GRP78 is a novel receptor initiating a vascular barrier protective response to oxidized phospholipids

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Abbreviations used: BAL, bronchoalveolar lavage; CEM, caveolin-enriched microdomain; DMPS, oxidation-resistant phosphatidyl serine; EC, endothelial cell; ER, endoplasmic reticulum; HPAEC, human pulmonary artery endothelial cells; HTV, mechanical ventilation at high tidal volume; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; OxPAPS, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine; OxPL, oxidized phospholipid; S1P-R1, sphingosine 1-phosphate receptor 1; TER, transendothelial electrical resistance.

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ABSTRACT Vascular integrity and the maintenance of blood vessel continuity are fundamental features of the circulatory system maintained through endothelial cell–cell junctions. Defects in the endothelial barrier become an initiating factor in several pathologies, including ischemia/reperfusion, tumor angiogenesis, pulmonary edema, sepsis, and acute lung injury. Better understanding of mechanisms stimulating endothelial barrier enhancement may provide novel therapeutic strategies. We previously reported that oxidized phospholipids (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine [OxPAPC]) promote endothelial cell (EC) barrier enhancement both in vitro and in vivo. This study examines the initiating mechanistic events triggered by OxPAPC to increase vascular integrity. Our data demonstrate that OxPAPC directly binds the cell membrane–localized chaperone protein, GRP78, associated with its cofactor, HTJ-1. OxPAPC binding to plasma membrane–localized GRP78 leads to GRP78 trafficking to caveolin-enriched microdomains (CEMs) on the cell surface and consequent activation of sphingosine 1-phosphate receptor 1, Src and Fyn tyrosine kinases, and Rac1 GTPase, processes essential for cytoskeletal reorganization and EC barrier enhancement. Using animal models of acute lung injury with vascular hyperpermeability, we observed that HTJ-1 knockdown blocked OxPAPC protection from interleukin-6 and ventilator-induced lung injury. Our data indicate for the first time an essential role of GRP78 and HTJ-1 in OxPAPC-mediated CEM dynamics and enhancement of vascular integrity.

INTRODUCTION The effects of oxidized phospholipids (OxPLs) described in vitro and in vivo demonstrate their relevance to different pathologies, acute inflammation, ischemia/reperfusion injury, atherosclerosis, lung injury, and many other conditions (Bochkov et al., 2010). Besides their role as pathogenic factors, full-length products of phospholipid oxidation, such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC) and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoserine (OxPAPS), exhibit potent barrier-enhancing and anti-inflammatory effects in vitro and in vivo (Walton et al., 2003; Birukova et al., 2007a; Oskolkova et al., 2010). Single intravenous injection of OxPAPC in animal models inhibited lung inflammation and lung vascular leak induced by intratracheal administration of bacterial lipopolysaccharide (Ma et al., 2004; Nonas et al., 2006) or mechanical ventilation at high tidal volume (HTV; Nonas et al., 2008).
We described potent barrier-enhancing effects of OxPAPC on vascular endothelium. The signaling mechanism involved recruitment of sphingosine 1-phosphate receptor-1 (S1P-R1) to the caveolin-enriched microdomains (CEMs, also known as lipid rafts or caveolae) and activation of serine/threonine kinases mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K) and Rac-specific nucleotide exchange factors Tiam1 and Vav2, which stimulated Rac1 GTPase signaling to cytoskeleton and cell junctions (Singleton et al., 2009; Birukova et al., 2011). A number of OxPL receptors (PAF receptor, CD36 scavenger receptor Bl, prostaglandin receptor EP2, vascular endothelial growth factor (VEGF) receptor-2, S1P-R1) have been identified that mediate different cellular effects of OxPLs, such as modulation of innate immunity, activation of anti-inflammatory gene expression, VEGF expression and signaling, expression of proatherosclerotic factors, proangiogenic effects, and increased permeability and OxPL uptake (Ishii et al., 2003; Bluml et al., 2005; Salomon, 2005; Imai et al., 2008; Bochkov et al., 2010). However, the primary receptor(s) mediating OxPAPC-induced endothelial cell (EC) barrier enhancement remain elusive.

GRP78 is a multifunctional regulator of endoplasmic reticulum (ER) homeostasis and stress response. It was initially identified as a key component of the cellular stress pathway induced by accumulation of unfolded proteins. GRP78 is mainly located in the ER, where it binds denatured or incorrectly folded polypeptides and initiates a cascade of protective reactions helping the cell to survive under conditions of stress induced by protein overload (He et al., 2011), viral infection (Shi-Chen Ou et al., 2011; Thongtan et al., 2012), and so on. Alterations in GRP78 tissue levels or activity have been implicated in cancer, immune regulation, aging, Alzheimer disease, and other diseases associated with ER stress (Misra et al., 2005; Pfaffenbach and Lee, 2011; Wei et al., 2012; Soejima et al., 2013).

HTJ-1, or its mouse homologue MTJ-1, is a partner of GRP78 in ER protein quality control (Chevalier et al., 2000). In addition to its role as an ER-localized cochaperone involved in the unfolded protein response, a second important function of HTJ-1/MTJ-1 is the translocation and anchoring of GRP78 to the cell plasma membrane, where GRP78 assumes a novel role. Cell surface–expressed GRP78 functions as a signal-transducing receptor or coreceptor for soluble ligands such as C6-macroglobulin (Misra et al., 2005), tumor differentiation factor (Sokolowska et al., 2012), and vasoconstriction (Nakatsuka et al., 2012), as well as for glycosylphosphatidylinositol–anchored proteins—for example, T-cadherin (Philippova et al., 2008) and teratocarcinoma-derived growth factor 1 (Cripto; Shani et al., 2008). The present study examines a role of surface-expressed GRP78 as an initiator of the OxPAPC-mediated barrier-enhancing signaling cascade in vitro and in vivo. Our data support the notion that GRP78 functions as a receptor for OxPLs that is critically important for their barrier-protective effects.

RESULTS
Inhibition of the cell membrane–associated GRP78 pool attenuates OxPAPC-induced EC barrier enhancement and F-actin remodeling

Inhibition of cell membrane–associated GRP78 was achieved by EC preincubation with GRP78- blocking antibody or by small interfering RNA (siRNA)–induced knockdown of GRP78 cofactor, HTJ-1, essential for GRP78 translocation to the cell membrane (Chevalier et al., 2000). Because cytosolic and endoplasmic reticulum–associated pools of GRP78 are important for other cell functions (Li and Lee, 2006; Wei et al., 2012), global knockdown of GRP78 was not used in this study. GRP78-blocking antibody binds the extracellular C-terminal GRP78 domain and blocks its interactions with extracellular ligands (Philippova et al., 2008; Misra et al., 2009). This antibody-attenuated increase in transendothelial electrical resistance (TER) reflects the OxPAPC-induced barrier enhancement response, whereas control immunoglobulin G (IgG) antibody is without effect (Figure 1A). Similarly, inhibition of GRP78 targeting to the cell membrane by HTJ-1 knockdown abolished OxPAPC-induced barrier enhancement (Figure 1B). In control experiments, HTJ-1 knockdown did not affect EC barrier enhancement induced by the prostacyclin stable analogue iloprost (Figure 1C), known to act via a distinct pathway mediated by prostacyclin receptor IP (Bos et al., 2004).

These results demonstrate specific involvement of the HTJ-1–GRP78 mechanism in the OxPAPC-induced EC barrier enhancement. OxPAPC-induced changes in TER were associated with increased EC monolayer barrier properties reflected by decreased penetration of fluorescein isothiocyanate (FITC)–labeled avidin through intercellular junctions, as measured by XPerT permeability assay (Dubrovskiy et al., 2013; Figure 1EF). This EC barrier enhancement response was attenuated by HTJ1 depletion.

GRP78–HTJ-1 association is stimulated by OxPAPC

GRP78 and HTJ-1 are expressed in microvascular and macrovascular endothelial cells (Figure 2A). Stimulation with 10 μg/ml OxPAPC increased GRP78–HTJ-1 association in cells, as determined by coimmunoprecipitation assay (Figure 2B). Of note, the oxidation-resistant phospholipid dimyristoylphosphatidylcholine (DMPC) had no effect on GRP78–HTJ-1 association.

GRP78 localizes at cell membrane and directly interacts with OxPAPC and OxPAPS

The foregoing experiments suggested the role of GRP78 in initiating the OxPAPC–induced EC cytoskeletal remodeling and barrier response. Direct interaction between GRP78 and OxPLs was tested by several approaches. First, cells preincubated with OxPAPC or nonoxidized control (DMPC) were used for immunoprecipitation with anti-OxPAPC antibody EO6 (Friedman et al., 2002). Cell incubation with OxPAPC significantly increased GRP78 content in EO6 immunoprecipitates (Figure 2C). In the lipid native gel mobility shift assay, interaction with phospholipid bearing a phosphoserine polar head group will increase net protein negative charge, leading to increased electrophoretic mobility of the GRP78 x phospholipid complex under nondenaturing conditions. GRP78 preincubation with OxPAPS increased electrophoretic mobility of GRP78 (Figure 2D, left). Preincubation with oxidation-resistant phosphatidyl serine (DMPS) did not cause a GRP78 mobility shift. Monoclonal antibody EO6 (Horkko et al., 1999) recognizes OxPAPC but not OxPAPS or nonoxidized PAPC species and may be a useful tool for detection of OxPAPC complexes. Purified GRP78 was preincubated with control buffer, OxPAPC, OxPAPS, or their respective nonoxidizable analogues, DMPC and DMPS. Immunoblotting with EO6 monoclonal antibody, which reacts with oxidized phosphatidyl choline species (Friedman et al., 2002), revealed strong immunoreactivity of purified recombinant GRP78 preincubated with OxPAPC but not with any other phospholipid (Figure 2D, right).

Interaction of recombinant GRP78 with phospholipids immobilized on plastic was further tested by enzyme-linked immunosorbent assay (ELISA). Because test experiments showed low adsorption of OxPAPC to polystyrene plates (unpublished data), for ELISAs, we used OxPAPC, which, similarly to OxPAPC, causes a barrier-protective response in pulmonary EC (Birukova et al., 2007a). The oxidation-resistant PAPS analog DMPS was used as negative control. Human recombinant GRP78 efficiently interacted with OxPAPS but not with DMPS (Figure 2E, left). Similar experiments were performed...
cell surface location was further verified by in situ biotinylation of cell surface proteins in control and OxPAPC-challenged EC, and the level of biotinylated GRP78 was assessed by Western blot. Increased GRP78 biotinylation was observed in cells treated with OxPAPC (Figure 3B). Increased levels of biotinylated VE-cadherin also illustrate OxPAPC-induced activation of VE-cadherin surface expression and adherens junction dynamics. EC treatment with 10 μg/ml OxPAPC did not affect the cell surface presentation of VEGF receptor-2 (VEGFR2). In complementary experiments, the CEMs (lipid rafts) were isolated from control and OxPAPC-stimulated EC after cell surface biotin labeling, followed by GRP78 immunoprecipitation. OxPAPC treatment increased the levels of biotinylated GRP78 detected in GRP78 precipitates (Figure 3C).

on plates with immobilized OxPAPS incubated with EC lysates containing endogenous GRP78. GRP78 immunoreactivity was significantly higher in OxPAPS-containing wells incubated with cell lysate (Figure 2E, middle). Similar to results of ELISA with purified protein, GRP78 endogenously expressed in endothelial cells interacted with immobilized OxPAPS but not with DMPS (Figure 2E, right). Together these data demonstrate the direct interaction of OxPAPS and OxPAPC with GRP78. GRP78 cell membrane localization was tested by an immunocytochemistry approach. Plasma membrane was counterstained with Alexa 576-conjugated wheat germ agglutinin. Confocal microscopy analysis showed that OxPAPC promoted GRP78 accumulation within the plasma membrane (Figure 3A). The OxPAPC-induced GRP78 cell surface location was further verified by in situ biotinylation of cell surface proteins in control and OxPAPC-challenged EC, and the level of biotinylated GRP78 was assessed by Western blot. Increased GRP78 biotinylation was observed in cells treated with OxPAPC (Figure 3B). Increased levels of biotinylated VE-cadherin also illustrate OxPAPC-induced activation of VE-cadherin surface expression and adherens junction dynamics. EC treatment with 10 μg/ml OxPAPC did not affect the cell surface presentation of VEGF receptor-2 (VEGFR2). In complementary experiments, the CEMs (lipid rafts) were isolated from control and OxPAPC-stimulated EC after cell surface biotin labeling, followed by GRP78 immunoprecipitation. OxPAPC treatment increased the levels of biotinylated GRP78 detected in GRP78 precipitates (Figure 3C).
accumulation of GRP78 and HTJ-1 (Figure 4A). SiRNA-induced knockdown of HTJ-1 abolished GRP78 recruitment and assembly of S1P-R1 signaling complex in the CEM (Figure 4B). Of importance, siRNA-based knockdown of caveolin-1 or disruption of the CEM by methyl-β-cyclodextrin (MβCD) inhibited GRP78 and HTJ-1 translocation after OxPAPC treatment (Figure 4C). Of note, the endoplasmic reticulum markers calnexin and calreticulin were not detected in the CEM preparation.

We further examined the mechanism by which GRP78 initiates S1P-R1 transactivation. CEM disruption by cell pretreatment with MβCD abolished OxPAPC-induced phosphorylation of S1P-R1 at Thr-236, which reflects its activation (Lee et al., 2001) but did not

Accumulation of S1P-R1 in lipid rafts and its transactivation by Src family kinases Src and Fyn triggers the Rac-dependent mechanism of EC barrier enhancement by OxPAPC (Singleton et al., 2009). However, the upstream mechanism that triggers an S1P-R1 signaling complex assembly by OxPAPC is unknown. We tested a role of OxPAPC-GRP78/HTJ-1 interaction as a molecular switch leading to S1P-R1 transactivation and EC barrier response. Cell stimulation with OxPAPC induced recruitment of S1P-R1 and phosphorylated Src, Fyn, and Rac1 to CEM, which was also accompanied by accumulation of GRP78 and HTJ-1 (Figure 4A). SiRNA-induced knockdown of HTJ-1 abolished GRP78 recruitment and assembly of S1P-R1 signaling complex in the CEM (Figure 4B). Of importance, siRNA-based knockdown of caveolin-1 or disruption of the CEM by methyl-β-cyclodextrin (MβCD) inhibited GRP78 and HTJ-1 translocation after OxPAPC treatment (Figure 4C). Of note, the endoplasmic reticulum markers calnexin and calreticulin were not detected in the CEM preparation.

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showed inhibition of OxPAPC-induced Rac activation in cells with HTJ-1 knockdown (Figure 5B).

Activation of Rac1 induces cortactin localization in lamellipodia and podosomes (Head et al., 2003) and regulates actin dynamics. In turn, impaired reestablishment of EC monolayer integrity in cells with inhibited Rac1 is accompanied by suppressed cortactin peripheral localization and decreased cortactin tyrosine phosphorylation (Birukova et al., 2012a). siRNA-induced knockdown of Rac-specific guanine nucleotide exchange factors Tiam1 and βPIX abolished OxPAPC-induced activation of Rac and its effector kinase PAK1 and attenuated OxPAPC-induced peripheral translocation of cortactin, peripheral actin cytoskeletal enhancement and EC barrier protective response (Birukova et al., 2007b). These data demonstrate a role of OxPAPC-induced cortactin phosphorylation and peripheral translocation in the mechanisms of OxPAPC-induced EC barrier enhancement. HTJ-1 knockdown (Figure 5C) or cell pretreatment with GRP78-blocking antibody (Figure 5D) attenuated OxPAPC-induced site-specific phosphorylation of the Src and Rac targets PAK1 and cortactin.

Enhancement of the cortical actin cytoskeleton is essential for barrier-protective effects of OxPAPC (Birukova et al., 2007b). Cortactin is an actin-binding protein that mediates Rac-dependent cortical actin polymerization (Weed and Parsons, 2001), and its accumulation at the cell periphery indicates formation of new F-actin networks. Pulmonary EC expressing green fluorescent protein (GFP)-tagged cortactin were depleted of HTJ-1 using specific siRNA, and OxPAPC-induced peripheral accumulation of GFP-cortactin reflecting cytoskeletal enhancement was monitored using live-cell microscopy. OxPAPC stimulation of control EC treated with nonspecific siRNA caused peripheral accumulation of cortactin, whereas depletion of HTJ-1 by specific siRNA abolished this effect (Figure 6B). OxPAPC-induced activation of peripheral cortactin dynamics was linked to enlargement of the peripheral F-actin rim (Figure 6C). Knockdown of HTJ-1 (Figure 6C, top) or incubation with GRP78 blocking antibody (Figure 6C, bottom) prevented OxPAPC-induced cytoskeletal remodeling.

Knockdown of mouse HTJ-1 homologue (MTJ-1) attenuates OxPAPC’s protective effects in animal models of acute lung injury

Inhibition of GRP78 membrane signaling by the knockdown of MTJ-1, the mouse homologue of HTJ-1, blunted lung-protective effects of OxPAPC in animal models of acute lung injury (ALI). Endogenous MTJ-1 was depleted in mouse lungs using the siRNA approach. Lung-specific knockdown of MTJ-1 was confirmed by real time (RT)-PCR analysis of lung samples and Western blot analysis of lung, heart, liver, and kidney tissues (Figure 7A). In the interleukin-6
At 24 h, collection of bronchoalveolar lavage (BAL) fluid was performed as described in Materials and Methods. IL-6 instillation significantly increased BAL protein concentration and cell count in untreated controls or mice treated with nonspecific RNA (Figure 7, B and C). These parameters were attenuated by OxPAPC administration (Figure 7B). In contrast, MTJ-1 knockdown abolished protective effects of OxPAPC against protein and cell accumulation in the BAL of the IL-6–treated group (Figure 7C). The effects of OxPAPC on the IL-6–induced lung vascular leak in control and MTJ1 knockdown mice were also evaluated by measurements of Evans blue extravasation into the lung tissue. OxPAPC significantly reduced IL-6–induced Evans blue accumulation in the lung parenchyma. MTJ1 knockdown abolished protective effects of OxPAPC against vascular leak caused by IL-6 (Figure 7D). In an alternative model of ALI induced by HTV (Nonas et al., 2008), control and si-MTJ-1–treated mice were exposed to HTV (30 ml/kg, 4 h) with or without OxPAPC administration. Measurements of BAL protein concentration and cell count in HTV-subjected animals showed no significant difference between nonspecific and si-MTJ-1–transfected groups (Figure 7E), whereas depletion of MTJ-1 abolished protective effects of OxPAPC against HTV-induced increases in BAL cell count and protein concentration as compared with controls transfected with nonspecific siRNA (Figure 7E).

**DISCUSSION**

A number of cell receptors interacting with oxidized phospholipids have been identified and shown to be involved in various cell responses, including proinflammatory and anti-inflammatory effects, immunity, angiogenesis, activation of gene expression, and lipid metabolism (Bluml et al., 2005; Salomon, 2005; Bochkov et al., 2010; Lee et al., 2012). However, receptor(s) mediating barrier-protective effects of low OxPAPC doses remain elusive. Here we present the first report of a heat shock protein family member as a cell surface receptor mediating signaling by oxidized phospholipids.

Traditionally, GRP78 function as a molecular chaperone was associated with recognition of misfolded polypeptides, leading to stimulation of GRP78 activity as molecular chaperone and initiation of the unfolded protein response (Li and Lee, 2006; Dudek et al., 2009). Alternative cell surface localization of GRP78 was documented by immunofluorescence staining and cell surface biotinylation (Philippova et al., 2008). However, GRP78 has never been considered as a mediator of phospholipid signaling.
OxPAPC triggered HTJ-1–assisted accumulation of GRP78 at the cell membrane, which turned on GRP78/HTJ-1–mediated assembly of other components of the S1P-R1 signaling complex and their recruitment to the CEM, which provided full activation of S1P-R1 and downstream Rac pathway of EC barrier enhancement. OxPAPC binding to EC plasma membrane–resident GRP78 promoted recruitment of GRP78 to CEM. This induced cytosolic HTJ1/GRP78 complexes to be recruited to the plasma membrane and led to further recruitment of GRP78 to CEM, which potentiated transactivation of S1P-R1 and led to EC barrier enhancement. Although we have not elucidated the complete mechanism(s) of how plasma membrane–activated GRP78 recruits cytosolic GRP78 to the plasma membrane, our results demonstrate that HTJ1 is crucial for this process. Precise mechanisms of signal complex assembly in the CEM driven by OxPAPC–activated GRP78 require further investigation, but these data strongly suggest that in addition to acting as a molecular chaperone, GRP78 regulates OxPAPC cytoskeletal remodeling and EC barrier function by assembling the S1P-R1–Akt–mTOR–Tiam1/Vav-2–Rac1 signaling complex.

Although we cannot exclude additional mechanisms of OxPLs barrier-protective effects—for example, disturbance of lipid bilayer structure or GRP78/HTJ-1 interactions with other receptors involved in EC barrier regulation—stable physical binding between GRP78 and OxPLs, which was demonstrated by different approaches (Figure 2), strongly supports the notion that GRP78 is a bona fide signaling receptor for OxPLs. We speculate that “hijacking” of the sphingosine-1-phosphate signaling mechanism by OxPAPC via prolonged transactivation of the S1P-R1 receptor defines the sustained endothelial barrier–enhancing response.

Potent anti-inflammatory effects of OxPAPC in models of acute inflammation have been associated with inhibition of inflammatory signaling induced by toll-like receptor 4 (TLR4) and TLR9. Administration of OxPAPC decreased inflammatory cell recruitment and even protected against endotoxin-induced lethal shock in animal models of acute inflammation (Bochkov et al., 2002; Ma et al., 2004; Nonas et al., 2006; Erridge et al., 2008; von Schlieffen et al., 2009; Oskolkova et al., 2010). This study is the first demonstration of the GRP78/HTJ-1–dependent mechanism of vascular barrier protection in two models of lung injury not directly associated with TLR receptor activation. Taken together, these findings expand our understanding of the role of OxPLs in innate immunity and control of tissue inflammation and show that OxPAPC activates at least two independent anti-inflammatory mechanisms—suppression of TLR-dependent inflammatory signaling (Bochkov et al., 2002; Walton et al., 2003) and GRP78–S1P-R1 mediated vascular barrier protection (present study).

In summary, we propose a mechanism of OxPAPC-induced EC barrier enhancement via GRP78/HTJ-1 acting as an OxPAPC/OxPAPS receptor (Figure 8). OxPAPC directly interacts with GRP78, which leads to membrane accumulation of the GRP78/HTJ-1 complex and its targeting to the CEM. Activated complex triggers Src/Fyn kinase activation, leading to assembly of Akt–PI3K–mTOR–S1P-R1 signalosome and transactivation of S1P-R1, which in turn activates the downstream Rac pathway, cortical actin cytoskeletal remodeling, and enhancement of the EC barrier. This study demonstrates a novel role of GRP78/HTJ-1 as a receptor mediating barrier-protective effects of oxidized phospholipids and engaged in assembly of CEM–associated signalosome, which is critical for maintenance of cell barrier homeostasis. These results provide a basis for further investigation into the roles of membrane-associated molecular chaperones in normal and pathological cell signaling.

MATERIALS AND METHODS
Reagents and cell culture
Unless specified, biochemical reagents were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies to phosphocortactin and cortactin were from Millipore (Billerica, MA); GRP78, VE-cadherin, calnexin, calreticulin, and HTJ-1 were from Santa Cruz.
Plasmid encoding GFP-tagged cortactin was from Addgene (Cambridge, MA). For in vivo experiments, a predesigned MTJ-11–specific mouse siRNA set of standard purity was purchased from Ambion. Polymer-based administration of nonspecific or MTJ-11–specific siRNA conjugated with polycation polyethyleneimine (PEI-22) was shown to promote lung-specific DNA and siRNA delivery (Thomas et al., 2005a,b) and was used as described in our previous studies (Singleton et al., 2009; Birukova et al., 2010b). The optimal concentration of siRNA was determined in the following series of preliminary experiments. PEI-22-siRNA polyplexes were formed at a ratio of 1:10 (1 μg of siRNA/10 μg of PEI-22), and siPAK1 was tested in the 0.1- to 5.0-mg/kg dose range. SiRNA at 1–4 mg/kg showed the most significant inhibition of the target gene after 72 h of transfection, as determined by RT-PCR analysis of lung tissues or Western blot analysis (Figure 6A). Treated mice showed no signs of nonspecific siRNA-induced inflammation. Nonspecific, nontargeting siRNA (Dharmacon, Lafayette, CO) was used as a control treatment for both in vitro and in vivo experiments.

**Lipid oxidation and analysis**
Nonoxidized PAPC was obtained from Avanti Polar Lipids. PAPC was oxidized by exposure to air for 36 h. The extent of oxidation was monitored by positive-ion electrospray mass spectrometry. Phospholipids were overlaid with argon and stored at −70°C.

**siRNA and DNA transfections**
Transfection of EC with siRNA was performed as previously described (Birukova et al., 2010a). Predesigned HTJ-11– and MTJ-11–specific siRNAs of standard purity were purchased from Ambion (Austin, TX). After 72 h of transfection, cells were used for experiments. Transient transfection was performed using PolyJet reagent (SignaGen, Rockville, MD) according to the manufacturer’s instructions. After 48 h of transfection, cells were treated with either vehicle or OxPAPC and used for live-imaging analysis.

Biotechnology (Santa Cruz, CA); phospho-PAK1, phospho-Src, Src, Fyn, caveolin-1, and VEGFR2 antibodies were from Cell Signaling (Beverly, MA); GRP78 and Rac1 were from BD Transduction Laboratories (San Diego, CA), EO6 monoclonal antibody recognizing oxidized phosphatidyl choline epitope was from Avanti Polar Lipids (Alabaster, AL). Human pulmonary artery endothelial cells (HPAECs) and human lung microvascular endothelial cells were obtained from Lonza (Allendale, NJ).

**Transendothelial electrical resistance**
TER across confluent human pulmonary artery endothelial monolayers was measured using an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY).
were then centrifuged, the supernatant was removed, and precipitated proteins were eluted with 0.75 M \( N\)-acetylglucosamine containing 0.1% NP-40.

**Surface protein biotinylation assay**

Cells stimulated with OxPAPC were washed with phosphate-buffered saline (PBS; 37°C) and incubated with Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL; 5 mM, 10 min, room temperature). Subsequently, cells were washed twice with 100 mM glycine/PBS, lysed in 1% Triton-100/PBS (30 min, on ice), and centrifuged (10,000 \( \times \) g, 10 min, 4°C). Equal amounts of lysates were analyzed. *p < 0.05 vs. IL-6; n = 4 (C). Vascular leak was assessed by measurements of Evans blue accumulation in the lung parenchyma. *p < 0.05 vs. nonspecific RNA; n = 4 (D). (E) Mice were transfected with nonspecific or MTJ1-specific siRNA, followed by HTV with or without OxPAPC intravenous injection (1.5 mg/kg). Protein concentration and cell count in BAL samples of control and HTV-exposed mice were analyzed. *p < 0.05 vs. HTV; n = 6.

**Plasma membrane isolation protocol**

Control and OxPAPC-treated endothelial cell lysates or CEM fractions were further purified to enrich plasma membrane–associated proteins by wheat germ agglutinin (WGA) affinity as previously described (Singleton et al., 2009). Commonprecipitation studies and Western blot analysis were performed using confluent HPAEC monolayers treated with vehicle or stimulated with OxPAPC. After stimulation, cells were lysed, and protein extracts were separated by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies.

**Analysis of GRP78 interaction with OxPLs by gel electrophoresis**

**Sample preparation.** Human recombinant GRP78 in PBS (30–100 ng) was mixed with OxPAPC, OxPAPS, DMPC, and DMPS (0.1–2 μg) in PBS/0.01% butylhydroxytoluene (total volume of 20 μl) overlaid with argon to prevent artificial oxidation. The mixture was incubated on a shaker for 90 min at 37°C.

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**GTPase activation, protein fractionation, immunoprecipitation, and immunoblotting**

Activation of Rac GTPase in pulmonary endothelial cell culture was analyzed using a GTPase in vitro pull-down assay kit available from Millipore. Confluent HPAECs were stimulated with OxPAPC, and cytosolic and membrane fractions were separated using S-PEK kit (EMD Chemicals, Gibbstown, NJ). CEMs were isolated from human lung EC as previously described (Singleton et al., 2009). Commonprecipitation studies and Western blot analysis were performed using confluent HPAEC monolayers treated with vehicle or stimulated with OxPAPC. After stimulation, cells were lysed, and protein extracts were separated by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies.
**Figure 8:** Proposed model of OxPAPC-induced human EC barrier enhancement. OxPAPC binds GRP78 and induces membrane localization of GRP78/HTJ-1 complex. OxPAPC-bound GRP78/HTJ-1 interacts with S1P-R1 and induces its activation via translocation to CEMs and consequent Src and Fyn tyrosine kinase-dependent phosphorylation of Akt, resulting in Akt-mediated S1P-R1 transactivation (threonine phosphorylation). Activated S1P-R1 receptor induces full activation of Akt via mTOR and PI3K-dependent serine and threonine phosphorylation required for Rac1 activation, cortical actin cytoskeletal rearrangement, and consequent OxPAPC-mediated EC barrier enhancement.

**Band-shift assay.** After addition of native PAGE sample buffer, samples were applied to a gradient 3–12% native PAGE gel (pH 7.5; Invitrogen) and run under nondenaturing conditions (50 mM Bis-Tris, 16 mM tricine, pH 7.3).

**GRP78 ELISA**

Microtiter 96-well plates (MaxiSorp; Nunc, Thermo Scientific, Rochester, NY) were coated with OxPAPs or DMPS (each 100 μg/ml in PBS containing 0.01% BHT) at 4°C overnight. Plates were washed with PBS and blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Recombinant human GRP78 (Prospect-Tany Technogene, East Brunswick, NJ; 1 μg/ml in PBS/1% BSA) or HPAEC cell lysates in PBS/0.05% Tween-20 were applied for 1 h at room temperature. After washing with PBS and sequential addition of anti-GRP78 and anti-goat horseradish peroxidase (HRP) antibodies, one-step Ultra-TMB substrate (Thermo Scientific) was added. Absorption was measured using a 2030 Multilabel Reader Victor X5 (PerkinElmer, Waltham, MA).

**Live-cell imaging and time-lapse tracking of cortactin dynamics**

Cells were plated on MatTek dishes (MatTek, Ashland, MA) and transfected with GFP-cortactin. Images were acquired with a 100x numerical aperture 1.45 oil objective in a 3I Marianas Yokogawa-type spinning disk confocal system equipped with a CO₂ chamber and a heated stage (Yokogawa, Tokyo, Japan). Time-lapse images were taken with 2-s intervals for 40-60 s.

**Animal models and mechanical ventilation protocol**

All experimental protocols involving the use of animals were approved by the University of Chicago Institutional Animal Care and Use Committee for the humane treatment of experimental animals. Ventilator-induced lung injury was performed as described previously (Birukova et al., 2010a). In brief, C57BL/6J mice (8- to 10-wk-old males) with weight 20–25 g (Jackson Laboratories, Bar Harbor, ME) were anesthetized and subjected to mechanical ventilation (Harvard Apparatus, Boston, MA) at high tidal volume (30 ml/kg) for 4 h. In the other experimental group, C57BL/6J mice were randomized to concurrently receive sterile saline solution or IL-6 (5 μg/kg, intrathecal, 24 h) with or without subsequent exposure to mechanical ventilation.

**Statistical analysis**

Results are expressed as mean ± SD of four to six experiments. Experimental samples were compared with controls by unpaired Student’s t test. For multiple-group comparisons, a one-way variance analysis and post hoc multiple comparisons tests were used. p < 0.05 was considered statistically significant.

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