC-ε−/− mice compared with PLC-ε +/+ mice challenged with LPS. Further support for the proinflammatory role of PLC-ε comes from previous reports...
showing a role of PLC-ε in skin and intestinal inflammation and tumorigenesis (24, 28).

Studies have shown that activation of NF-κB in EC plays an important role in ALI and other inflammatory diseases by its ability to control the expression of proinflammatory genes (53). Conditional blockade of NF-κB signaling in the endothelium is sufficient to reduce multiple-organ (including lung) inflammation, prevent vascular leak, and improve survival in murine models of sepsis (53). Similarly, EC-specific inhibition of NF-κB protects mice from atherosclerosis (19). Hence, we addressed the possibility that PLC-ε contributes, at least in part, to lung inflammatory injury via activation of NF-κB in EC. Indeed, our data show that PLC-ε contributes, at least in part, to lung inflammatory injury via activation of NF-κB in the endothelium. Indeed, our data show that PLC-ε is an important mediator of NF-κB activation in EC. Furthermore, PLC-ε-mediated activation of NF-κB was noted for several major physiological proinflammatory mediators, such as thrombin, LPS, and TNF-α, an effect that may allow for enhanced NF-κB signaling in the endothelium. Analysis of NF-κB signaling pathway revealed that PLC-ε regulates thrombin-induced NF-κB activation by a dual mechanism whereby it mediates Ik-κB degradation–dependent nuclear uptake and DNA binding of RelA/p65 and also phosphorylation of RelA/p65 to increase its transcriptional capacity. Consistent with its role in NF-κB activation, PLC-ε knockdown was associated with a significant decrease in VCAM-1 levels following thrombin challenge of EC. Together, these data identify PLC-ε as an important mediator of EC inflammation by its ability to control NF-κB signaling.

Our data also support a previously unrecognized role of PLC-ε in regulating endothelial barrier function. EC depleted of PLC-ε showed a decrease in EC barrier disruption caused by thrombin. We found that PLC-ε mediates EC barrier disruption by inducing loss of VE-cadherin from AJs and contractile forces generated by actin–myosin interaction (actin stress fiber formation). Together, these data underscore the importance of PLC-ε in causing EC inflammation.

Fig. 5. PLC-ε regulates NF-κB activity by promoting degradation of Ik-κBα, nuclear DNA binding, and phosphorylation of RelA/p65. HPAEC were transfected with si-Con or si-PLC-ε. After 36 h, cells were treated with thrombin (5 U/ml) for 1.5 h. A: total cell lysates were prepared and immunoblotted with an anti-Ik-κBα antibody. RelA/p65 levels were used to monitor loading. B: nuclear extracts were prepared and analyzed for RelA/p65 nuclear DNA binding using Cayman’s NF-κB transcription factor assay kit as described in MATERIALS AND METHODS. C: total cell lysates were immunoblotted with an anti-phospho-RelA/p65 (Ser536) antibody. RelA/p65 levels were used to monitor loading. Data are means ± SE (n = 6–8 for each condition). *P < 0.05 vs. si-Con untreated controls; #P < 0.05 vs. si-Con thrombin-treated controls.

Fig. 6. PLC-ε regulates endothelial barrier disruption by decreasing cell surface VE-cadherin and increasing actin stress fiber formation. HPAEC were transfected with si-Con or si-PLC-ε. A: after 24 h, cells were reseeded on gold electrode plates and cultured for an additional 48 h. The confluent monolayers were then treated with thrombin, and endothelial barrier disruption was determined by measuring transendothelial electrical resistance (TER). Data are means ± SE (n = 3–5 for each condition). *Difference between si-Con thrombin-treated vs. si-PLC-ε thrombin-treated controls (P < 0.05). B: after 36 h, cells were challenged with thrombin for 5 min, and immunofluorescence was performed using VE-cadherin antibody to visualize adherens junctions. Arrows indicate the sites of disruption of VE-cadherin staining. C: after 36 h, cells were challenged with thrombin for 15 min and labeled with Alexa 488-labeled phalloidin to visualize the actin stress fibers by fluorescence microscopy. Results are representative of 2–3 experiments.

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and permeability by its ability to induce NF-κB signaling and disassembly of AJ.

There are several possible mechanisms by which PLC-ε may control EC inflammation and permeability. First, the facts that PLC-ε, unlike other PLC isoforms, is an important effector of Rho and Ras superfamily of GTPases (8, 9, 26, 41) and that these GTPases are implicated in EC inflammation and barrier disruption (1, 27, 31) raise the possibility that PLC-ε may serve to link small GTPases to NF-κB activation and EC barrier disruption. One possible link may involve generation of second messengers by PLC-ε such as DAG and subsequent PKC activation (25, 41), which has been shown to be important in mediating EC inflammation and permeability (31, 37, 46). Relative to PLC-β, which transduces physiological responses primarily via rapid IP3-mediated calcium mobilization (2, 12, 48), PLC-ε appears to be involved in longer-term second messenger generation and may be primarily involved in chronic DAG production (9, 12, 25, 55). These observations raise the interesting possibility that PLC-β is engaged to initiate, whereas PLC-ε serves to prolong, EC dysfunction caused by GPCRs such as proteinase-activated receptor 1. Moreover, because PKC can also mediate activation of small GTPases (31, 32), the other possibility that PLC-ε may act upstream of these GTPases to induce NF-κB activation and EC barrier disruption cannot be excluded. Thus PLC-ε may contribute to NF-κB activation and EC barrier disruption by multiple pathways; however, elucidation of the precise mechanism underlying PLC-ε-mediated EC inflammation and permeability requires additional comprehensive studies.

In summary, our integrated in vitro and in vivo studies establish PLC-ε as a critical regulator of EC dysfunction (inflammation and permeability) and lung inflammatory injury. Several lines of evidence are consistent with an important role of endothelial PLC-ε in lung vascular inflammation and injury. These include 1) in vitro evidence showing the importance of PLC-ε in EC inflammation and barrier disruption, 2) in vivo results showing protection against LPS-induced decrease in VE-cadherin levels, which is exclusively expressed in the endothelium, and 3) reduction in LPS-induced expression of adhesion molecules in PLC-ε−/− lungs, particularly that of VCAM-1, whose expression is largely restricted to the endothelium. However, it should be emphasized that the present study does not exclude the involvement of PLC-ε derived from other cells, particularly epithelial and inflammatory cells, in this model of lung inflammation. Although our preliminary experiments show that depletion of PLC-ε in AS49 lung epithelial cells fails to inhibit TNF-α-induced NF-κB activity (data not shown), a role of PLC-ε in causing inflammation in some epithelial cells, particularly keratinocytes, has been reported (21). Moreover, PLC-ε also reduces the levels of cytokines/chemokines (TNF-α, MCP-1, KC, MIP-2, and GM-CSF) that are also produced by epithelial and inflammatory cells, in addition to EC. However, PLC-ε is highly expressed in lung and poorly expressed in tissues rich in immune cells such as spleen or thymus (26). Nevertheless, addressing the precise contribution of PLC-ε derived from endothelial vs. epithelial or inflammatory cells in ALI requires additional comprehensive studies using mice with cell-specific deletion of PLC-ε.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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