Akt-Mediated Transactivation of the S1P₁ Receptor in Caveolin-Enriched Microdomains Regulates Endothelial Barrier Enhancement by Oxidized Phospholipids

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Abstract—Endothelial cell (EC) barrier dysfunction results in increased vascular permeability, leading to increased mass transport across the vessel wall and leukocyte extravasation, the key mechanisms in pathogenesis of tissue inflammation and edema. We have previously demonstrated that OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) significantly enhances vascular endothelial barrier properties in vitro and in vivo and attenuates endothelial hyperpermeability induced by inflammatory and edemagenic agents via Rac and Cdc42 GTPase dependent mechanisms. These findings suggested potential important therapeutic value of barrier-protective oxidized phospholipids. In this study, we examined involvement of signaling complexes associated with caveolin-enriched microdomains (CEMs) in barrier-protective responses of human pulmonary ECs to OxPAPC. Immunoblotting from OxPAPC-treated ECs revealed OxPAPC-mediated rapid recruitment (5 minutes) to CEMs of the sphingosine 1-phosphate receptor (S1P₁), the serine/threonine kinase Akt, and the Rac1 guanine nucleotide exchange factor Tiam1 and phosphorylation of caveolin-1, indicative of signaling activation in CEMs. Abolishing CEM formation (methyl-β-cyclodextrin) blocked OxPAPC-mediated Rac1 activation, cytoskeletal reorganization, and EC barrier enhancement. Silencing (small interfering RNA) Akt expression blocked OxPAPC-mediated S1P₁ activation (threonine phosphorylation), whereas silencing S1P₁ receptor expression blocked OxPAPC-mediated Tiam1 recruitment to CEMs, Rac1 activation, and EC barrier enhancement. To confirm our in vitro results in an in vivo murine model of acute lung injury with pulmonary vascular hyperpermeability, we observed that selective lung silencing of caveolin-1 or S1P₁ receptor expression blocked OxPAPC-mediated protection from ventilator-induced lung injury. Taken together, these results suggest Akt-dependent transactivation of S1P₁, within CEMs is important for OxPAPC-mediated cortical actin rearrangement and EC barrier protection. (Circ Res. 2009;104:978-986.)

Key Words: OxPAPC  Akt  S1P receptor  caveolin-enriched microdomain  endothelial barrier enhancement

Endothelial cells (ECs) provide a semiselective barrier between the blood and underlying tissue interstitium with barrier disruption resulting in increased vascular permeability and organ dysfunction. Therefore, agents that enhance EC barrier function are a desirable therapeutic strategy for a variety of inflammatory diseases, tumor angiogenesis and atherosclerosis. We have recently described that oxidized phospholipids (OxPLs) derived from OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) exhibited potent barrier-protective effects toward human pulmonary endothelial monolayers. However, the underlying signaling mechanisms by which OxPAPC increases vascular integrity remains poorly understood.

In endothelial cells, as in many other cell types, there exist specialized sterol- and sphingolipid-enriched domains, called lipid rafts, that have been implicated in OxPAPC signaling. In addition, there exists a subset of lipid rafts which are 50- to 100-nm plasma membrane microdomains containing a specific scaffolding protein called caveolin-1. OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine) exhibited potent barrier-protective effects toward human pulmonary endothelial monolayers. However, the underlying signaling mechanisms by which OxPAPC increases vascular integrity remains poorly understood.

Our published data indicate that certain EC barrier enhancing stimuli including hyaluronan and hepatocyte growth factor. Furthermore, we have demonstrated that CEMs are critical for Rac1 activation of barrier enhancing stimuli including hyaluronan and hepatocyte growth factor. However, the role of CEMs in OxPAPC-mediated EC barrier regulation is incompletely defined.

The sphingosine-1-phosphate (S1P) receptor S1P₁ resides in CEMs and is critically involved in EC barrier enhancement. Our published data indicate that certain EC barrier enhancing stimuli including hyaluronan induce S1P₁ transac-
ivation (threonine phosphorylation), which regulates Rac1 activation (Rac1-GTP formation) and rearrangement of the cortical actin cytoskeleton. We examined the role(s) of S1P1 receptor transactivation, CEMs, and Akt on OxPAPC-mediated cytoskeletal regulation and EC barrier enhancement in this study.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture and Reagents
Human pulmonary artery ECs were obtained from Clonetics (Walkersville, Md) and cultured as previously described.16 Unless otherwise specified, reagents were obtained from Sigma (St Louis, Mo).

Lipid Oxidation and Analysis
PAPC (nonoxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine) was obtained from Avanti Polar Lipids (Alabaster, Ala) and oxidized and analyzed as previously described.17

CEM Isolation
CEMs were isolated from human lung ECs using Triton X-100 insolubility and centrifugation, as we have described previously.13–15

Measurement of EC Electric Resistance
ECs were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and transendothelial cell electric resistance measurements performed using an electric cell-substrate impedance sensing system obtained from Applied Biophysics (Troy, NY), as described previously in detail.16

Delivery of Small Interfering RNA in Mice
Adult male C57BL/6J mice, 8 to 10 weeks old, with an average weight of 20 to 25 g (The Jackson Laboratory, Bar Harbor, Me) were bred at the University of Chicago animal care center. All experimental protocols involving the use of animals were approved by the University of Chicago Institutional Animal Care & Use Committee for the humane treatment of experimental animals. Small interfering (si)RNAs from Dharmacon (Lafayette, Colo) had the following sequences: siCaveolin1: 5′-ACGUAGACUCCGAGGCA-3′; siS1P1 receptor: 5′-CUUGCUAACUAUUUGGAAA-3′; control siRNA (Luciferase): 5′-UAAGGCUAUGAAGAGAUA-3′. Polyethyleneimine-22, which provides preferential RNA targeting to the lung,18 was used as a carrier in the in vivo experiments with siRNA-induced caveolin and S1P1 receptor knockdown in vivo. Obtained polyethyleneimine-22–siRNA polyplexes (400 μL) were injected into the jugular vein of the 8- to 10-week-old C57BL/6 male mice under anesthesia. After 72 hours, the mice were subjected to mechanical ventilation or Evan’s blue dye and euthanized; lungs, livers, and hearts were collected and homogenized as previously described.19

Results
CEMs Regulate OxPAPC-Induced Human EC Barrier-Enhancing Events
OxPLs derived from OxPAPC promote a variety of important EC biological functions including vascular barrier enhance-
ECs.13,20 OxPAPC challenge of human ECs induces recruitment of CEMs in OxPAPC-mediated signaling and EC barrier function (Figure 1E). These data demonstrate a crucial involvement of CEMs in OxPAPC-mediated effects including EC barrier function (Figure 1B), Rac1 activation (Figure 1C), phosphorylation of caveolin-1 and the downstream Rac1 effector, PAK1 (Figure 1D), and actin cytoskeletal rearrangements (Figure 1E). These data demonstrate a crucial involvement of CEMs in OxPAPC-mediated signaling and EC barrier function.

OxPAPC Induces S1P1 Receptor Transactivation

Activation of S1P1, which resides in CEMs, regulates EC barrier enhancement.13 Therefore, we examined whether OxPAPC is able to transactivate the S1P1 receptor within CEM structures. Isolation of CEMs from control and OxPAPC-treated human ECs followed by immunoblot analysis revealed that OxPAPC induces recruitment of S1P1 receptor and Rac1 to CEMs and OxPAPC-induced S1P1 receptor transactivation (threonine phosphorylation) within CEMs (Figure 2A and 2B).

We and other have reported that the serine/threonine kinase Akt1 mediates S1P1 receptor transactivation in human ECs.13,20 OxPAPC challenge of human ECs induces recruitment of tyrosine phosphorylated Akt to CEMs, which is abolished with the Src family tyrosine kinase inhibitor protein phosphatase (PP2) (Figure 3A). Silencing Src family members present in human pulmonary ECs21 revealed that Src and Fyn are responsible for OxPAPC-mediated Akt tyrosine phosphorylation (Figure 3B). In addition, Akt1 silencing (siRNA) in human ECs indicate that Akt1 expression is required for OxPAPC-mediated S1P1 receptor threonine phosphorylation (Figure 3C and 3D).

Akt1 and S1P1 Receptor Regulate OxPAPC-Mediated Rac1 Activation

As stated previously, OxPAPC-mediated EC barrier function is dependent on activation of the small G protein Rac1.4,5 Therefore, we examined whether Akt1 and/or the S1P1 receptor are upstream regulators of OxPAPC-mediated Rac1 activation. Silencing of Akt1 (Figure 3A) or the S1P1 receptor (Figure 4A) dramatically decreased OxPAPC-mediated activation of Rac1 (Rac1-GTP formation) in human ECs (Figure 4B).

OxPAPC and S1P1 Receptor Regulate Akt Phosphorylation and EC Barrier Enhancement

Our results in Figures 3 and 4 indicate that Akt1 is tyrosine phosphorylated in CEMs and is required for OxPAPC-mediated S1P1 receptor transactivation and Rac1 activation. Akt is a serine/threonine kinase that is fully activated by serine, threonine, and tyrosine phosphorylation.22–24 Therefore, we examined the contributions of OxPAPC and the S1P1 receptor to the phosphorylation state of Akt. Interestingly, our results in Figure 5A indicate that OxPAPC-mediated tyrosine phosphorylation of Akt (by Src and Fyn) (Figure 3B) is S1P1 receptor-independent. However, serine and threonine phosphorylation of Akt is mediated by mTOR and the phosphatidylinositol (PI3)-kinase pathway and is S1P1 receptor-dependent (Figure 5B). Therefore, our results suggest that partial activation (tyrosine phosphorylation) of Akt appears to be sufficient for S1P1 receptor transactivation. However, full activation (serine, threonine, and tyrosine phosphorylation) of Akt appears to be required for Rac1 activation and EC barrier function (Figures 4 and 5). Measurements of EC barrier function in vitro (transendothelial cell electric resistance) revealed that silencing (siRNA) the expression of Akt1 (Figure 5C) or the S1P1 receptor (Figure 5D) reduced OxPAPC-induced EC barrier enhancement. In addition, silencing Src, Fyn, or mTOR or treatment with S1P1 receptor or PI3-kinase inhibitors attenuated OxPAPC-mediated EC barrier enhancement (Figure 5E).

Caveolin-1 and S1P1 Receptor Regulate OxPAPC-Mediated Protection From Ventilator-Induced Lung Injury

We used a ventilator-induced lung injury (VILI) model to test the role of CEMs and the S1P1 receptor on OxPAPC-mediated protection from vascular hyperpermeability associated with acute lung injury (ALI) in vivo. Our results in Figure 6 indicate that selective lung silencing of caveolin-1 expression (intravenous administration of caveolin-1 siRNA/polyethyleneimine-22 polyplexes18; Figure 6A) blocked OxPAPC-mediated protection from VILI as measured by Evans blue dye leakage (Figure 6B) and bronchoalveolar lavage (BAL) total cell count and protein content (Figure 6C). In addition, using the same procedures as above with S1P1
receptor siRNA indicate that selective pulmonary silencing of the S1P1 receptor blocked OxPAPC-mediated protection from VILI, as measured by BAL total cell count (Figure 7A) and BAL protein content (Figure 7B). These findings indicate the importance of the S1P1 receptor and CEMs in OxPAPC-mediated protection of pulmonary vascular integrity.

**Discussion**

Agents that exhibit the capacity to reverse increases in vascular permeability, a prominent feature in diverse inflammatory syndromes, tumor angiogenesis, and atherosclerosis, have obvious therapeutic applications.25,26 OxPAPC decreases EC permeability both in vitro and in vivo.5-27 As the upstream mechanisms of OxPAPC-mediated GTPase regulation and endothelial barrier protection remain poorly understood, we examined the role of CEMs and S1P1 receptor transactivation in OxPAPC-mediated signaling and human EC barrier regulation. Our novel results indicate that OxPAPC induces partial activation (Src- and Fyn-dependent tyrosine phosphorylation) of Akt, resulting in Akt-mediated S1P1 receptor transactivation (threonine phosphorylation) in CEMs. Activated S1P1 receptor induces full activation (mTOR and PI3-kinase--dependent serine and threonine phosphorylation) of Akt required for Rac1 activation, cortical actin cytoskeletal rearrangement, and consequent OxPAPC-mediated EC barrier enhancement (Figure 8).

CEMs, a subset of lipid rafts containing caveolin-1, have been implicated in EC migration, proliferation, adhesion, endocytosis, cholesterol, and calcium regulation and signal transduction.11,12,28 Deletion of caveolin-1 expression in mice inhibits CEM (caveolae) formation in ECs and promotes lung fibrosis and microvascular hyperpermeability.29 We observed that OxPAPC requires the existence of CEM fractions for Akt-mediated S1P1 receptor transactivation, Rac1 signaling, and EC barrier enhancement. Targeted use of siRNA to differentially reduce expression of either S1P1 or Akt1 revealed that OxPAPC transactivation of S1P1 is responsible for subsequent signaling to the EC cytoskeleton and barrier enhancement.

Our data indicate that Akt activation is required for OxPAPC-mediated EC barrier function. Activation of Akt1 can occur through threonine phosphorylation (T308) in the
catalytic domain by PI3-kinase–dependent PDK-1 and by serine phosphorylation (S\(^{273}\)) in the hydrophobic motif by various kinases including mTOR.\(^{30–33}\) In addition, Akt can be activated by tyrosine phosphorylation by Src family kinases,\(^{34}\) an event we observe with OxPAPC treatment of human ECs. Activated Akt can directly phosphorylate threonine residues within the S1P\(_1\) receptor (T\(^{236}\)),\(^{20}\) which promotes S1P-mediated EC Tiam1/Rac1 activation and cortical actin reorganization and migration. Our data indicate that activated S1P\(_1\) receptor is required for OxPAPC-mediated serine and threonine phosphorylation of Akt1. Because full activation of Akt occurs with serine, threonine, and tyrosine phosphorylation,\(^{30–34}\) our data indicate that Src and Fyn are responsible for OxPAPC-mediated Akt tyrosine phosphorylation, whereas S1P\(_1\) receptor–dependent activation of mTOR and PI3-kinase pathways promote Akt serine/threonine phosphorylation

Using pulmonary targeting of caveolin-1 and S1P\(_1\) receptor siRNA, our data indicate that OxPAPC protection from VILI is dependent on caveolin-1 and S1P\(_1\) expression. Furthermore, our intravenous administration of OxPAPC and intravenous pulmonary targeting of caveolin-1 siRNA suggest OxPAPC-induced protection from VILI act via a preferential endothelial, rather than epithelial, mechanism.\(^{18,35,36}\)

OxPLs play a dual role in vascular inflammation.\(^{4}\) In hyperlipidemic states, OxPLs contained in minimally modified LDL activate monocyte adhesion and transmigration through EC monolayer, stimulate production of chemotactic and inflammatory mediators, promote foam cell formation, and lead to progression of atherosclerotic vascular inflammation.\(^{37,38}\) In turn, various models of acute sepsis or inflammation (lipopolysaccharide [LPS]-induced lung injury, CpG, VILI, necrotizing pancreatitis) show potent antiinflammatory effects of transient OxPAPC elevation via blocking of signaling by nuclear factor κB and stress kinases, and direct barrier-protective effects on vascular endothelium.\(^{27,39–41}\) These reports show that antiinflammatory effects of OxPLs (OxPAPC or OxPAPS) administered at specific doses intravenously, subcutaneously, or intratracheally far exceed potential adverse proinflammatory effects.

Because OxPLs may possess beneficial or detrimental effects under different circumstances, these conditions need to be clearly defined. Protective effects of exogenously oxidized synthetic PAPC include: (1) inhibition of “sterile” ALI and sepsis induced by viral and bacterial derived Toll-like receptor (TLR)4/TLR9 ligands\(^{27,40}\); (2) inhibition of “aseptic” ALI induced by injurious mechanical ventilation\(^{27}\); (3) inhibition of stress signaling, inflammation, and tissue injury in the model of chemically induced acute necrotizing pancreatitis\(^{41}\); and (4) inhibition of dendritic cell maturation,\(^{42}\) which may prevent excessive immune reactions.

One protective mechanism of OxPAPC is via antagonistic interaction with the LPS coreceptors LPS-binding protein and CD14, leading to competitive blockage of the ability of LPS to bind its receptor, TLR4.\(^{43}\) Such inhibition blunts the nuclear factor κB–mediated inflammatory cascade. OxPAPC administration decreased inflammatory cell recruitment and even protected against LPS-mediated lethal shock.\(^{39}\)

In agreement with our previous report using aseptic rat and murine models of VILI,\(^ {27}\) the protective effects reported in this study do not appear to be mediated by an inhibition of LPS action. What are the mechanisms of OxPAPC protective effects in VILI model? One such mechanism involves direct protective effects of OxPAPC on vascular endothelial monolayers and Rac/Cdc42-dependent attenuation of barrier-disruptive Rho signaling.\(^ {4,5}\) Indeed, OxPAPC attenuated Rho pathway of barrier disruption in pulmonary ECs subjected to thrombin and high-magnitude cyclic stretch in vitro, promoted Rac-dependent barrier recovery, and markedly reduced lung barrier dysfunction in mice exposed to high tidal volume mechanical ventilation and Rho activator TRAP (thrombin receptor activating peptide) in vivo.\(^ {27}\) Similar protective effects were achieved by intravenous injection of Rho kinase inhibitor Y-27632.\(^ {27}\) The results of the present study further support this model and strongly suggest a CEM/S1P\(_1\)-
mediated pathway of Rac activation underlying OxPAPC barrier protective effects in vitro and in vivo.

Another possible protective mechanism of OxPAPC in aseptic ALI models including VILI may be inhibition of TLR-mediated inflammatory signaling triggered by endogenous TLR ligands generated in the course of ALI. For example, murine model of bleomycin-induced ALI showed increased generation of low-molecular-mass hyaluronan, which engaged MyD88 and both TLR4 and TLR2 and initiated inflammatory responses in the lung. The following negative OxPAPC effects in acute sepsis and lung injury models were reported: (1) high doses of exogenous OxPAPC caused pulmonary endothelial barrier disruption and increased lung elastance as a parameter of rapid impairment of lung function; (2) products of advanced endogenous phospholipid oxidation such as 4-hydroxy-2-nonenal, ozone-oxidized surfactant phospholipids stimulate inflammatory reactions, cause dysfunction of lung mechanical properties and lung endothelial permeability, and fragmented PAPC oxidation products induce endothelial barrier disruption in vitro; (3) OxPAPC suppressed bacterial phagocytosis and pinocytosis by peritoneal macrophages and neutrophils in the model of bacterial peritonitis, leading to propagation of bacteremia.

A study by Imai et al showed accumulation of oxidized phosphatidylcholine products in human and animal lungs infected with SARS (severe acute respiratory syndrome), anthrax, and H5N1 avian influenza virus and in a mouse model of acid-induced lung injury judged by increased immunoreactivity with monoclonal EO6 antibody. In vitro, BSA-conjugated PAPC oxidation products stimulated interleukin-6 production by alveolar macrophages in a TLR4/TRIF-dependent fashion.

Apparently conflicting reports regarding a role of OxPL in acute inflammation and lung barrier function may be explained by differences in composition and concentrations of OxPLs used in different studies. Immunologic OxPL detection using EO6 antibody has certain limitations. EO6 specifically binds to OxPL containing the PC head group, such as POVP, but not to native nonoxidized PLs. EO6 recognizes products of aldol condensation of PAPC oxidation.

**Figure 5.** OxPAPC and S1P1 receptor regulate Akt phosphorylation and human EC barrier enhancement. A. ECs were either treated with scrambled siRNA or S1P1 receptor siRNA, grown to confluence, serum-starved for 1 hour, and either left untreated (control) or treated with 20 µg/mL OxPAPC for 5 minutes. ECs were then solubilized and analyzed using immunoblotting with anti-phospho-tyrosine (Y326) Akt (a), anti-phospho-serine (S473) Akt (b), anti-phospho-threonine (T308) Akt (c), or anti-Akt (d) antibody. B. Graphic representation of the ratio of tyrosine phosphorylated Akt to total Akt in EC lysates treated with 20 µg/mL OxPAPC (5 minutes) with or without scrambled siRNA, S1P1 receptor siRNA, or S1P1 receptor antagonist W146 (250 nmol/L, 1 hour), mTOR siRNA, or PI3-kinase inhibitor LY294002 (10 µmol/L, 1 hour). C. ECs were plated on gold microelectrodes, treated with scrambled siRNA, or Akt1 siRNA, grown to confluence, serum-starved for 1 hour, and either left untreated (control) or treated with 20 µg/mL OxPAPC. The arrow indicates the time of OxPAPC addition. E. Graphic representation of the percentage maximal OxPAPC-induced transendothelial cell electric resistance (y axis) using siRNA and inhibitors (x axis) as we have described in Figure 3B and Figure 5B through 5D.
products, such as P(POVPC)VPC, diLysoPC-C9, and di-OVPC. This antibody also reacts with Schiff bases forming covalent bonds between protein lysine residues and aldehyde groups of fragmented OxPLs such as POVPC-BSA. Therefore, EO6 does not discriminate between fragmented and oxygenated products of PAPC oxidation, and specific profiles of OxPLs generated in various ALI models remain to be determined.

OxPL exhibits dose-dependent, biphasic effects on the endothelial permeability. Low OxPL concentrations protect endothelial barrier, whereas high concentrations of the same OxPLs induced barrier-disruptive effects. Previous in vitro studies have described caveolar disruption with Ox-PAPC at 50 μg/mL. Because the present studies demonstrate that the S1P1 transactivation leading to EC barrier enhancement depends on intact caveolae, such caveolae disruption at high OxPAPC concentrations may help explain barrier-disruptive effects of OxPAPC at higher concentrations.

A similar explanation can be applied to the animal models. The OxPAPC doses used for intratracheal instillation by Imai et al (20 μg/g body weight) were 5 to 10 times higher compared to the protective doses of intratracheal OxPAPC against of LPS- and CpG DNA–induced ALI. Whether these OxPL treatments quantitatively and qualitatively represent endogenous OxPL generation during acid-, SARS-,
anthrax-, or H5N1 influenza–induced lung injury in vivo is a subject of further studies. It also appears that, if administered intravenously, even higher OxPAPC doses (up to 40 mg/kg) may be well tolerated and exhibit protective effects in animal models of LPS-induced lung injury, VILI, and acute necrotizing pancreatitis.

Thus, careful analysis of tissue OxPL levels using more elaborate techniques such as mass spectrometry is essential for precise characterization of the composition and amounts of endogenous OxPL generated in different pathological conditions. These studies will allow better understanding of the OxPL role in the pathogenesis of ALI.

In summary, although putative receptor(s) mediating OxPAPC barrier protection remain elusive, we now show that OxPAPC-mediated Rac1 activation, cortical actin rearrangement, and barrier regulation are critically dependent on S1P1 transactivation within CEMs. The recruitment of S1P1, Akt1, and Rac GTPases within CEM fractions may be a common feature of barrier enhancing stimuli. These results further indicate that OxPAPC may serve as a potentially useful therapeutic treatment for diseases characterized by high permeability states.

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**Disclosures**

None.

**References**


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Materials and Methods

Cell Culture and Reagents – Human pulmonary artery EC were obtained from Clonetics (Walkersville, MD) and cultured as previously described in EBM-2 complete medium (Clonetics) at 37°C in a humidified atmosphere of 5% CO₂, 95% air, with passages 6-10 used for experimentation. Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Reagents for SDS-PAGE electrophoresis were purchased from Bio-Rad (Richmond, CA), Immobilon-P transfer membrane from Millipore (Millipore Corp., Bedford, MA), and gold microelectrodes from Applied Biophysics (Troy, NY). Rabbit anti-caveolin-1 antibody and rabbit anti-phospho-tyrosine caveolin-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-S1P₁ receptor antibody was purchased from Affinity Bioreagents (Golden, CO). Anti-Akt and anti-phospho-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-Rac1 antibody, mouse anti-phospho-tubulin and mouse anti-phospho-PAK1 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Texas Red-conjugated phallodin was purchased from Molecular Probes (Eugene, OR). LY294002 was purchased from EMD Chemicals (Gibbstown, NJ). The S1P₁ Antagonist, W146, (R)-3-Amino-4-(3-hexylphenylamino)-4-oxobutylphosphonic acid, was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).
**Lipid Oxidation and Analysis** - Non-oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) was obtained from Avanti Polar Lipids (Alabaster, AL). PAPC was oxidized by exposure to air for 72 h. The extent of oxidation was measured by positive ion electrospray mass spectrometry (ESI-MS) as previously described.\(^2\) After completion of oxidation, the phospholipids were stored at -70°C dissolved in chloroform and used within 2 weeks after mass spectrometry testing. All oxidized and non-oxidized phospholipid preparations were analyzed by the limulus amebocyte assay (BioWhittaker, Frederick, MD) and shown negative for endotoxin.

**Caveolin-enriched Microdomain (CEM) Isolation** – CEMs were isolated from human lung EC as we have previously described.\(^3\,^4\,^5\) Briefly, EC were scraped in PBS, centrifuged at 2,000 rpm at 4°C and lysed with 0.2 ml of TN solution [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, protease inhibitors, 10% sucrose, 1% Triton X-100] for 30 min on ice. Triton X-100-insoluble materials were then mixed with 0.6 ml of cold 60% Optiprep™ and overlaid with 0.6 ml of 40%, 30% and 20% Optiprep™ in TN solution. The gradients were centrifuged at 35,000 rpm in SW60 rotor for 12 h at 4°C and different fractions were collected. Cellular proteins or lipids associated with each fraction were precipitated according to the procedures described previously and analyzed by SDS-PAGE plus immunoblotting and/or immunoprecipitation.
Immunoprecipitation and Immunoblotting – Cell lysates and/or cellular materials associated within the 20% Optiprep™ fractions (CEM fraction) were incubated with IP buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% Triton X-100, 0.1% SDS, 0.4 mM Na₃VO₄, 40 mM NaF, 50 μM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3). The samples were then immunoprecipitated with anti-S1P₁ receptor followed by SDS-PAGE in 4-15% polyacrylamide gels, transfer onto Immobilon™ membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Construction and Transfection of siRNA against S1P₁ and Akt1 - The siRNA sequence(s) targeting human S1P₁ and Akt1 were generated using mRNA sequences from Gen-Bank™ (gi:13027635 and gi:62241010 respectively) as we have previously described. For each mRNA (or scramble), two targets were identified. Specifically, S1P₁ target sequence 1 (5'-AAGCTACACAAAAAGCCTGGA-3’), S1P₁ target sequence 2 (5’-AAAAAGCCTGGATCACTCATC-3’), Akt1 target sequence 1 (5’-AATTATGGGTCTGTAACCACC-3’), Akt1 target sequence 2 (5’-AAATGAATGAACCAGATTCATC-3’), scrambled sequence 1 (5’-AAGCTACACAAAAAGCCTGGA-3’), and scramble sequence 2 (5’-AAGAGAAATCGAAACCGAAAA-3’) were utilized. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies, Inc.
Supplement Material

construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). Human lung EC were then transfected with siRNA using siPORTamine™ as the transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~ 40% confluent) were serum-starved for 1 hour followed by incubated with 3 μM (1.5 μM of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 hours in serum-free media. The serum-containing media was then added (1% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted. The siRNA for Src, Fyn, Yes, Lyn, Blk, mTOR and S1P1 siRNA 2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were utilized according to the manufacturer's specifications.

Determination of threonine phosphorylation of S1P₁ – Solubilized CEM proteins in IP buffer (see above) were immunoprecipitated with rabbit anti-S1P₁ antibody followed by SDS-PAGE in 4-15% polyacrylamide gels and transfer onto Immobilon™ membranes (Millipore Corp., Bedford, MA). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-S1P₁ antibody or rabbit anti-phospho-threonine antibody followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).
Rac1 Activation Assay – Rac activity in human lung EC was performed as previously described.³

Measurement of EC Electrical Resistance – EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and TER measurements performed using an electrical cell-substrate impedance sensing system obtained from Applied Biophysics (Troy, NY) as previously described in detail.¹ TER values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± S.E.

Immunofluorescence Microscopy – Polymerized actin rearrangement was assessed with Texas Red-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) and analyzed using a Nikon Eclipse TE 300 microscope as we have described.¹,³

Delivery of siRNA in Mice - Adult male C57BL/6J mice, 8-10 weeks old, with average weight 20-25 grams (Jackson Laboratories, Bar Harbor, ME) were bred at University of Chicago animal care center. All experimental protocols involving the use of animals were approved by the University of Chicago Institutional Animal Care & Use Committee for the humane treatment of experimental animals. siRNAs from Dharmacon (Lafayette, CO) had the following sequences - siCaveolin1: 5'-ACGUAGACUCCGAGGGACAUU-3'; siS1P₁ receptor: 5'-CUUGCUAACUAUUUGGAAA-3'; control siRNA (Luciferase): 5'-UAAGGCUAUGAAGAGAUA-3'. Polyethylenimine-22 (PEI22) that provides
preferential RNA targeting to the lung\textsuperscript{6} was used as a carrier in the \textit{in vivo} experiments with siRNA-induced caveolin and S1P\textsubscript{1} receptor knockdown \textit{in vivo}. PEI22/siRNA ratio (N/P=10:1) used in these experiments represents ratio of PEI22 nitrogen to RNA phosphate. Required amounts of PEI22 were brought to 200 µl with 5% aqueous glucose and added to the equal volume of the glucose solution containing corresponding amounts of siRNA to reach 4 mg/kg, 6 mg/kg and 10 mg/kg siRNA dosage \textit{in vivo}. The resulting polyplexes were incubated at room temperature for 10 min. Obtained PEI22-siRNA polyplexes (400 µl) were injected into jugular vein of 8- to 10-week-old C57BL/6 male mice, with weight of 20-25 grams (Jackson Laboratory, Bar Harbor, ME) under anesthesia. After 72 h, the mice were sacrificed; their lungs, livers and hearts were collected and homogenized in 1 ml of SDS lysis buffer containing protease inhibitor cocktail set III (Calbiochem, NJ).

\textit{Mechanical Ventilation of Mice} - After 72 h of siRNA delivery in mice with siCaveolin or nsRNA at dose of 6 mg/kg, mice were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg). Tracheotomy was performed and the trachea was cannulated with a 20-gauge one inch catheter (Penn-Century Inc., Philadelphia, PA), which was tied into place to prevent air leak. The animals were placed on mechanical ventilator (Harvard Apparatus, Boston, MA) for 4 hours with high tidal volume (30 ml/kg, 75 breaths per minute and 0 PEEP, HTV) ventilation. Mice were randomized to concurrently receive sterile saline solution or OxPAPC (1.5 mg/kg, i.v. via jugular vein) to yield 4 groups: nsRNA VILI, nsRNA VILI + OxPAPC, siRNA VILI and
siRNA VILI + OxPAPC. After the experiment, animals were sacrificed by exsanguination under anesthesia. BAL was performed using 1 ml of sterile Hanks Balanced Salt Buffer or not for histological examination. The BAL protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific Inc, IL). BAL inflammatory cell count was performed with a hemacytometer.

Assessment of Pulmonary Vascular Leakage by Evans Blue - Two hours prior to the termination of HTV, Evans blue was injected intravenously at dose of 30 mg/kg. At the end of ventilation, thoracotomy was performed, and the lungs were perfused free of blood with PBS containing 5 mM EDTA. Both left lung and right lung were excised and imaged by Kodak digital camera. After imaging lungs were blotted dry, weighed and homogenized in PBS (1 ml/100 µg tissue). Homogenized tissue was incubated with 2 volume formamide (18 h, 60°C), centrifuged at 12,000 g for 20 min. Optical density of the supernatant was determined by spectrophotometry at 620 nm and 740 nm. Extravasated EBD concentration (micrograms of Evans blue dye per g lung) in lung homogenates was calculated against a standard curve. Mean value of nsRNA VILI group was considered as 100% injury, the other three groups injury were assessed by the ratios of mean values to the mean value of nsRNA VILI group.
References


