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


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 reviews10–12).

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 coflin 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...
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).

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 intercellular 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 PEPC representing major peaks (Figure 3A, right panel), induced prominent increases in TER (Figure 3B, right panel), thus mimicking barrier protective effects of low concentra-
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 Ox-PAPC,13,16,25 we next tested effects of synthetic POVPC, PGPC, and lysoPC 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 inhibi-
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 Rac⁴ 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
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. 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. 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, 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
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) 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.
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, or exposure to ozone. Increased levels of oxidized phospholipids have been shown in murine lung tissue 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.

Although the kinetics of OxPAPC-mediated intracellular signaling, 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, PAF itself does not mimic barrier-protective OxPAPC effects (K. Birukov, unpublished observations, 2004), and instead is a well-recognized edemagenic agent. 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.

Coordinated remodeling of the actin cytoskeleton, focal adhesions, and adherens junctions is precisely controlled by small GTPases. Activated Rho, Rac, and Cdc42 induce the formation of stress fibers, lamellipodia, and filopodia, respectively. 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. 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. 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 and formation of novel focal adhesion contacts, which involves PAK, GIT2, and paxillin, activated Cdc42 triggers N-WASP-induced filopodia and microspike formation, as well as assembly of paxillin-PAK-GIT1-GIT2 focal adhesion protein complexes. 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. Activation of both Rac and Cdc42 is involved in cell spreading after adhesion to thrombospondin-1. 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 therapeutic 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, HL73994, HL76259), the Dr David Marine Endowment for J.G.N.G., NIH and Blood Institute (HL58064, HL69340, HL67307, HL73994), and N.L.

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Circ Res. 2004;95:892-901; originally published online October 7, 2004; doi: 10.1161/01.RES.0000147310.18962.06

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Epoxycyclopentenone-containing oxidized phospholipids restore endothelial barrier function via Cdc42 and Rac.

Abbreviations

EC - endothelial cells
ECIS - electrical cell substrate impedance-sensing system
HPAEC - human pulmonary artery endothelial cells
OxPAPC - oxidized 1-Palmitoyl-2-Arachidonoyl-sn-Glycero-3-Phosphocholine
S1P - sphingosine 1-phosphate
TER - transendothelial electrical resistance
GIT1 – G protein coupled receptor interacting protein 1
GIT2 – G protein coupled receptor interacting protein 2

Materials and methods

Materials. All biochemical reagents including mouse monoclonal pan-MLC antibody were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Mouse monoclonal anti-GIT1, PKL/GIT2, FAK, and paxillin antibodies (Transduction Laboratories, Lexington, KY), Rho-kinase inhibitor Y-27632 was obtained from Tocris (Ellisville, MO). Toxin B was purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal phospho-cofillin antibodies were obtained from Santa Cruz (Santa Cruz, CA). Mouse monoclonal anti-FAK and anti-paxillin antibodies were obtained from BD Pharamingen (San Diego, CA).
Lipid oxidation and analysis. 1-Palmitoyl-2-Arachidonoyl-sn-Glycero-3-Phosphocholine (PAPC), 1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphocholine (lysoPPC), 1-Palmitoyl-2-Linoleoyl-sn-Glycero-3-Phosphocholine (PLPC), 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC), 1-Palmitoyl-2-Arachidonoyl-sn-Glycero-3-[Phospho-L-Serine] (PAPS), 1-Palmitoyl-2-Arachidonoyl-sn-Glycero-3-Phosphate (phosphatidic acid, PAPA) and 1-Palmitoyl-2-Arachidonoyl-sn-Glycero-3-Phosphoethanolamine (PAPE) were obtained from Avanti Polar Lipids (Alabaster, AL). Phospholipids were oxidized by exposure of dry lipid to air as previously described. The extent of oxidation was monitored by positive ion electrospray mass spectrometry (ESI-MS) as described previously. POVPC and PGPC were synthesized from PAPC or lysoPC, correspondingly. Lipids were stored at –70°C in chloroform and used within 2 weeks after mass spectrometry testing. Presence of endotoxin in PAPC and OxPAPC preparations was tested by the Limulus amebocyte assay (BioWhittaker, Frederick, MD).

Chromatographic separation of lipids. OxPAPC was fractionated by preparative layer chromatography on 2-mm thick Kieselgel 60 plates (Merck). A mixture of PAPC, POVPC, lysoPPC and PGPC standards was applied at the sides of the plates. After development in chloroform/methanol/water = 10:5:1, sides of the plate were cut off and stained with 10% CuSO₄ in 8% H₃PO₄. Silica gel was scraped from the rest of the plate as two fractions. Fraction 1 contained lipids migrating between the origin and POVPC (Rf 0.28), and fraction 2 – between
POVPC and PAPC (Rf 0.41). The lipids were eluted from silica by chloroform/methanol/water = 65:25:4, dried and stored at –70°C.

HPLC separation of fraction 2 obtained by preparative layer chromatography was performed by reversed phase HPLC/MS on a LiChroCART 250-4 HPLC-cartridge containing LiChrospher 100 RP-8 sorbent (Hewlett Packard) using a linear gradient of 80 to 100% methanol in water as described 5. Eluted substances were monitored by ESI-MS and UV absorbance at 250 nm. Fractions were collected according to peaks of optical density.

Cell culture. Human pulmonary artery endothelial cells were obtained from Clonetics, BioWhittaker Inc. (Frederick, MD). Cells were maintained in complete culture medium consisting of Clonetics EBM basic medium containing 10% bovine serum and supplemented with a set of non-essential amino acids, endothelial cell growth factors, and 100 units/ml penicillin/streptomycin provided by Clonetics, BioWhittaker Inc., and incubated at 37°C in humidified 5% CO₂ incubator. Cells were used for experiments at passages 6-8.

TER measurements. To measure agonist-induced changes in transendothelial electrical resistance (TER) in EC cultures, the electrical cell substrate impedance-sensing (ECIS) system available from Applied BioPhysics, Inc. (Troy, NY) was used as described elsewhere 6-9. Briefly, cells were cultured on small gold electrodes (10⁻² mm²), and culture media were used as electrolyte. The total electrical resistance was measured dynamically across the monolayer and was
determined by the combined resistance between the basal surface of the cell and the electrode, reflective of focal adhesion, and the resistance between cells. As cells adhere and spread out on the microelectrode, the transmonolayer electrical resistance (TER) increased (maximal at confluence), whereas cell retraction, rounding, or loss of adhesion was reflected by a decrease in TER. The small gold electrodes and the larger counter electrodes (100 mm²) were connected to a phase sensitive lock-in amplifier (5301A; EG&G Instruments, Princeton, NJ) with a built in differential preamplifier (5316A; EG&G Instruments). A 1-V, 4,000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant-current source. Voltage and phase data were stored and processed with PC that controlled the output of the amplifier and relay switches to different electrodes. Experiments were conducted only on wells that achieved >1,000 Ω (10 microelectrodes/well) of steady-state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized resistance value.

Protein fractionation of EC lysates. For separation of cytosolic and membrane/cytoskeletal fractions, EC monolayers grown in D35 plates were lysed in 200 µL extraction buffer (20 mmol/L Tris-HCl (pH 7.4), 125 mmol/L sucrose, 50 mmol/L NaCl, 2 mmol/L EGTA, 1 mmol/L PMSF) and collected in microtubes. After ultracentrifugation at 100,000g, 30 min, +4°C, supernatants containing cytosolic proteins, and pellets containing particulate fraction were separated,
solubilized in 3X SDS sample buffer, and subcellular protein fractions were analyzed by western blot.

*Rac, Cdc42 and Rho activation assays* were performed using commercially available assay kits purchased from Upstate Biotechnology (Lake Placid, NY). EC stimulated with OxPAPC, thrombin, or sphingosine 1-phosphate were lysed, and Rac, Cdc42, and Rho activities in the cell lysates were tested according to manufacturer's protocols.

*Western immunoblotting.* Protein extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (30 V for 18 h or 90 V for 2 h), and the membranes were incubated with specific antibodies of interest. Immunoreactive proteins were detected with the enhanced chemiluminescent detection system (ECL) according to the manufacturer's directions (New England BioLabs, Beverly, MA).

*Plasmids.* For transient transfection experiments, we used Rac and Cdc42 mutants: dominant negative Rac, constitutively active Rac, dominant negative Cdc42 and constitutively active Cdc42 subcloned into pCDNA3.1 vectors. These plasmids have been described elsewhere \(^\text{10}\) or were kindly provided Dr. M. Nicolic (Harvard Medical School, MA).
**Transfection protocol.** EC seeded in petri dishes or in 12-well plates with gelatin-coated glass coverslips were transfected at 70% confluency. Each well was incubated with 2 mL of OPTI-MEM medium containing 2 µg DNA and 20 µL of Fugene 6 (Boehringer Mannheim) for 6 hrs in CO₂ incubator at 37°C. Following washing (DMEM + 10% FCS) cells were incubated for additional 24 h and used for experiments with agonist stimulation. Control transfections were performed with empty vectors.

**Depletion of endogenous Rac, Cdc42 and Rho in EC.** To reduce the content of endogenous Rac, Cdc42 or Rho proteins, HPAEC were treated with Rac-, Cdc42 or Rho-specific small interfering RNA (siRNA) duplex oligonucleotides, which guide sequence-specific degradation of the homologous mRNA (Elbashir, 2001 #1065). Pre-designed siRNAs of standard purity were ordered from Ambion, (Austin, TX) in purified, desalted, deprotected and annealed double strand form. The following 21 base pair duplexes of siRNA were used: for Rac1: sense 5’-GGAGAUUGGUGCUGUAAAAtt-3’ and antisense 5’-UUUUACAGCACCAUCCUCCtt-3’, for RhoA: sense 5’-GGUGGAGGAAAGCAGGUAtt-3’ and antisense 5’-UACCUGCUUUCACCAUCACc-3’, for Cdc42: sense 5’-GGGCAAGAGGAUUAUGACAtt-3’ and antisense 5’-UGUCAUAUCCUUGCCTgc-3’. Non-specific, non-silencing FI-Luciferase GL2 duplex fluorescently labeled on the sense strand with 5’-Fluorescein (Dharmacon Research, Lafayette, CO) was used as a control treatment. HPAEC were grown to 70% confluence, and the transfection of siRNA (final concentration
100 nM) was performed using GeneSilencer™ transfection reagent (Gene Therapy Systems, San Diego, CA) according to manufacturer’s protocol, as we have recently described 11. Forty-eight hours later cells were used for the measurements of transendothelial electrical resistance, and siRNA-induced specific small GTPase depletion was confirmed by western blot.

**Immunofluorescent staining.** EC grown on glass coverslips after treatment were fixed in 3.7% formaldehyde solution in PBS for 10 min at 4°C, washed three times with PBS, permeabilized with 0.2% triton X-100 in phosphate buffered saline (PBS), pH 7.4 containing 0.05% Tween-20 (PBST) for 30 min at room temperature, and blocked with 2% BSA in PBST for 30 min. Incubation with antibodies was performed in blocking solution for 1 hour at room temperature. Alexa 488-, Alexa 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used for immunodetection, and actin filaments were stained with Texas Red- conjugated phalloidin (Molecular Probes) for 1 hour at room temperature. After immunostaining, the glass slides were prepared using mounting medium (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and analyzed using Nikon video-imaging system (Nikon Instech Co., Japan) consisting of a inverted microscope Nikon Eclipse TE300 with epi-fluorescence module using 60XA/1.40 oil objective connected to SPOT RT monochrome digital camera (temperature of 37°C) and image processor (Diagnostic Instruments, Sterling Heights, MI). The images were acquired using SPOT 3.5 acquisition software (Diagnostic Instruments) and processed with Adobe
Photoshop 7.0 (Adobe Systems, San Jose, CA) and Adobe Illustrator CS (Adobe Systems) software.

Statistical analysis. ANOVAs with a Student-Newman-Keuls test were used to compare the means of two or more different treatment groups. Results are expressed as means ± SE. Differences between two groups were considered statistically significant when P < 0.05.
References


5. Watson AD, Subbanagounder G, Welsbie DS, Faull KF, Navab M, Jung ME, Fogelman AM, Berliner JA. Structural identification of a novel pro-


Figure 1S. Effects of OxPAPC on cytoskeletal regulatory proteins. A – Immunofluorescent detection of the OxPAPC-induced peripheral translocation of cortactin, p21Arc, Arp3, N-WASP, and accumulation of phospho-cofilin. Cells were treated with OxPAPC (20 µg/mL, 30 min), and intracellular localization of proteins of interest was examined by immunofluorescent staining with the appropriate antibody, as described in Materials and Methods. B – Translocation of cortactin, p21Arc, Arp3, N-WASP and phosphor-cofilin to the membrane/cytoskeletal fraction in response to OxPAPC (20 µg/mL, 30 min) was detected by western blot analysis of soluble and particulate fractions, as described in Materials and Methods. Results are representative of three independent experiments. Bar = 10 µm.