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Circ. Res. 2004;95;892-901; originally published online Oct 7, 2004;
DOI: 10.1161/01.RES.0000147310.18962.06
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0009-7330. Online ISSN: 1524-4571

Epoxycyclopentenone-Containing Oxidized Phospholipids Restore Endothelial Barrier Function via Cdc42 and Rac

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Abstract—After an acute phase of inflammation or injury, restoration of the endothelial barrier is important to regain vascular integrity and to prevent edema formation. However, little is known about mediators that control restoration of endothelial barrier function. We show here that oxidized phospholipids that accumulate at sites of inflammation and tissue damage are potent regulators of endothelial barrier function. Oxygenated epoxyisoprostane-containing phospholipids, but not fragmented oxidized phospholipids, exhibited barrier-protective effects mediated by small GTPases Cdc42 and Rac and their cytoskeletal, focal adhesion, and adherens junction effector proteins. Oxidized phospholipid-induced cytoskeletal rearrangements resulted in a unique peripheral actin rim formation, which was mimicked by coexpression of constitutively active Cdc42 and Rac, and abolished by coexpression of dominant-negative Rac and Cdc42. Thus, oxidative modification of phospholipids during inflammation leads to the formation of novel regulators that may be critically involved in restoration of vascular barrier function. **(*Circ Res*. 2004;95:892-901.)**

Key Words: endothelial permeability ■ mildly oxidized phospholipids ■ small GTPases ■ actin cytoskeleton ■ thrombin

Increased vascular leakage is associated with numerous life threatening diseases such as acute lung injury, sepsis, and acute respiratory distress syndrome (ARDS). Increased lung vascular permeability results in excessive leukocyte infiltration, alveolar flooding, and pulmonary edema. Thus, restoration of endothelial barrier function is essential for successful resolution of acute injury and inflammatory processes. However, mechanisms and mediators involved in recovery of endothelial barrier function are poorly understood. A critical role for small GTPases Rac and Cdc42 in endothelial barrier protection has been indicated in recent reports. Rac-dependent mechanisms have been described for rearrangements of actin cytoskeleton, focal adhesions, and adherens junctions associated with sphingosine-1-phosphate (S1P)–mediated barrier protection,[1–3] and the role for Cdc42 in restoration of compromised endothelial barrier function has been suggested.[4]

Enhanced lipid peroxidation and formation of oxidized phospholipids were observed in acute pathological conditions, including ARDS, ventilator-induced lung injury, and asthma.[5,6] As a result of tissue injury and apoptosis associated with acute lung injury, cardiac ischemia, acute coronary syndrome, and platelet activation,[7,8] membrane vesicles containing oxidized phospholipids are released by various cell types into the blood circulation.[7,9] Cells respond to these newly formed stress signals with activation of pro- and antiinflammatory cascades (see reviews[10–12]).

Structures of several biologically active oxidized phospholipids derived from oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC) have been identified, and a role for these compounds in the pathogenesis of chronic inflammatory diseases was suggested.[12–14] Moreover, oxidized phospholipids also exhibit antiinflammatory properties and inhibit innate immune responses via blocking LPS binding to toll-like receptor 4 and blunting the NF-κB–mediated expression of inflammatory genes. These effects represent a possible feedback mechanism to downregulate acute inflammation.[15,16]

We have shown previously the effects of OxPAPC on the activation of the cytoskeletal protein cofilin and the focal adhesion proteins paxillin and FAK, all of which are involved in endothelial cell (EC) remodeling and barrier regulation.[17] A number of signaling molecules potentially involved in effects of OxPAPC on cytoskeletal activation, such as protein

Original received May 11, 2004; revision received September 21, 2004; accepted September 28, 2004.

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This manuscript was sent to Elizabeth Nabel, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000147310.18962.06**

kinases A and C, Erk1,2, and p38 MAP kinases and p60Src have been described.[12,16–18]

In this study, we show that defined phospholipid oxidation products are capable of increasing EC barrier function via signaling mechanisms mediated by small GTPases Rac and Cdc42 leading to EC cytoskeletal remodeling and barrier restoration. We identify specific components of oxidized phospholipids with barrier protective properties, which will allow structure-based drug design and may reveal new therapeutic strategies for treatments of acute lung injury syndromes and other diseases associated with vascular leakage.

Materials and Methods

Human pulmonary endothelial cells (HPAECs) were cultured and transfected with cDNAs as described previously.[19] Sources of reagents and details of procedures are provided in the expanded Materials and Methods section in the online data supplement available at http://circres.ahajournals.org. Lipid oxidation and analysis of oxidation products by positive ion electrospray mass spectrometry (ESI-MS) was performed as described previously.[13,16,18] Measurements of transendothelial electrical resistance were performed using electrical cell substrate impedance-sensing (ECIS) system as described elsewhere.[1,20] Transient transfections and siRNA-based protein depletion of small GTPases were performed as described elsewhere.[19–21] Rac, Cdc42 and Rho activation assays were performed using assay kits from Upstate Biotechnology.[1,20] Subcellular protein fractionation, Western blot analyses, and densitometric analyses were performed from at least 3 experiments as described.[20] Immunofluorescent staining of HPAECs was performed as previously described.[19,20] ANOVA and a post hoc Student-Newman-Keuls test were used to compare the means of two or more different treatment groups. Results were expressed as the mean±SE. Differences between two groups were considered statistically significant with a value of $P<0.05$.

Results

Effects of Oxidized Phospholipids on Endothelial Barrier Function

OxPAPC caused dose-dependent increases in transendothelial electrical resistance (TER) across the EC monolayers with maximal response to 20 μg/mL OxPAPC (Figure 1A and 1F). Barrier-protective responses were dependent on oxidative modification of the PAPC, because nonoxidized PAPC or other nonoxidized phosphatidylcholines, palmitoyl-linoleate phosphatidyl choline (PLPC) and dimyristoyl phosphatidyl choline (DMPC), did not exhibit significant effects on TER, and oxidized PLPC also did not affect TER (Figure 1B and 1C). Preincubation of OxPAPC with butylated hydroxytoluene (BHT) (5 μmol/L, 10 minutes), a free radical quencher, before EC stimulation did not affect OxPAPC-induced TER increase (Figure 1C), suggesting that the barrier-protective effect of oxidized phospholipids was not mediated by free radicals present in OxPAPC preparations.

Effects of OxPAPC on Thrombin- and Sphingosine 1-Phosphate–Induced TER Changes

Thrombin treatment of pulmonary EC caused abrupt decrease in TER followed by barrier recovery. Cumulative data from five independent experiments suggest that addition of OxPAPC (20 μg/mL) to EC challenged with thrombin (50 nmol/L) not only decreased TER recovery time more than two-fold (40 minutes after maximal TER decline versus 115 minutes with thrombin stimulation alone), but also brought TER levels above the baseline observed in nonstimulated ECs (Figure 1D and 1E), suggesting further barrier enhancement. Barrier-protective effects of sphingosine 1-phosphate (S1P) are mediated via G-protein–coupled Edg1 and Edg3 receptors and involve activation of small GTPase Rac.[1] S1P induced rapid concentration-dependent TER increase within maximal barrier protective effect at 1 μmol/L (Figure 1F). OxPAPC-induced barrier-protective response reached a peak at 20 minutes of stimulation with maximal barrier-protective effect of OxPAPC at 20 μg/mL (Figure 1F). Combined stimulation of pulmonary ECs with OxPAPC and S1P at concentrations, which cause maximal barrier protection by each agonist alone (20 μg/mL and 1.5 μmol/L, respectively) revealed additive effect of combined OxPAPC and S1P treatment on TER increase (Figure 1G). These results strongly indicate distinct but additive mechanisms underlying barrier protection induced by these lipid mediators.

Unique EC Cytoskeletal Rearrangement Induced by OxPAPC

Regulation of EC barrier integrity is critically dependent on cytoskeletal elements and cell contacts.[22] OxPAPC (20 μg/mL) induced significant reduction in central F-actin stress fibers and remodeling of cortical cytoskeleton (Figure 2A), characterized by a pronounced enhancement of peripheral F-actin staining (5 minutes) followed by appearance of peripheral F-actin structures (15 minutes), which resembled microspikes normally observed in single cells with activated small GTPases Rac and Cdc42 or PI3-kinase.[23,24] On completion of F-actin remodeling by 30 minutes of OxPAPC stimulation, HPAECs formed of a strong peripheral actin rim with disappearance of central stress fibers. Higher magnification images of cell-cell interface areas (Figure 2B) revealed formation of unique zip-like actin projections that formed an intercollated peripheral actin cytoskeletal structures not previously observed in the S1P model of EC barrier enhancement (Figure 2B, right panel).

Oxygenated, but not Fragmented Phospholipids Increase TER

In contrast to barrier protective effects exhibited by OxPAPC at 20 μg/mL, higher OxPAPC concentrations (100 μg/mL) caused barrier-disruptive effect (Figures 1F and 3B, left panel), which may reflect adverse effects of barrier-disruptive compounds present in OxPAPC. To further characterize biologically active molecules in OxPAPC, we separated OxPAPC by TLC into two fractions containing either fragmented (m/z <782,7, Fraction #1), or oxygenated (m/z >782,7, Fraction #2) *sn*-2 residues (Figure 3A). ESI-MS-analysis demonstrated that Fraction #1 was enriched in lysoPC, POVPC and PGPG (Figure 3A, middle panel). Fraction #1 dose-dependently decreased barrier function (Figure 3B, middle panel). In contrast, Fraction #2, which was enriched in oxygenated compounds with PEIPC and PECPC representing major peaks (Figure 3A, right panel), induced prominent increases in TER (Figure 3B, right panel), thus mimicking barrier protective effects of low concentra-

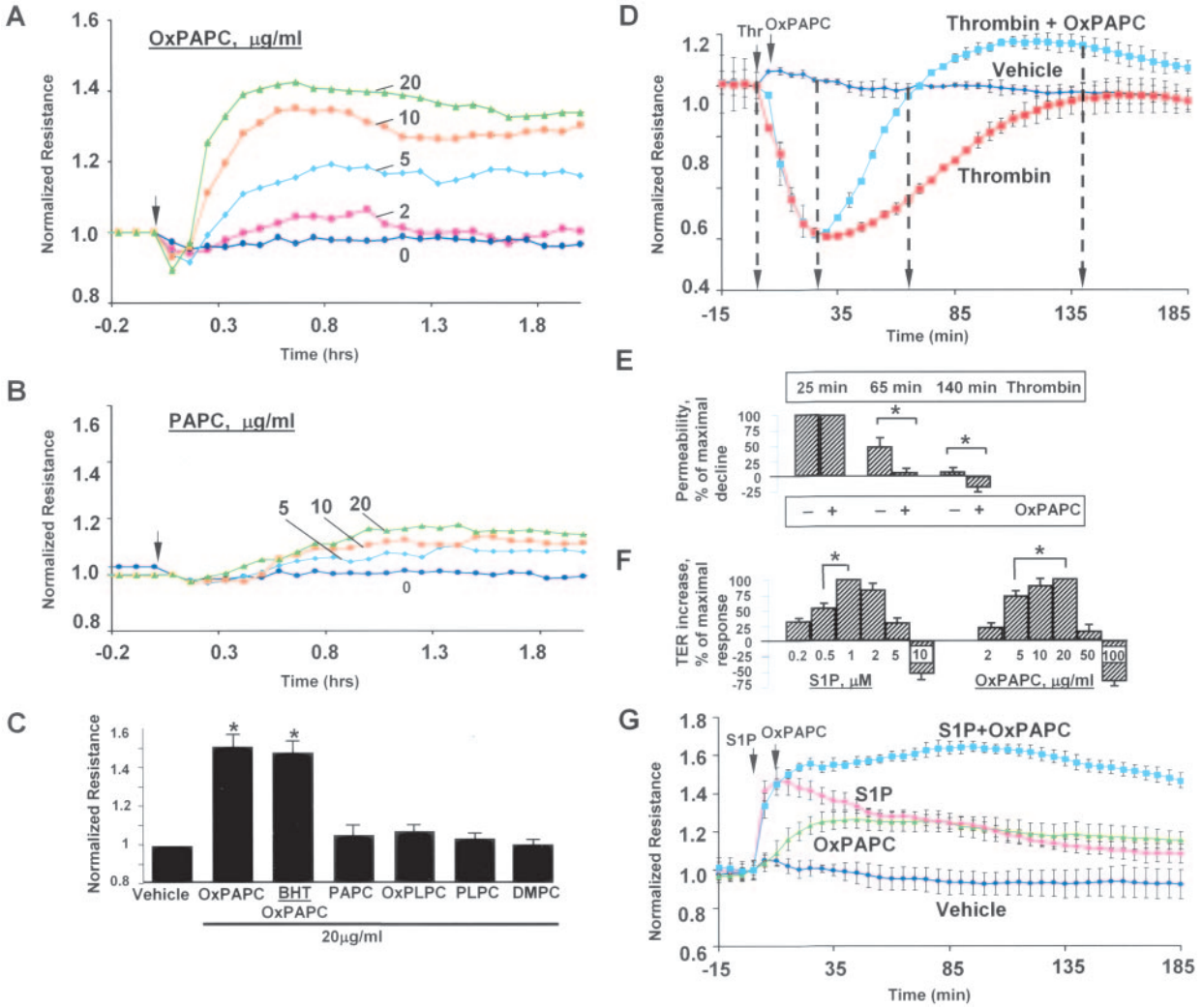

Figure 1. Effects of oxidized phospholipids on transendothelial electrical resistance (TER) changes in human pulmonary endothelial cells. A, Cells were treated with 0, 5, 10, and 20 μg/mL OxPAPC. B, Effects of native PAPC on TER changes in HPAECs. Cells were treated with 0, 5, 10, and 20 μg/mL PAPC. C, Effects of OxPAPC, PAPC, PLPC, OxPLPC, and DMPC treatment on TER changes in endothelial cells. Each phospholipid was used at 20 μg/mL. In selected experiments, OxPAPC was pretreated with butylated hydroxytoluene (BHT, 5 μmol/L, 10 minutes). D, Effect of OxPAPC on EC barrier recovery after thrombin stimulation. HPAECs were challenged with thrombin (50 nmol/L) followed by OxPAPC addition (20 μg/mL) as indicated by arrows. Control cells were stimulated with thrombin alone. Shown are cumulative data from five independent experiments. E, Quantitation of OxPAPC barrier-protective effects against thrombin-induced EC barrier compromise. TER measurements at the time points indicated by dotted arrows in Panel D are expressed as percent of maximal permeability in EC monolayers after 15 thrombin stimulation (50 nmol/L, 15 minutes). Results are mean±SD of five independent experiments. *$P<0.05$. F, Concentration-dependent effects of S1P and OxPAPC on TER changes. HPAEC monolayers were treated with phospholipids at indicated concentrations, and TER were measured 15 minutes after stimulation. Data are presented as percent of maximal TER increase. Results are mean±SD of five independent experiments. *$P<0.05$. G, Additive effect of OxPAPC and S1P on TER increase. HPAECs were treated with OxPAPC (20 μg/mL) and S1P (1 μmol/L) alone, or administered together. Control cells were left untreated. Results are mean±SD of five independent experiments.

tions of OxPAPC. Importantly, barrier-protective effects of Fraction #2 were associated with enhancement of peripheral actin cytoskeleton also observed in OxPAPC-stimulated cells (Figure 3C, right panel), whereas barrier-disruptive effects of Fraction #1 were accompanied by gap formation, and distinct pattern of cytoskeletal remodeling with appearance of random stress fibers (Figure 3C, middle panel). Because OxPAPC contains several oxidized phospholipids bearing a fragmented acyl chain at the *sn*-2 position, such as POVPC, PGPC, and lysoPC, and they are all present in OxPAPC,[13,16,25] we next tested effects of synthetic POVPC, lysoPC, and PGPC on EC barrier properties. All three compounds, POVPC, PGPC, and lysoPC, prepared by chemical synthesis significantly and concentration-dependently decreased TER (Figure 3D). These results clearly demonstrate barrier-disruptive effects of fragmented oxidation products and lysoPC on the pulmonary EC monolayers.

Effects of OxPAPC on Activation of Small GTPases Rac, Rho, and Cdc42

Previous studies have stressed out a critical role for Rho and Rac in specific cytoskeletal responses associated with endothelial barrier regulation.[1,20,26] Figure 4A shows that OxPAPC-induced increases in TER were attenuated by inhi-

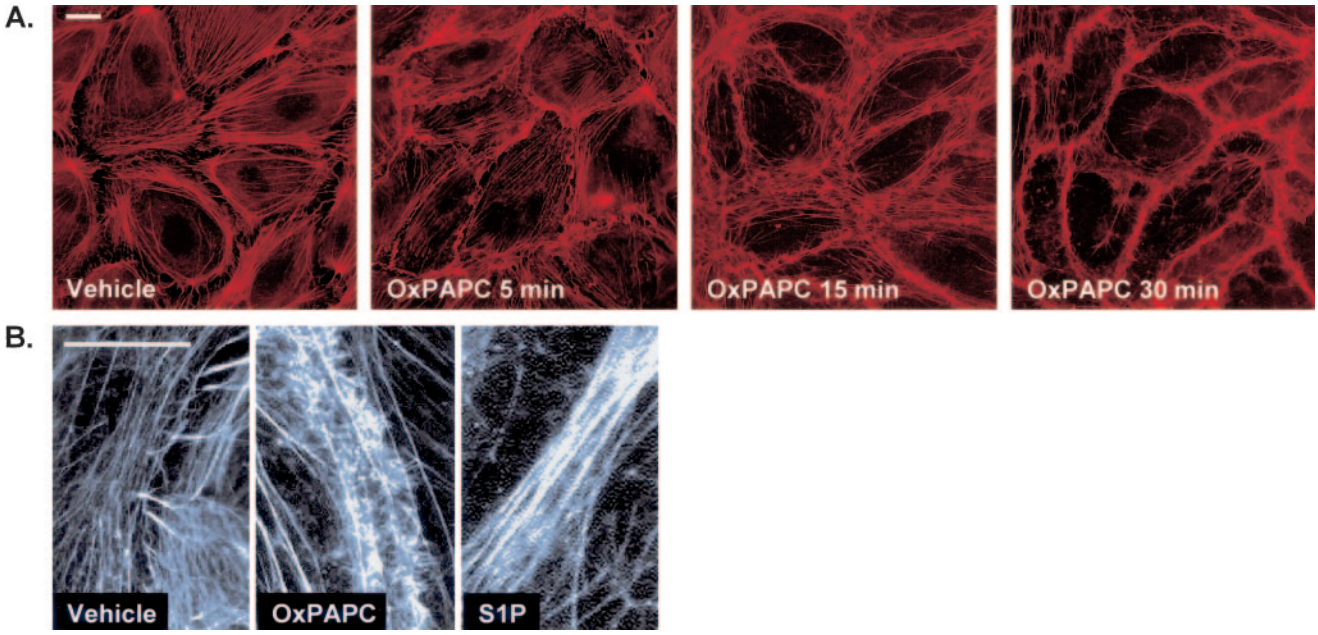

Figure 2. Time-dependent effects of OxPAPC on the HPAEC actin cytoskeleton. A, Cells were treated with OxPAPC (20 μg/mL) for the indicated periods of time. B, F-actin structure at the cell-cell interface of HPAECs stimulated with OxPAPC (20 μg/mL) and S1P (1 μmol/L). OxPAPC induces unique actin microspike formation. Shown are representative results of three independent experiments. Bar=5 μm.

bition of Rac, Cdc42 and Rho activities using toxin B (100 ng/mL), but not by HPAEC pretreatment with Rho-kinase inhibitor Y27632 (5 μmol/L, 1 hour). These results strongly suggest an involvement of Rac and Cdc42, but not Rho in the barrier protective effects of oxidized phospholipids. Measurements of OxPAPC-induced small GTPase activation (Figure 4B) revealed transient activation of Rac with peak at 5 minutes and a decline after 15 minutes. Furthermore, OxPAPC-induced Cdc42 activation reached a peak at 5 minutes and remained elevated above the basal level until 30 minutes of stimulation. In contrast, Rho activity was not affected by OxPAPC (Figure 4B, lower panels). Importantly, HPAEC stimulation with OxPAPC Fraction #2, which exhibited barrier-protective properties (Figure 3B, right panels) induced Rac and Cdc42 activation without effects on Rho activity, whereas OxPAPC Fraction #1, which contained fragmented phospholipids and did not reveal barrier-protective properties showed no significant Rac and Cdc42 activation (Figure 4B, right panels). Subcellular fractionation studies indicated OxPAPC-induced translocation of Cdc42, Rac, and the Rac effector PAK1 (αPAK) from the cytosol to the membrane (Figure 4C), whereas intracellular distribution of Rho remained unchanged.

Effects of Rac and Cdc42 Activities on OxPAPC-Induced Cytoskeletal Remodeling

To test a role of coordinated Rac and Cdc42 activation in the unique cytoskeletal remodeling observed in OxPAPC-stimulated cells, HPAECs were transiently transfected with constitutively active or dominant-negative Rac and Cdc42 mutants. Expression of constitutively active L61Cdc42 caused significant filopodia formation and cell retraction, whereas expression of constitutively active V12Rac stimulated cell spreading and enhanced cortical actin rim formation (Figure 5A). Expression of V14Rho caused intense central stress fiber formation, the cytoskeletal effect distinct from the pattern of OxPAPC-induced actin remodeling (Figure 5A). Because the unique OxPAPC-induced peripheral cytoskeletal remodeling was associated with activation of both Rac and Cdc42, ECs were next cotransfected with V12Rac and L61Cdc42. Coexpression of activated Rac and Cdc42 induced peripheral actin cytoskeletal remodeling that resembled OxPAPC-induced effects (Figure 5B). Finally, cotransfection of human pulmonary ECs with dominant-negative N17Rac and N17Cdc42 mutants completely abolished enhancement of peripheral actin cytoskeleton induced by OxPAPC or its barrier-protective Fraction #2 (Figure 5C, upper panels), as compared with OxPAPC-stimulated cells transfected with empty vector (Figure 5C, lower panels). HPAEC transfection with dominant-negative Rac abolished OxPAPC-induced enhancement of continuous peripheral F-actin staining observed in nontransfected cells, but did not affect formation of microspike-like structures (data not shown). Importantly, S1P stimulation of HPAECs overexpressing dominant-negative Rac[1] did not reveal formation of microspike-like structures observed in OxPAPC stimulated cells, again suggesting that Cdc42 activation is unique to OxPAPC-stimulated endothelial cells. We next tested effects of specific small GTPase depletion on OxPAPC-induced TER changes using siRNA-mediated knockdown of Rac, Cdc42, or Rho. Depletion of Rac and Cdc42 protein expression significantly attenuated TER increase induced by OxPAPC and TLC Fraction #2 (Figure 5D), whereas depletion of Rho or treatment with nonspecific RNA duplex oligonucleotide were without effect. Depletion of target proteins on treatment with corresponding siRNA was confirmed by immunoblotting with appropriate antibody (Figure 5E). Cell

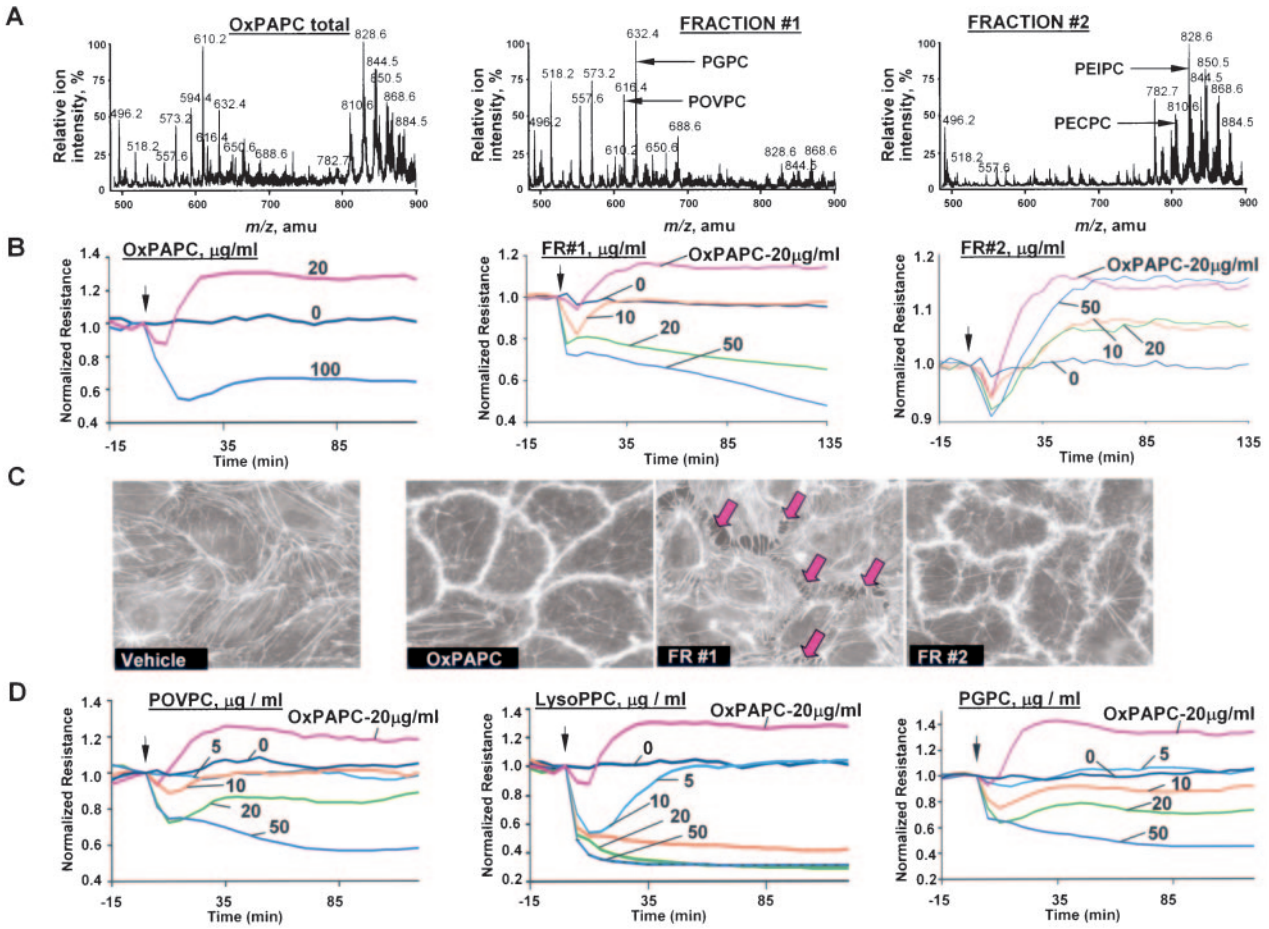

Figure 3. Oxygenated, but not fragmented, phospholipids exhibit barrier-protective effect. A, Mass-spectra of OxPAPC and Fractions #1 and #2 obtained by preparative thin layer chromatography, as described in Materials and Methods. Arrows indicate peaks corresponding to the major phospholipid products present in Fractions #1 and #2. B, Effects of OxPAPC and Fractions #1 and #2 on TER. Concentrations indicated in the Figure for Fractions #1 and #2 (10 μg/mL, 20 μg/mL, and 50 μg/mL) correspond to the amount of OxPAPC from which Fractions #1 and #2 were obtained. OxPAPC at 100 μg/mL exhibits barrier-disruptive effect compared with prominent barrier-protective effect observed at 20 μg/mL. The results are representative of three experiments using two preparations of Fractions #1 and #2. C, Effects of OxPAPC and Fractions #1 and #2 on actin cytoskeleton. Cells were treated with OxPAPC, OxPAPC Fraction #1, or OxPAPC Fraction #2 (20 μg/mL, 20 minutes). F-Actin was visualized by Texas Red phalloidin staining. Shown are representative results of three independent experiments. Bar=5 μm. D, Dose-dependent effects of synthetic POVPC, LysoPC, and PGPC on endothelial monolayer TER. Cells were treated with indicated concentrations of synthetic phospholipids. Shown are representative results of three independent experiments.

treatment with nonspecific RNA duplex oligonucleotide did not affect small GTPase expression.

Increased phosphorylation of Rac-dependent regulator of actin polymerization cofilin stimulates peripheral actin polymerization and can be induced by OxPAPC and S1P.[1,17] OxPAPC stimulation of EC monolayers induced peripheral translocation of the regulators of actin polymerization preferentially activated by Rac (cortactin, p21Arc), Cdc42 (N-WASP), and Rac/Cdc42 (Arp3, phosphocofilin) (online Figure 1S available in the online data supplement). Subcellular fractionation and Western blot analysis validated the results of immunofluorescent analysis with membrane translocation of cortactin, p21Arc, Arp3, N-WASP, and phosphocofilin in response to OxPAPC stimulation (online Figure 1S). Taken together, these data demonstrate essential role for Cdc42- and Rac-mediated signaling pathways in OxPAPC-induced endothelial barrier regulation and unique cytoskeletal remodeling driven by Rac/Cdc42 cytoskeletal effector proteins.

Molecule With *m/z* 810 (PECPC) Coelutes With Biological Activity in HPLC-MS

Among oxygenated derivatives of PAPC, PEIPC (*m/z* 828) and PECPC (*m/z* 810) have been structurally identified and shown to exert biological activities.[13,16,25] Because TER-increasing activity is present in the fraction containing oxygenated PCs, we further separated the TLC Fraction 2 using reversed phase HPLC-MS, which separates these compounds into several isomers,[13] and tested effects of individual fractions on EC barrier properties. We found three major fractions with barrier protective activities eluted at 18 minutes, 21.5 minutes, and 25.5 minutes (Figure 6A). Single ion tracing for PEIPC and PECPC (*m/z* 810 and 828, respectively) revealed that the molecule with *m/z* 810 coeluted with the fraction exhibiting major barrier-protective activity (25.5 minutes) (Figure 6B and 6C). ESI-MS analysis of this fraction demonstrated that PECPC (*m/z* 810.5, $[M+Na^+]$ 832.5) was the major component of this fraction, whereas

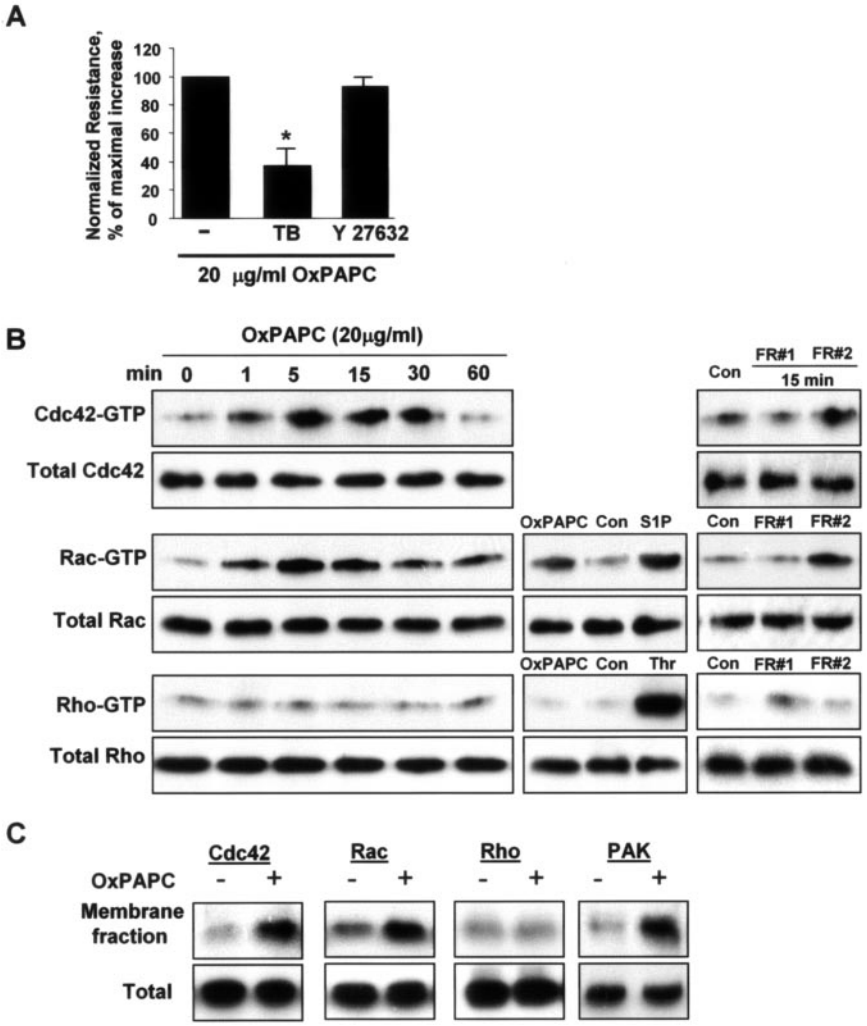

Figure 4. OxPAPC activates Rac and Cdc42. A, Effect of inhibitors on OxPAPC-mediated EC barrier regulation. Cells were preincubated with the *C. difficile* toxin B (1 ng/mL) or Y27632 (5 μmol/L) 30 minutes before OxPAPC (20 μg/mL) challenge. Results are expressed as percent of TER increase at 30 minutes in response to OxPAPC. Results are mean±SD of three independent experiments. *$P<0.05$. B, Effects of OxPAPC and OxPAPC Fraction #1 and Fraction #2 on Cdc42, Rac, and Rho activity. Activated GTP-bound forms of Rac, Cdc42, and Rho after OxPAPC (20 μg/mL) stimulation for indicated periods of time were isolated using pulldown assays. Effects of OxPAPC fractions equal to 20 μg/mL of OxPAPC on Rac and Rho activation (right panels) were measured after 15 minutes of stimulation. Total Rac, Cdc42, and Rho content in cell lysates was verified by immunoblotting. S1P (0.5 μmol/L, 5 minutes) and thrombin (50 nmol/L, 5 minutes) stimulation were used as positive controls for Rac and Rho activation, respectively. C, Translocation of Cdc42, Rac, and PAK, but not Rho, to the membrane/cytoskeletal fraction after OxPAPC stimulation was detected by subcellular fractionation followed by Western blot analysis, as described in Materials and Methods.

minor components (m/z 828, 830, 844) were also present (Figure 6D).

Discussion

Precise regulation of endothelial semiselective barrier is critically important for mass transport and metabolic exchange between blood and peripheral tissue. Edemagenic and proinflammatory agents including thrombin and cytokines compromise endothelial barrier leading to extravasation of fluid and blood cells, which is a hallmark of inflammation and edema formation. In contrast to mechanisms involved in barrier dysfunction, mechanisms of EC barrier recovery are not well understood. In addition, little is known about bioactive compounds that are released during injury or inflammation and promote resealing of the endothelial monolayer, which is an important aspect in resolution of inflammation.

Our results show that specific phospholipid oxidation products induce concentration-dependent and sustained barrier-protective effects (Figures 1, 3, and 6), counteracting thrombin-induced EC barrier disruption (Figure 1). These effects were specific for oxidized forms of phospholipids, because nonoxidized phospholipids in the same concentration range did not significantly affect EC permeability (Figure 1). Structure-function analysis revealed that the barrier protective effect was independent of the phospholipid head group, because oxidized phosphatidylserine, -ethanolamine, and phosphatidic acid also increased TER (data not shown). Oxidation products of arachidonic acid-, but not linoleic acid–containing phospholipids exhibited barrier-protective properties (Figure 1), and we show that *sn*-2-oxygenated, but not *sn*-2-fragmented phospholipids, are responsible for the induction of barrier protective effects (Figure 3). Analysis of these oxygenated products using HPLC-MS revealed that a molecule with *m/z* 810 corresponding to 1-palmitoyl-2-(epoxycyclopentenone)-*sn*-glycero-3-phosphorylcholine (PECPC)[14] and a molecule with *m/z* 828 corresponding to another epoxyisoprostane-containing phospholipid, 1-palmitoyl-2-(epoxyisoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC), coeluted with TER increasing activity (Figure 6). Along with PECPC and PEIPC, several other not yet identified compounds that are present in the oxygenated fraction of OxPAPC may contribute to the overall barrier protective effect (Figure 6). It will be the goal of future studies to identify the chemical structures of these compounds.

Oxidized lipids appear in several lung disorders. For example, in acute lung injury there is leakage of native lipoproteins from serum into the alveolar space where they

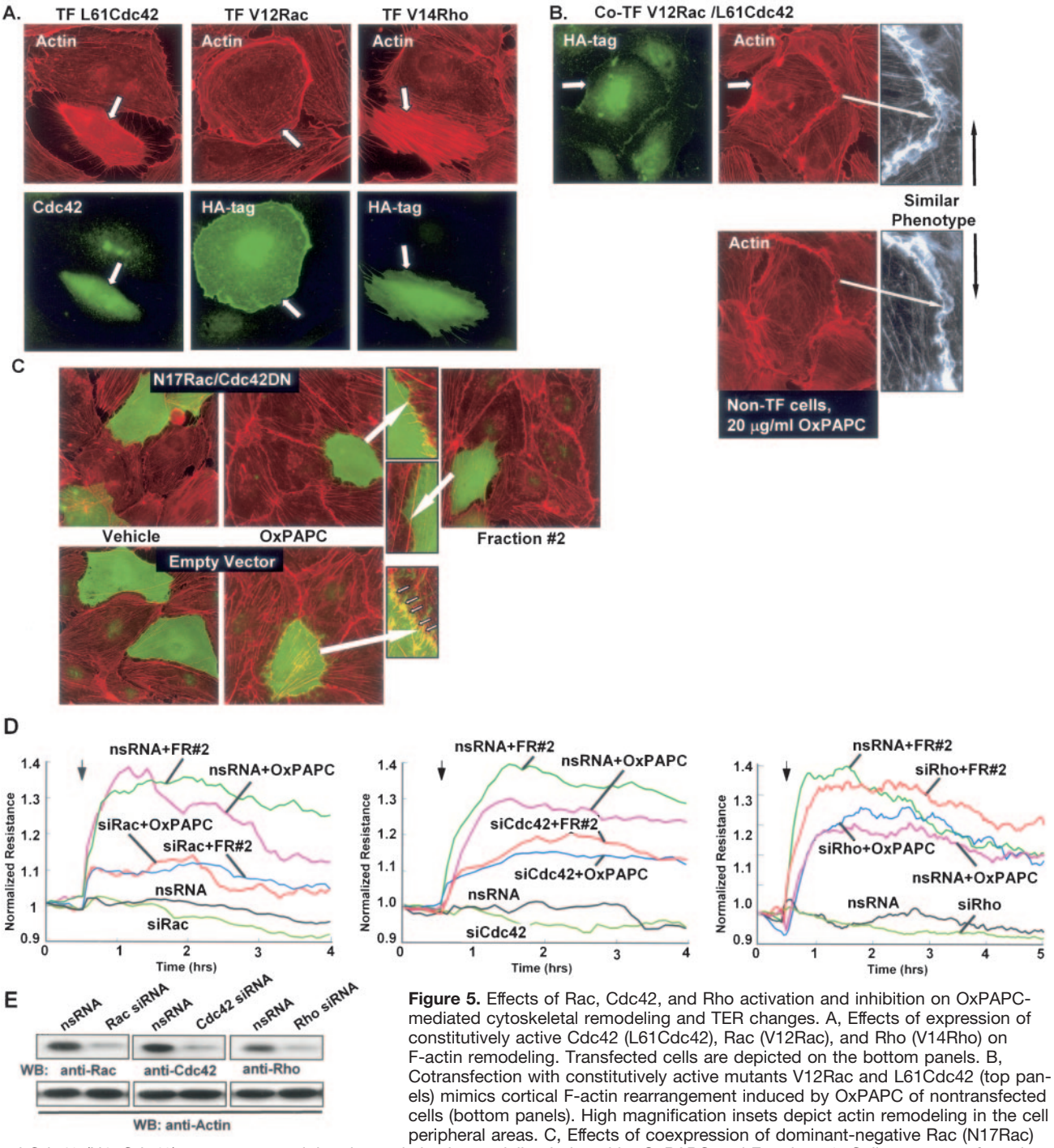

Figure 5. Effects of Rac, Cdc42, and Rho activation and inhibition on OxPAPC-mediated cytoskeletal remodeling and TER changes. A, Effects of expression of constitutively active Cdc42 (L61Cdc42), Rac (V12Rac), and Rho (V14Rho) on F-actin remodeling. Transfected cells are depicted on the bottom panels. B, Cotransfection with constitutively active mutants V12Rac and L61Cdc42 (top panels) mimics cortical F-actin rearrangement induced by OxPAPC of nontransfected cells (bottom panels). High magnification insets depict actin remodeling in the cell peripheral areas. C, Effects of coexpression of dominant-negative Rac (N17Rac) and Cdc42 (N17Cdc42) mutants on peripheral cytoskeletal remodeling induced by OxPAPC and Fraction #2. Cells were transfected with empty vector (bottom panels) or were cotransfected with N17Rac and N17Cdc42 (top panels) followed by stimulation with OxPAPC or Fraction #2 (20 μg/mL, 20 minutes, right panels). Shown are merged immunofluorescent images stained with Texas red phalloidin to visualize F-actin (red) and anti–myc tag Ab for detection of Rac/Cdc42-overexpressing cells. Insets depict magnified areas of cell-cell interface (F-actin staining in transfected cells after merging appears as yellow). Arrows point to the cortical actin band in OxPAPC-treated cells. Shown are representative results of three independent experiments. D, HPAECs grown on gold microelectrodes were incubated with siRNA to Rac1, Cdc42, Rho, or treated with nonspecific RNA duplexes, as described in Materials and Methods and used for TER measurements. Cells were stimulated with OxPAPC or Fraction #2 (20 μg/mL) in the time marked by arrow. E, Cells grown in D35 culture plates were incubated with siRNA to Rac1, Cdc42, Rho, or treated with nonspecific RNA duplex oligonucleotide, and target protein depletion was examined by immunoblotting with corresponding antibody. Control blots represent β-actin expression in ECs treated with siRNA. Shown are representative results of three independent experiments.

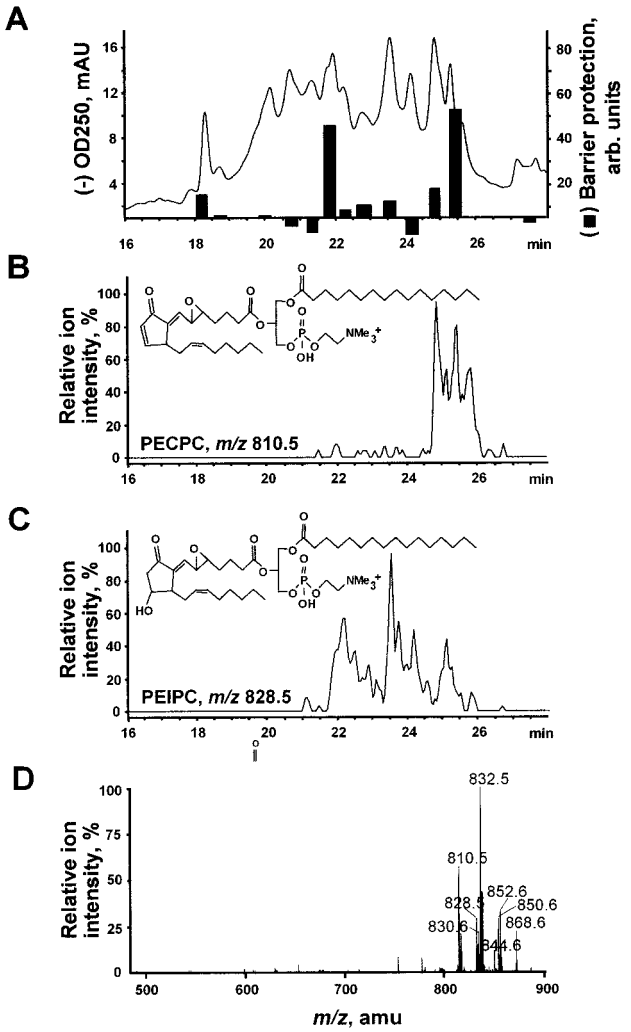

Figure 6. A molecule with *m/z* 810 (PECPC) coelutes with biological activity. A, Fraction #2 obtained by preparative thin layer chromatography was further separated by reversed-phase HPLC as described in the Materials and Methods section. Fractions corresponding to peaks of optical density at 250 nm (line, left axis) were collected and tested for effects on TER (bars, right axis). B and C, Elution profile of PECPC and PEIPC was monitored by online ESI-MS at *m/z* values of 810.5 and 828.5, respectively. D, Mass-spectrum of the fraction eluting at 25.5 minutes, which demonstrated the highest TER-increasing activity.

are oxidatively modified. Oxidative stress, intrinsic to lung injury, results from impaired antioxidant defense, the presence of reactive oxidant species, and exposure to hyperoxia during mechanical ventilation,[27] or exposure to ozone.[28] Increased levels of oxidized phospholipids have been shown in murine lung tissue[29] and may also appear in lung circulation in pathological settings of acute injury, sepsis, and inflammation, all of which are also associated with platelet activation and increased release of S1P by platelets. Our data demonstrate additive effects of oxidized phospholipids and S1P on EC barrier protection (Figure 1). Importantly, OxPAPC and S1P trigger distinct intracellular signaling pathways with preferential activation of Cdc42 and Rac-mediated signaling and cytoskeletal remodeling by OxPAPC and Rho and Rac-mediated signaling by S1P.[1,3]

Although the kinetics of OxPAPC-mediated intracellular signaling,[9,15,17,18,30] cytoskeletal remodeling and barrier regulation (Figures 1 and 2) suggest a receptor-mediated cellular response, a specific receptor for OxPAPC has not yet been identified. Although some specific effects of OxPAPC can be partially inhibited by platelet activating factor (PAF) receptor antagonists,[30–32] PAF itself does not mimic barrier-protective OxPAPC effects (K. Birukov, unpublished observations, 2004), and instead is a well-recognized edemagenic agent.[33] These observations suggest a potential structural homology of a putative OxPAPC receptor with the PAF receptor and do not exclude the potential for several receptors capable of binding different components of OxPAPC and triggering OxPAPC-mediated signal transduction.[16]

Coordinated remodeling of the actin cytoskeleton, focal adhesions, and adherens junctions is precisely controlled by small GTPases.[34–36] Activated Rho, Rac, and Cdc42 induce the formation of stress fibers, lamellipodia, and filopodia, respectively.[37] Whereas Rho functions mostly by reorganizing preexisting actin filaments, Rac and Cdc42 promote new actin polymerization at the cell cortical layer, either by stimulating the uncapping or severing of actin filaments.[38] Our results demonstrate for the first time that OxPAPC induces specific activation of Rac- and Cdc42 (Figure 4), which govern a unique cytoskeletal rearrangement (Figures 2 and 3) characterized by an enhanced peripheral actin cytoskeleton and formation of F-actin structures at the cell-cell interface that resemble microspikes in single cells with activated Rac/Cdc42 cascade.[23] These cytoskeletal changes were linked to the accumulation of Arp3, p21-Arc, cortactin, N-WASP and phosphocofilin in the cortical layer (online Figure 1S). Although activated Rac promotes lamellipodia formation via local activation of Arp2/3-cortactin–dependent actin polymerization[39,40] and formation of novel focal adhesion contacts, which involves PAK, GIT2, and paxillin,[41] activated Cdc42 triggers N-WASP-induced filopodia and microspike formation, as well as assembly of paxillin-PAK-GIT1-GIT2 focal adhesion protein complexes.[34,37,41] Moreover, Cdc42 and Rac control cadherin-mediated cell-cell adhesion and formation of novel adherens junction complexes via modulation of interactions between α-catenin and cadherin-catenin complex.[36] Activation of both Rac and Cdc42 is involved in cell spreading after adhesion to thrombospondin-1.[42] Thus, the specific cytoskeletal rearrangement induced by OxPAPC may well be a result of combined activation of Rac and Cdc42.

An essential role for the combined Rac and Cdc42 activation in OxPAPC-mediated cytoskeletal remodeling was further supported by our results showing that only the coexpression of constitutively active Rac and Cdc42 induced the unique cytoskeletal rearrangement that was observed in OxPAPC-stimulated EC monolayers (Figure 5) and which was different from S1P-induced actin remodeling (Figure 2B). Moreover, coexpression of dominant-negative Rac and Cdc42 abolished peripheral actin cytoskeletal remodeling induced by OxPAPC, and siRNA-based depletion of endogenous Rac and Cdc42 pools attenuated EC barrier-protective response induced by OxPAPC and its barrier-protective Fraction #2 containing oxygenated phospholipids PECPC and

PEIPC (Figures 5 and 6). Taken together, these data suggest that Rac and Cdc42 may serve as integrating signaling systems that mediate specific rearrangements of actin cytoskeleton and cell contacts leading to OxPAPC-mediated barrier protection in endothelial monolayers.

Based on our studies, we propose a role for oxidized phospholipids in resolution of acute inflammation involving vascular leakage. Excessive accumulation of short chain oxidized phospholipids is associated with early stages of acute lung injury characterized by high levels of oxidative stress and may compromise EC barrier function, thus contributing to edema formation. However, at later phases diminished oxidative stress in the areas of tissue injury leads to the formation of oxygenated phospholipids to the levels that would enhance EC barrier function, which would represent a feedback mechanism leading to EC barrier recovery. This protective effect can be further potentiated by S1P generated by activated platelets, which acts in additive fashion with oxidized phospholipids. These findings suggest an interesting possibility of controlled administration of exogenous barrier-protective oxidized phospholipids, which may be potentially considered as a new therapeutical approach in the treatment of acute lung injury syndromes.

In summary, our results demonstrate for the first time barrier-protective properties of biologically active oxidized phospholipids in endothelial cells. We show that OxPAPC-induced barrier protection involves a unique cytoskeletal remodeling mediated by combined activation of the small GTPases Cdc42 and Rac. The characterization of structurally defined components of OxPAPC with the potent barrier protective effects forms a basis for targeted drug design of a novel class of antiedemagenic and antiinflammatory therapeutic agents and provides new insights into the role of oxidized phospholipids in the compensatory mechanisms of endothelial barrier protection under life-threatening conditions, such as acute lung injury and inflammation.

Acknowledgments

This work was supported by grants from the National Heart Lung and Blood Institute (HL58064, HL69340, HL67307, HL73994, HL76259), the Dr David Marine Endowment for J.G.N.G., NIH grant 1PO1 CI000095-01 for G.M.B., and Fonds zur Förderung der wissenschaftlichen Forschung (Austria) Project P16086 for V.N.B. and N.L.

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