Cross talk between paxillin and Rac is critical for mediation of barrier-protective effects by oxidized phospholipids

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Submitted 2 April 2008; accepted in final form 29 July 2008

Birukova AA, Alekseeva E, Cokic I, Turner CE, Birukov KG. Cross talk between paxillin and Rac is critical for mediation of barrier-protective effects by oxidized phospholipids. Am J Physiol Lung Cell Mol Physiol 295: L593–L602, 2008. First published August 1, 2008; doi:10.1152/ajplung.90257.2008.—We previously reported that the barrier-protective effects of oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) on pulmonary endothelial cells (ECs) delineate the role of Rac- and Cdc42-dependent mechanisms and described the involvement of the focal adhesion (FA) protein paxillin in enhancement of the EC barrier upon OxPAPC challenge. This study examined a potential role of paxillin in the feedback mechanism of Rac regulation by FAs in OxPAPC-stimulated ECs. Our results demonstrate that OxPAPC induced Rac-dependent, Rho-independent peripheral accumulation of paxillin-containing FAs and time-dependent paxillin phosphorylation. Molecular inhibition of Rac decreased association of paxillin with the Rac-specific guanine nucleotide exchange factor β-PIX. Molecular inhibition of paxillin also attenuated OxPAPC-induced enhancement of adherens junctions critical for the EC barrier-protective response, accumulation of vascular endothelial cadherin in the membrane fractions, and decreased activation of Rac and its effector p21-activated kinase (PAK1). Expression of paxillin with a mutated PAK1-dependent phosphorylation site (S273A) attenuated OxPAPC-induced PAK1 activation and the EC barrier-protective response. These results suggest that PAK1-specific paxillin phosphorylation at Ser273 is critically involved in the positive-feedback regulation of the Rac-PAK1 pathway and may contribute to sustained enhancement of the EC barrier caused by oxidized phospholipids.

PAK1: small GTPases; cytoskeleton; pulmonary endothelium; permeability

OXIDIZED PHOSPHOLIPIDS (OxPLs) may appear in the lung circulation as components of oxidized low-density lipoproteins, cell membrane vesicles, or microparticles released by activated platelets, vascular cells, or injured tissues (19, 26, 28, 35, 39, 51). Circulating phospholipids undergo oxidation by enzymatic and nonenzymatic mechanisms (19). In the lungs, phospholipid oxidation may occur as a result of acute lung injury (ALI), lung inflammation, acute respiratory distress syndrome, ventilator-induced lung injury, systemic inflammatory response syndrome, and sepsis (25, 29, 56). Under these conditions, lung vascular barrier function is largely compromised. Oxidation of phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), generates a group of bioactive OxPL species that demonstrate a wide spectrum of physiological effects. In chronic settings, OxPLs activate monocyte adhesion to endothelial cells (ECs) and contribute to chronic vascular inflammation and atherosclerosis (2, 30). However, in conditions associated with ALI, PAPC oxidation products are potent inhibitors of tissue inflammation triggered by compounds derived from gram-negative and gram-positive bacteria (2, 18, 19, 32).

We previously described potent barrier-protective effects of oxidized PAPC (OxPAPC) on the human pulmonary endothelium (3) that have been further substantiated in animal models of ALI and vascular hyperpermeability (10, 37, 38). Beneficial effects of OxPAPC in the models of ALI may involve coordinated reactions by various cell types residing in the lung. Major effects of OxPAPC include barrier-protective responses by ECs and acute anti-inflammatory effects on a variety of cells, including alveolar epithelium, lung macrophages, fibroblasts, circulating monocytes, neutrophils, and dendritic cells (10, 16, 19, 32). The mechanism of OxPAPC-induced anti-inflammatory effects is inhibition of signaling by Toll-like receptors and blunting of the NF-κB inflammatory cascade (17). Thus, OxPAPC may coordinate signaling by lung macrophages, epithelial cells, and ECs under conditions of ALI by anti-inflammatory action on circulating neutrophils, macrophages, lung microphages, and alveolar epithelium (16, 19, 32) and by barrier-protective effects on lung vascular endothelium (3, 10). Together, these OxPAPC activities lead to decreased inflammatory activation of neutrophils, macrophages, and alveolar epithelial cells. In turn, increased barrier properties of lung vascular endothelium induced by OxPAPC prevent transvascular flux of fluid and macromolecules, which further improve lung barrier function and attenuate lung inflammation, the major hallmarks of ALI. This study investigated molecular mechanisms of OxPAPC barrier-protective effects on pulmonary endothelium mediated by cross talk between Rac GTPase and the focal adhesion (FA)-associated protein paxillin.

Barrier-protective effects of OxPAPC had been linked to the activation of small GTPases, Rac and Cdc42, which mediated enhancement of the peripheral actin cytoskeleton and increased interactions between the FAs and adherens junction complexes essential for endothelial barrier maintenance (3, 11, 12). Importantly, protective effects of OxPLs on the EC monolayers exposed to barrier-disruptive agents have been linked to Rac-mediated downregulation of the Rho-dependent pathways (10). FAs are formed by transmembrane extracellular receptors, integrins, which interact with the intracellular FA protein complex connected to the actin cytoskeleton. Regulation of FA dynamics is a complex process mediated by different GTPase families, which, nonetheless, converge on a few key regulatory
proteins within the FA complex containing scaffold/signaling proteins such as paxillin and p21-activated kinase (PAK)-interactive exchange factor (PIX) and regulatory proteins including Rac and Rho effectors: PAK, G protein-coupled receptor kinase-interacting protein (GIT)-1, and paxillin kinase linker (PKL)/GIT2 (24, 50, 54, 57).

Paxillin is a multidomain adapter FA protein that functions as a molecular scaffold for protein recruitment to FAs and, thereby, facilitates protein networking and efficient signal transmission (48, 50). The NH2-terminal domain of paxillin contains five copies of LD motifs (28), which function as binding sites for other FA-associated proteins. The COOH-terminal half of paxillin contains four LIM domains involved in protein-protein interactions: the LIM-3 domain targets paxillin to FAs, whereas the LIM-2 domain plays a minor role. These LIM domains engage binding partners to direct and/or tether paxillin in FAs, but the exact LIM domain binding partners have not been identified. Through the multiple SH2- and SH3-binding domains, LIM domains, and LD motifs, paxillin interacts with the signaling proteins FA kinase (FAK),

Fig. 1. Time-dependent effects of oxidized 1-palmitoyl-2-arachidonoyl--sn-glycero-3-phosphocholine (OxPAPC) on paxillin redistribution. Endothelial cells (ECs) grown on glass coverslips were stimulated with OxPAPC (20 μg/ml) for 0–45 min. Phosphorylation of paxillin was analyzed by immunoblotting of cell lysates with a panel of phosphospecific antibodies. Equal protein loading was verified by membrane reprobing with paxillin antibody. Results of quantitative analysis of site-specific paxillin phosphorylation are shown as relative densitometric units (RDU). Values are means ± SD of 3 independent experiments. *P < 0.05.

Fig. 2. OxPAPC induces time-dependent site-specific paxillin phosphorylation. Human pulmonary artery ECs were treated with OxPAPC (20 μg/ml) for 0–45 min. Phosphorylation of paxillin was analyzed by immunoblotting of cell lysates with a panel of phosphospecific antibodies. Equal protein loading was verified by membrane reprobing with paxillin antibody. Results of quantitative analysis of site-specific paxillin phosphorylation are shown as relative densitometric units (RDU). Values are means ± SD of 3 independent experiments. *P < 0.05.
p60 Src kinase, Crk, Csk, Pyk2, and integrin-linked kinase and the structural FA-associated proteins vinculin, actopaxin, and tubulin (48, 53). Multiple paxillin phosphorylation sites, including the major tyrosine phosphorylation sites Tyr31 and Tyr118 regulated by FAK, have been identified (1, 49). These Crk-binding sites may provide docking motifs for recruitment of other signaling molecules to FAs. The role of serine/threonine phosphorylation sites remains less explored. Activation of Raf/MEK/MAPK signaling results in phosphorylation of paxillin at Ser126, whereas JNK1 and Cdc2 are able to phosphorylate paxillin at Ser178 and, therefore, may be involved in cell migration (23). Recent studies show that paxillin phosphorylation within the LD4 motif, including a PAK-dependent Ser273 site, is critical for Rac activation, cell adhesion, and protrusion (36). These findings indicate novel mechanisms of Rac regulation by FAs.

We previously reported site-specific paxillin phosphorylation and agonist-specific interactions of paxillin with its binding partners GIT1, GIT2, and FAK, which were linked to differential EC barrier responses (12, 44, 45). However, the precise mechanisms of EC barrier regulation by FA complexes remain to be investigated. In the present study, we examined the effects of OxPAPC on Rac-dependent redistribution and site-specific phosphorylation of paxillin and investigated the involvement of paxillin in positive-feedback stimulation of Rac activity and further EC barrier enhancement exhibited by OxPLs.

**MATERIALS AND METHODS**

Reagents and cell culture. Antibodies to paxillin and vascular endothelial (VE)-cadherin were obtained from BD Transduction Laboratories (San Diego, CA); phosphospecific paxillin antibodies from Biosource (Invitrogen, Carlsbad, CA); Rac, hemagglutinin (HA) tag, β-PIX, and p115 Rho guanine nucleotide exchange factor (GEF) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); and horseradish peroxidase-linked anti-mouse and anti-rabbit IgG, phos-
phorylated PAK1 (Thr423), and PAK1 antibodies from Cell Signaling (Beverly, MA). All reagents used for immunofluorescence staining were purchased from Molecular Probes (Eugene, OR). Unless specified, all biochemical reagents, including PAPC, were obtained from Sigma (St. Louis, MO). PAPC (Avanti Polar Lipids, Alabaster, AL) was oxidized by exposure of dry lipid to air, as previously described (20, 31, 52). The extent of oxidation was monitored by positive-ion electrospray-mass spectrometry, as described previously (52). Human pulmonary artery ECs (HPAECs) were obtained from Lonza (Allendale, NJ) and used at passages 5–9, as previously described (4).

Expression plasmids, transient transfection, and nucleofection protocol. Plasmids encoding constitutively active (CA) Rac and Rho and dominant-negative (DN) Rac and Rho bearing an HA tag and paxillin wild-type (PXN-wt), phosphorylation-deficient paxillin (PXN-S273A), and paxillin lacking the LD4 motif (PXN−/LD4) bearing a green fluorescent protein (GFP) tag have been previously described (3, 13, 36, 55). ECs were used for transient transfections according to a protocol described previously (3, 13). Control transfections were performed with empty vectors. For more effective introduction of cDNA into the cell, we used the Nucleofector kit (Amaxa Biosystems, Gaithersburg, MD). Optimized protocol of nucleofection provided by the manufacturer was used with minor modifications described previously (6).

Depletion of paxillin and Rac in ECs. To deplete endogenous paxillin or Rac, we treated HPAECs with gene-specific small interfering (siRNA) duplexes, as described elsewhere (3, 11, 12). Pre-designed siRNAs of standard purity were ordered from Ambion (Austin, TX), and transfection of ECs with siRNA was performed as described previously (3, 7). Nonspecific, nontargeting siRNA was used as a control treatment. After 48 h of transfection, cells were used for experiments or harvested for Western blot verification of specific protein depletion.

Measurement of transendothelial electrical resistance. EC barrier properties were assessed by measurements of transendothelial electrical resistance (TER) across confluent HPAEC monolayers using an

![Fig. 4. Role of paxillin regulation of OxPAPC-induced Rac signaling. Human pulmonary ECs were transfected with Rac-specific (A) or paxillin-specific (B–E) small interfering (si) RNA and then stimulated with OxPAPC (20 μg/ml). Control transfections were performed using nonspecific (ns) RNA. Depletion of target proteins induced by specific siRNA duplexes was confirmed by immunoblotting with appropriate antibodies compared with treatment with nsRNA. Immunoblot with β-tubulin antibodies was used as a normalization control. Veh, vehicle. Results of scanning densitometry of Western blots are shown as RDU. Results are representative of 3 independent experiments. Values are means ± SD. *P < 0.05. A: after cell lysis, protein complexes were immunoprecipitated with paxillin antibodies, and β-PIX was detected by Western blotting. Equal protein loading was confirmed by membrane reprobing with paxillin antibodies. B: Rac activation was determined in control and OxPAPC-stimulated ECs using pull-down assay. Rac content in EC lysates is shown as RDU. C: OxPAPC-induced PAK1 phosphorylation was determined in total lysates using phosphorylated PAK1-specific antibody. D: OxPAPC-induced distribution of vascular endothelial (VE)-cadherin and p115 Rho GEF in cytosolic and membrane fractions was analyzed by Western blot with corresponding antibodies. E: effects of paxillin knockdown on redistribution of vinculin-containing FAs in control and OxPAPC-treated monolayers were analyzed by immunofluorescence staining with vinculin antibodies. Arrows, areas of peripheral vinculin accumulation.

AJP-Lung Cell Mol Physiol • VOL 295 • OCTOBER 2008 • www.ajplung.org
electrical cell-substrate impedance-sensing system (Applied Biophysics, Troy, NY), as previously described (3, 4, 7).

**Immunofluorescence staining and image analysis.** ECs grown on glass coverslips were stimulated with OxPAPC or left untreated, and immunofluorescence staining for proteins of interest was performed using corresponding antibodies, as described elsewhere (6, 7, 9). Images were processed with Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Quantitative analysis of peripheral paxillin accumulation was performed according to a previously described algorithm (4, 7, 14). The 16-bit images were analyzed using MetaVue 4.6 software (Universal Imaging, Downingtown, PA). Cell areas within 5 μm from the cell-cell interface were manually outlined, and images were differentially segmented between paxillin-positive FAs and background. Paxillin immunoreactivity was captured on the basis of image gray-scale levels, and immunofluorescence signal intensity was measured using MetaVue 4.6 software. Peripheral paxillin translocation was expressed as paxillin immunofluorescence signal intensity relative to signal intensity in the outlined peripheral area. Values were statistically processed using Sigma Plot 7.1 software (SPSS Science, Chicago, IL).

**Differential protein fractionation and coimmunoprecipitation assays.** EC monolayers were stimulated with OxPAPC, and cytosolic and membrane fractions were isolated as described previously (12, 15). Coimmunoprecipitation studies were performed using confluent HPAECs, as described previously (12, 44, 46). Protein extracts were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies of interest. The relative intensities of immunoreactive protein bands were quantified by scanning densitometry using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

**Statistical analysis.** Values are means ± SD of three to five independent experiments. Stimulated samples were compared with controls by unpaired Student’s t-tests. For multiple-group comparisons, a one-way ANOVA was followed by the Fisher’s post hoc test.

**RESULTS**

**OxPAPC-induced paxillin translocation and phosphorylation in pulmonary ECs.** To examine the time-dependent paxillin redistribution induced by OxPAPC, we stimulated pulmonary EC monolayers with OxPAPC for 5, 15, 30, or 45 min and then performed immunofluorescence analysis of cytoskeletal remodeling using actin staining (Fig. 1A, left) and paxillin translocation using anti-paxillin antibody (Fig. 1A, right). After 5 min of OxPAPC stimulation, ECs exhibited pronounced lamellipodia formation and the onset of FA remodeling. In agreement with previous observations (3, 12), at later time points (15–30 min), the peripheral actin cytoskeleton formed ziplike structures at the cell-cell interfaces. These cytoskeletal changes were associated with recruitment of FAs to the cell periphery (Fig. 1A, right). Quantitative analysis of peripheral paxillin immunofluorescence intensity showed time-dependent accumulation of paxillin-positive FAs at the cell periphery upon OxPAPC stimulation (Fig. 1B). Cortical actin remodeling and peripheral accumulation of FAs were complete after 30–45 min of OxPAPC stimulation and remained unchanged for ≥2 h after addition of OxPAPC (data not shown). At these time points, OxPAPC dramatically increased TER, reflecting enhancement of the EC barrier, and remained elevated for several hours (3, 10).

Among multiple signaling cascades activated in the pulmonary endothelium by OxPAPC (5, 8), Rac/Cdc42-dependent pathways and Src-dependent signaling mechanisms play a critical role in the mediation of OxPAPC-induced barrier enhancement, whereas MAPK and JNK cascades, although activated, were not involved in the OxPAPC-mediated barrier-protective response (8). In the next experiments, we analyzed...
site-specific phosphorylation of paxillin in response to OxPAPC. The paxillin phosphorylation sites specific for signaling kinases activated by OxPAPC have been selected for analysis (Fig. 2). OxPAPC-induced time-dependent phosphorylation of paxillin at ERK1,2- and JNK-specific sites (Ser126 and Ser178) and phosphorylation at Tyr31 and Tyr118, the major docking sites involved in paxillin interaction with other proteins. Importantly, OxPAPC induced paxillin phosphorylation at Ser273, the PAK-dependent site residing within the LD4 motif. Quantitative analysis of paxillin phosphorylation shows major phosphorylation at Tyr31 and Tyr118 that reached maximal levels at 15 min and remained elevated 45 min after OxPAPC stimulation. In turn, phosphorylation at Ser178 was significantly elevated after 15 min and reached maximum at 30 min of OxPAPC challenge. The maximal phosphorylation at Ser178 and Ser126 was observed at later time points (30–45 min) of OxPAPC treatment and was consistent with the sustained phase of the EC barrier-protective response to OxPAPC.

OxPAPC-induced redistribution of paxillin is Rac dependent. We previously described a major role of Rac/Cdc42-dependent pathways in the OxPAPC-mediated barrier-protective responses (3). In the next experiments, we addressed a role for Rac- and Rho-dependent signaling in the peripheral translocation of paxillin upon OxPAPC challenge. Lung ECs were transiently transfected with CA Rac or Rho GTPases (Fig. 3, A and B). Alternatively, DN Rac or Rho was introduced into EC monolayers, which were then stimulated with OxPAPC (Fig. 3, C–E). After treatment, cells were subjected to immunofluorescence double staining, and paxillin redistribution was analyzed in the nontransfected controls or in two adjacent cells expressing Rac or Rho mutants. Staining for the HA tag was performed to detect transfected cells. Overexpression of CA Rac caused translocation of FAs to the cell periphery and accumulation of paxillin at the areas of cell-cell junctions (Fig. 3, A and inset). In contrast, overexpression of CA Rho caused formation of large FAs randomly distributed within the cell. Importantly, no paxillin accumulation was observed at the areas of cell-cell junctions (Fig. 3, B and inset). In agreement with these results, ectopic expression of DN Rac significantly attenuated OxPAPC-induced peripheral translocation of paxillin in transfected adjacent cells compared with nontransfected controls (Fig. 3, C and Fig. 3E, insets 1 and 2), whereas overexpression of DN Rho did not alter patterns of paxillin distribution after OxPAPC stimulation (Fig. 3D and Fig. 3E, insets 1 and 3). These data clearly demonstrate that small

Fig. 6. Phosphorylation of paxillin at Ser273 is critical for OxPAPC-induced PAK1 autophosphorylation, cytoskeletal remodeling, and recovery of EC monolayer integrity. A: EC monolayers were subjected to nucleofection with PXN-wt or PXN-S273A bearing GFP tags. After 24 h of transfection, cells were stimulated with OxPAPC (20 μg/ml). OxPAPC-induced PAK1 phosphorylation was determined in total lysates using phosphorylated PAK1-specific antibody. Results of scanning densitometry of Western blots are shown as RDU. Values are means ± SD of 3 independent experiments. *P < 0.01 vs. nonstimulated control. **P < 0.05 vs. OxPAPC-stimulated EC transfected with PXN-wt. B: subconfluent ECs were transiently transfected with GFP-PXN-S273A and then treated with OxPAPC (20 μg/ml). Cells were fixed and subjected to immunofluorescence staining with Texas red-phalloidin for detection of F-actin. Transfected cells are shown in outlined area. Arrows, remaining gaps between GFP-PXN-S273A-expressing cells after OxPAPC stimulation. Results are representative of 3 independent experiments.
Paxillin is required for full Rac activation in response to OxPAPC. Previous reports indicate the important role of paxillin-GIT-β-PIX-PAK complex formation in signal transmission from FAs (23, 34, 36, 41, 43, 48, 57). Whether this complex may modulate OxPAPC-mediated Rac signaling in the lung endothelium is not clear. Our previous study demonstrated the role of the Rac-specific nucleotide exchange factor β-PIX in OxPAPC-induced Rac activation (11). To test the hypothesis that paxillin may be involved in the positive-feedback loop of Rac activation in response to OxPLs, we 1) investigated the effect of OxPAPC on formation of the paxillin-GIT-β-PIX-PAK complex and 2) explored the role of Rac in the regulation of this association. After stimulation of pulmonary ECs with OxPAPC, paxillin was immunoprecipitated under nondenaturing conditions (Fig. 4A). OxPAPC increased paxillin association with β-PIX, whereas siRNA-based Rac knockdown suppressed these interactions. These results show that, in the model of EC barrier protection induced by OxPLs, Rac is not only an upstream regulator of paxillin, but it may also serve as a downstream target for paxillin-mediated signaling.

We further studied the effects of paxillin downregulation on Rac activation and barrier enhancement in response to OxPAPC. Pulmonary ECs were transfected with paxillin-specific or nonspecific siRNA and then stimulated with OxPAPC. Measurements of Rac activation in control and stimulated ECs showed that, in contrast to cells transfected with nonspecific RNA, depletion of paxillin dramatically reduced Rac activation in response to OxPAPC (Fig. 4B). Consistent with these results, paxillin-depleted ECs exhibited decreased Rac-dependent autophosphorylation of the downstream Rac target PAK1 after OxPAPC challenge (Fig. 4C). Membrane accumulation of VE-cadherin is a critical step of adherens junction assembly, leading to enhancement of cell-cell adhesions and increased EC barrier function. Downregulation of paxillin significantly decreased OxPAPC-induced accumulation of the adherens junction marker VE-cadherin in the membrane fraction (Fig. 4D). VE-cadherin was predominantly localized in the membrane fraction, and its changes in the cytosolic fraction were below the level of detection in these experiments. Control experiments showed that subcellular localization of the Rac-specific nucleotide exchange factor p115 Rho GEF unrelated to this pathway did not change upon OxPAPC stimulation or paxillin depletion.

To test whether paxillin knockdown affects OxPAPC-induced peripheral redistribution of FAs, ECs treated with nonspecific or paxillin-specific siRNA were stimulated with OxPAPC, and distribution of another FA-targeted protein, vinculin, was monitored by immunofluorescence staining. Paxillin knockdown abolished OxPAPC-induced translocation of vinculin to the cell periphery (Fig. 4D), suggesting inhibition of OxPAPC-induced localization of FAs to the areas of cell-cell junctions. Taken together, these results demonstrate the involvement of paxillin in the regulation of Rac-dependent signaling and adherens junction assembly stimulated by OxPLs.

**Paxillin phosphorylation at Ser**273** is critical for OxPAPC-induced endothelial barrier enhancement.** Recent reports indicate a critical role of the paxillin LD4 motif in the regulation of paxillin interactions with GIT, β-PIX, and PAK (24, 55), and paxillin phosphorylation at Ser**273** within the LD4 motif can be involved in the regulation of Rac-dependent processes, such as cell spreading and migration (36). In the present study, we examined the involvement of the LD4 motif and paxillin phosphorylation at Ser**273** in the mechanisms of OxPAPC-induced EC barrier protection. Using the nucleofection technique, which allows high transfection efficiency (6), we introduced PXN-wt, PXN-ΔLD4, and PXN-S273A into pulmonary ECs (36). Control transfections were performed with empty GFP vector. Successful transfection was confirmed by Western blot analysis with GFP antibodies (data not shown). Transfected EC monolayers were stimulated with OxPAPC, and then EC permeability was measured. Consistent with our previous results (3, 10), OxPAPC induced a dramatic increase in TER in pulmonary ECs transfected with empty vector (Fig. 5A). Ecotropic expression of PXN-wt did not compromise OxPAPC-mediated EC barrier enhancement (Fig. 5A). In contrast, expression of PXN-ΔLD4 or PXN-S273A decreased OxPAPC-induced elevation of EC resistance (Fig. 5, B and C). Complementary experiments showed that ectopic expression of PXN-S273A dramatically attenuated OxPAPC-induced PAK1 autophosphorylation (Fig. 6A), indicative of downregulation of Rac activity (21). Next, we tested effects of PXN-S273A ectopic expression on the “sealing” of intercellular gaps observed in subconfluent EC monolayers treated with OxPAPC and reflective of OxPAPC barrier-restoring properties. Expression of PXN-S273A did not affect cell morphology in quiescent subconfluent EC culture. However, in contrast to surrounding nontransfected cells, ECs transfected with PXN-
S273A were incapable of sealing the gaps upon OxPAPC stimulation (Fig. 6B). Expression of PXN-S273A also suppressed cortical actin polymerization and lamellipodia formation after OxPAPC challenge. Taken together, these data suggest an essential role of paxillin phosphorylation at Ser273 in OxPAPC-induced pulmonary endothelial barrier protection.

**DISCUSSION**

A role for Rac and Rho GTPases and the Rac downstream effector PAK1 in FA remodeling is well recognized. However, the precise mechanisms of Rho- and Rac-mediated FA remodeling and downstream pathways of paxillin signaling remain to be determined. The results of this study demonstrate that OxPAPC-induced paxillin translocation and peripheral FA formation are mediated by Rac-dependent mechanisms and essential for OxPAPC-induced EC barrier enhancement. We demonstrate for the first time that molecular inhibition of paxillin reduces OxPAPC-induced Rac activation and inhibits membrane translocation of a key adherens junction component, VE-cadherin, indicating a Rac-mediated cross talk between cell-substrate and cell-cell adhesions essential for the maintenance of EC monolayer integrity and barrier properties. Our data show that paxillin phosphorylation at the PAK-dependent site Ser273 within the LD4 motif is required for a full activation of Rac signaling by OxPAPC, therefore providing a novel positive-feedback mechanism of Rac activation essential for EC barrier protection. Interestingly, ectopic expression of PXN-ΔLD4 or PXN-S273A mutants did not delay the initial phase of barrier enhancement but did reduce the maximal levels of the OxPAPC-induced EC-barrier-protective response. These partial effects of the paxillin Ser273 mutant may be explained by preserved paxillin interactions with other signaling partners controlled by paxillin phosphorylation at Tyr31 and Tyr118 (1, 43).

Tyrosine phosphorylation of paxillin creates binding sites for SH2 domain-containing signaling proteins. Tyr31, Tyr118, and Tyr181 are embedded in high-affinity SH2 domain-binding sites, which upon phosphorylation by FAK, Src, or Pyk become docking sites for paxillin binding partners. SH2 interactions include association of p85 phosphatidylinositol 3-kinase with paxillin phosphorylated at Tyr31, Tyr40, and Tyr118; association of Crk/CrkL with paxillin phosphorylated at Tyr31, Tyr118, and Tyr181; and association of Csk, Chk, and p120 Ras GAP with phosphorylated paxillin at Tyr118 (23, 43, 47, 50, 57). Mutation of paxillin at Tyr31 and Tyr118, as well as deletion of the LD4 motif, also perturbs cellular motility and Rac signaling (22, 23, 43, 57). Paxillin phosphorylation at Ser273 causes assembly of a GIT-PIX-PAK1 signaling complex and promotes its localization near the leading edge (36). Thus, Tyr31, Tyr118, and Ser273 appear to be important phosphorylation sites involved in the regulation of paxillin-dependent peripheral FA translocation and motility. Our results show early paxillin phosphorylation at Tyr31 and Tyr118, which coincides with peripheral translocation of paxillin-containing FAs. These events also appear to be dependent on PAK1-induced phosphorylation of paxillin at Ser273 located within the LD-4 motif, since PXN-ΔLD4 and PXN-S273A attenuate these effects likely via displacement of the GIT-β-PIX-PAK1 complex from paxillin.

It is well recognized that FAs are important regulators of the endothelial barrier. Changes in barrier function may result from altered FA turnover kinetics, specific tyrosine phosphorylation events, and reassignment of FA constituents to new subcellular arrays (42). Recent reports by us and other groups (27, 33, 40, 44) showed an important role of FAK in lung endothelial barrier regulation. In particular, FAK activation promotes barrier recovery after transient hyperpermeability responses (33, 40). Since FAK is a key regulator of paxillin phosphorylation, we speculate that inhibition of FAK function may also attenuate the OxPAPC-induced EC barrier protective response. Our unpublished data show that downregulation of Src activity by its pharmacological inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) significantly attenuated the OxPAPC-induced increase in TER. On the other hand, GIT1 and GIT2 are critical regulators of paxillin association with the Rac-specific guanine exchange factor β-PIX (23). Our recent study showed that knockdown of β-PIX in the pulmonary endothelium abolished the ability of OxPAPC to induce barrier-protective effects in pulmonary ECs (11). Thus, although EC permeability changes may occur upon inhibition of different FA-associated proteins, paxillin plays a specific role in EC barrier regulation and signal transduction as a scaffold for other FA-associated proteins and an integrator of small GTPase signaling.

In summary, on the basis of previous reports and the results of the present study, we propose a hypothetical mechanism of EC barrier regulation by OxPAPC via a paxillin-mediated loop of Rac activation, which further contributes to the endothelial barrier enhancement (Fig. 7). OxPAPC-induced activation of Rac signaling leads to peripheral translocation of paxillin and integration of FA and adherens junction complexes, therefore providing a structural basis for enhancement of the EC barrier. In addition, PAK-dependent phosphorylation of paxillin at Ser273 within the LD4 motif promotes paxillin-GIT-β-PIX-PAK1 complex assembly and further β-PIX-mediated Rac activation, thus providing a positive-feedback loop of Rac regulation by peripheral FAs. This local Rac activity contributes to the sustained phase of the pulmonary endothelial barrier-protective response to OXPLs.

**ACKNOWLEDGMENTS**

We thank Alan Rick Horwitz (Univ. of Virginia) for providing plasmids encoding paxillin S273 mutants.

**GRANTS**

This study was supported by National Heart, Lung, and Blood Institute Grants HL-076259, HL-075349, and HL-58064; an American Lung Association Career Investigator Grant (to K. G. Birukov); a National Scientist Development Grant from the American Heart Association and an American Lung Association Biomedical Research Grant (to A. A. Birukova); and National Institute of General Medical Sciences Grant GM-47607 (to C. E. Turner).

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