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REVIEW

Lipid mediators in the regulation of endothelial barriers

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ABSTRACT

Lipid mediators play a critical role in the development and resolution of vascular endothelial barrier dysfunction caused by various pathologic interventions. The accumulation of excess lipids directly impairs endothelial cell (EC) barrier function that is known to contribute to the development of atherosclerosis and metabolic disorders such as obesity and diabetes as well as chronic inflammation in the vascular endothelium. Certain products of phospholipid oxidation (OxPL) such as fragmented phospholipids generated during oxidative and nitrosative stress show pro-inflammatory potential and cause endothelial barrier dysfunction. In turn, other OxPL products enhance basal EC barrier and exhibit potent barrier-protective effects in pathologic settings of acute vascular leak caused by pro-inflammatory mediators, barrier disruptive agonists and pathologic mechanical stimulation. These beneficial effects were further confirmed in rodent models of lung injury and inflammation. The bioactive oxidized lipid molecules may serve as important therapeutic prototype molecules for future treatment of acute lung injury syndromes associated with endothelial barrier dysfunction and inflammation. This review will summarize recent studies of biological effects exhibited by various groups of lipid mediators with a focus on the role of oxidized phospholipids in control of vascular endothelial barrier, agonist induced EC permeability, inflammation, and barrier recovery related to clinical settings of acute lung injury and inflammatory vascular leak.

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Introduction

The endothelial barrier provides a selective permeability to fluids and solutes between the blood and tissues and any alteration in this precisely regulated barrier integrity leads to many pathological conditions including lung edema, pulmonary hypertension, acute lung injury (ALI) and its most severe form acute respiratory distress syndrome (ARDS). A role of lipids in endothelial dysfunction is supported by the findings that accumulation of high levels of lipids in the circulation damage vascular tissues and their function.[1] Lipid-induced endothelial barrier dysfunction and its role in pathology of atherosclerosis and exacerbation of metabolic disorders such as obesity, diabetes has been described elsewhere and is not the scope of this review.[2–7] Rather, we will focus here on the endothelial barrier enhancing effects of bioactive phospholipids that have demonstrated protective effects in septic and aseptic models of ALI and inflammation *in vitro* as well as *in vivo*.

The endothelial barrier is a dynamic structure that constantly undergoes remodeling in response to mechanical forces or edemagenic and inflammatory agonists. The barrier disruptive and protective agents regulate endothelial permeability by altering the expression of cell-cell junction proteins or cell surface adhesion molecules, and reorganization of adherens junction (AJ), tight junction (TJ) and focal adhesion (FA) complexes.[8] The endothelial barrier function is regulated by a wide variety of signaling pathways, second messengers, kinases, phosphatases and small GTPases that determines cytoskeleton arrangements, cell contractility, and organization of cell junctions and focal adhesion (see [9–12] for review). Among these, small GTPases, namely Rho and Rac, take the central stage in regulating endothelial barrier function which have opposing effects in controlling endothelial permeability. Many agonists including thrombin induce Rho activation that triggers actomyosin

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contraction and actin stress fiber formation leading to increased endothelial permeability.[10,13,14] In turn, barrier protective compounds including certain lipids activate Rac that decreases endothelial permeability and enhances barrier function by inducing the formation of cortical actin, stabilization of cell-cell junctions and inhibition of paracellular gaps formation.[13,15,16] A dynamic negative cross talk between Rho and Rac regulates overall endothelial permeability and inflammatory activation. For instance, Rho can inhibit the activation of Rac via Rho-associated kinase (ROCK) and Rac can also suppress Rho activity by activating p190RhoGAP.[17,18] In this line of Rho-Rac cross talk in determining EC barrier function, we have shown that activation of Rap1, which can also activate Rac signaling pathway of EC barrier enhancement, mediates agonist-induced EC barrier recovery by down-regulating Rho.[19,20]

A direct link exists between the endothelial barrier dysfunction and inflammation.[21,22] Inflammatory agents increase the expression of endothelial cell-surface adhesion molecules- vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) which promote increased neutrophil adhesion and transmigration, resulting in lung inflammation. The interactions between neutrophils and endothelium cause cytoskeletal rearrangement resulting in increased cellular gaps that leads to vascular permeability.[23–25] However, recent studies have shown that neutrophils extravasation does not necessarily always result in vascular permeability.[26,27] In addition to the potent endothelial barrier protective effects discussed below, anti-inflammatory properties of phospholipids underscore their potential importance as prototype molecules for development of future therapeutic interventions.

Barrier protective oxidized phospholipids

In response to tissue injury, various cell types release membrane vesicles containing oxidized phospholipids that can act as both pro- and anti-inflammatory mediators.[28–30] Enhanced production of oxidized phospholipids has been described in ARDS and asthma.[31,32] The lipid modification also occurs through inflammation-induced reactive nitrogen species.[33,34] For example, the non-enzymatic free radical-induced formation of isoprostanes has been detected in various lung diseases including ALI, ARDS, asthma, cystic fibrosis and pulmonary hypertension, and the measurement of isoprostanes levels has been proposed as an index of oxidant stress.[35–38] These lipid mediators modulate endothelial barrier function at different levels: by regulating the gene expression, by activating cell surface receptors and signaling pathways, and by modifying cytoskeleton remodeling and binding of immune cells to vascular endothelium.[7,39-45]

Among various cellular phospholipids, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) is one of the major plasma membrane phospholipids which undergoes oxidation to generate a group of compounds with various modified residues at the *sn*-2 position of the phospholipid. Initial studies suggested that oxidized PAPC (OxPAPC) triggers inflammatory cascades and is present in atherosclerotic lesions.[5,30,41,46,47] OxPAPC was also shown to stimulate endothelial cells to bind to monocytes and the levels of these oxidized phospholipids were elevated in atherosclerosis.[48] However, other simultaneous studies showed that OxPAPC plays a protective role against lipopolysaccharide (LPS)-induced inflammatory tissue damage.[49,50] The inhibitory effects of OxPAPC against inflammation were attributed to its ability to block the interaction of LPS with LPS-binding protein and CD14.[49] Likewise, OxPAPC also inhibited LPS or CpG DNA-induced tumor necrosis factor-α (TNF-α) production in mice by blocking the activation of p38 MAPK and the NF-κB pathway.[50] In consistence with these anti-inflammatory properties of OxPAPC, several reports demonstrated OxPAPC-induced enhancement of endothelial barrier function, protection from agonist-induced EC hyperpermeability and LPS-caused barrier disruption.[51,52] These mechanisms will be discussed in the next section.

OxPAPC, a potent endothelial barrier protective agent

Pioneer studies by our group have described potent and sustained barrier-enhancing responses in human pulmonary artery endothelial cells (HPAECs) to low OxPAPC concentrations (5–20 μg/ml) and attenuation of thrombin-induced EC barrier disruption.[51] These barrier protective effects of OxPAPC were mediated by the activation of small GTPases Cdc42 and Rac. Later, our studies also showed that OxPAPC protects against LPS-induced acute lung injury both *in vitro* and in *vivo*.[52] OxPAPC reduced LPS-induced inflammatory cytokines production, and, also attenuated LPS-caused endothelial barrier dysfunction by actin cytoskeletal and focal adhesion

rearrangement.[52] OxPAPC also showed beneficial effects in ventilator-induced lung injury in rats and in mechanochemical-stimulated HPAECs.[53] Here, OxPAPC significantly reduced high tidal volume-induced protein and inflammatory cell accumulation in bronchoalveolar lavage (BAL) fluid and lung tissues in rats. In HPAECs, OxPAPC attenuated pathologic cyclic stretch (18%)-enhanced and thrombin-induced paracellular gaps by activating Rac.[53] With our continued interest in investigating the endothelial barrier protective activities of OxPAPC, we found that OxPAPC also protects against heat-killed *Stapylococcus aureus*-induced lung injury and endothelial permeability.[54] These cumulative findings strongly suggest that specific bioactive compounds generated during PAPC free radical oxidation show a promising therapeutic potential and may be used to mitigate acute lung injury caused by a wide range of agonists (Fig. 1). In addition to oxidation state, the polar head groups (phosphocholine, phosphoserine, phosphoethanolamine) contained in the PLs have been shown to be essential for barrier-protective effects of oxidized phospholipids. Interestingly, even among these structurally different species they showed different levels of protection against EC permeability caused by thrombin and other inflammatory agonists, while non-oxidized phospholipids did not exhibit any barrier protective effects.[55] To better establish the therapeutic potential of OxPAPC, we extensively studied the molecular and cellular mechanism(s) of its barrier protective and anti-inflammatory actions which will be described in the following section.

Intracellular signaling pathways involved in OxPAPC-induced barrier protection

OxPAPC activated protein kinase C (PKC), protein kinase A (PKA) and Raf-MEK1, 2-Erk 1, 2 mitogen-activated protein kinase (MAPK) cascade in HPAECs.[56] The same study also showed that OxPAPC induced tyrosine phosphorylation in EC and activated focal adhesion regulatory proteins cofillin, paxillin and focal adhesion kinase (FAK). The endothelial barrier enhancing and cytoskeletal remodeling effects of OxPAPC were mediated by the activation of Cdc42 and Rac.[51] Further studies revealed that Rac/Cdc42-specific guanine nucleotide exchange factors (GEFs) Tiam1 and betaPIX play a critical role in OxPAPC-induced Rac activation.[57] A cross talk between paxillin and Rac is essential for mediating barrier protective effects of OxPAPC which is

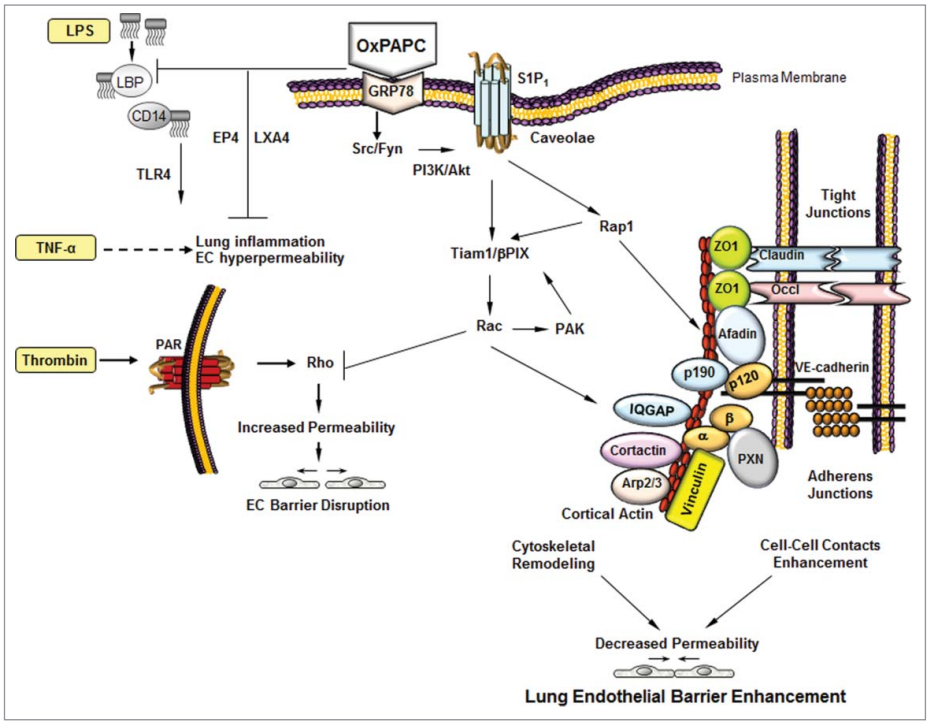

Figure 1. Mechanisms of OxPAPC-induced EC barrier protection. OxPAPC induces the activation of multiple signaling pathways that leads to the activation of Rap1 and Rac. The cytoskeletal remodeling facilitated by the cortical actin formation and assembly of tight junctions and adherens junctions proteins enhances lung endothelial barrier. In addition, EP4 receptor and lipoxin A4 also mediate the barrier protective effects of OxPAPC against LPS/TNFα-induced inflammation and lung injury *in vitro* and *in vivo*.

achieved by p21-activated kinase (PAK1) dependent phosphorylation of paxillin that serves as a positive feedback loop and contributes to sustained barrier protection by OxPAPC.[58] On further characterizing the Rac-mediated endothelial barrier protective effects of OxPAPC, we found that inhibition of PKA, PKC, tyrosine phosphorylation of Src, FAK and paxillin as well as inhibition of small GTPase with toxin B abolished the OxPAPC-induced Rac activation and decreased paxillin and FAK phosphorylation.[59] The interaction between AJ and FA complexes mediated by the association between β-catenin and paxillin was also critical for OxPAPC-induced EC barrier regulation.[60] Similarly, Rap1-mediated association between AJ and TJ proteins plays an important role in OxPAPC-induced EC barrier enhancement.[61] Briefly, OxPAPC activates Rap1 and increases the peripheral accumulation of AJ proteins VE-cadherin, p120- and β-catenin as well as TJ proteins ZO-1 and occludin. Furthermore, the barrier protective effects of OxPAPC depend on Rap1-mediated assembly of AJ-TJ protein complexes by promoting the interaction of VE-cadherin with p120-catenin, β-catenin and ZO-1. Afadin, an AJ-associated Rap1 effector, also plays a crucial role in mediating OxPAPC-induced EC barrier protection. OxPAPC enhances the accumulation of afadin in cell periphery in a Rap1-depedent manner and also increases the association of afadin with p120-catenin and ZO-1 which is essential for counterbalancing thrombin-induced EC barrier disruption.[62] OxPAPC activates caveolin-enriched microdomains (CEMs) and rapidly recruits shingosine 1-phosphate receptor ($S1P_1$), Akt kinase, Rac and Tiam1 to CEMs.[63] The depletion of Akt blocks OxPAPC-induced S1P1 activation and silencing of $S1P_1$ inhibits Tiam recruitment to CEMs and Rac activation, ultimately abolishing OxPAPC-induced cortical cytoskeletal remodeling, assembly of cell junction protein complexes and barrier enhancement. These findings indicate that Akt-dependent transactivation of $S1P_1$ in CEMs is essential for preserving the barrier protective effects of OxPAPC.[63] Further investigation revealed that OxPAPC-induced activation of $S1P_1$ requires its binding to plasma membrane localized chaperone protein GRP78.[64] Briefly, binding of OxPAPC to GRP78 in the cell membrane induces GRP78 trafficking to CEMs which subsequently activates $S1P_1$, Src and Fyn tyrosine kinases, and Rac1 GTPase that is essential for maintaining barrier protective actions of OxPAPC.[64]

With our persistent focus to better understand the intracellular signaling pathways involved in mediating OxPAPC effects in EC, we explored molecular basis of OxPAPC-induced downregulation of RhoA signaling. It was found that p190RhoGAP, plays a critical role as a negative regulator of Rho GTPase, and its depletion abolishes the protective effects of OxPAPC against ventilator-induced lung injury.[65] The interaction of p190RhoGAP with intracellular protein p120-catenin was necessary for its membrane translocation and to mediate the barrier protective effects of OxPAPC by activating Rac1 and inhibiting Rho.[8] Furthermore, afadin-controlled interactions between AJ protein p120-catenin and TJ protein ZO-1 is also crucial for OxPAPC-induced endothelial barrier enhancement.[62] Vinculin, a protein involved in transmitting the mechanical forces signaling in EC, also plays a role in mediating barrier protective effects of OxPAPC.[66] OxPAPC-induced association of vinculin with VE-cadherin is essential for EC barrier enhancement whereas the association of vinculin with FA protein talin following thrombin treatment causes an increase in EC permeability. Likewise, IQ domain containing GTPase-activating protein (IQGAP1), an effector of Rac1/Cdc42, acts as a molecular transducer of OxPAPC-induced EC barrier enhancement signals by targeting the additional activation of Rac1/Cdc42, thereby providing a positive feedback loop of Rac1/Cdc42 signaling.[67] Our studies showed the essential role of IQGAP1 in mediating barrier protective effects of OxPAPC since its knockdown abolishes OxPAPC-induced barrier protection against thrombin due to the reduced Rac1 and Cdc42 activation as well as decreased membrane localization of cortactin, VE-cadherin and p120-catenin.[67] In conclusion, OxPAPC appears to activate a wide arrays of signaling cascades that cumulatively result in Rac-mediated cytoskeleton remodeling leading to EC barrier protection (Fig. 1). The signaling pathways involved in OxPAPC-mediated barrier protection *in vivo* are less known and one of our study has shown that Rap1 plays a critical role in mediating the protective effects of OxPAPC against mechanical ventilation-induced lung injury.[61] Since our *in vitro* studies have established that Rap1 activates Rac via Tiam1, it can be speculated that the same signaling axis may exist *in vivo*. Moreover, Rac-mediated activation of p190RhoGAP, a negative regulator or Rho, mediated the protective effects of OxPAPC against ventilator-induced lung injury in

mice, indicating the direct involvement of Rac pathway. Future studies will determine the definite role of the Rac pathway in mediating OxPAPC-induced EC barrier enhancement *in vivo*.

Role of receptors in barrier protective effects of OxPAPC

OxPLs exert their biological effects by activating wide varieties of receptors including platelet activating factor (PAF) receptor, prostaglandin receptors E2 and D, scavenger receptor CD36, vascular endothelial growth factor receptor-2 (VEGFR-2), $S1P_1$, toll-like receptor 4, and peroxisome proliferator-activated-receptors (PPARα and PPARγ).[63,68-73] In consistent with these reports, our most recent study revealed a novel role of a prostanoid receptor in mediating the sustained barrier enhancing effects of OxPAPC that was associated with the activation of Rac. By employing the specific pharmacological inhibitors and gene silencing with small interfering RNA, we showed that prostaglandin E receptor-4 (EP4) plays a critical role in maintaining the EC barrier protective effects during the sustained phase but has no effect on rapid phase of barrier enhancement by OxPAPC.[74] Consistently, EP4 receptor did not play any role in suppressing thrombin-induced EC barrier dysfunction due to its acute nature but was involved in mediating the protective effects of OxPAPC against TNF-α-induced sustained EC barrier dysfunction and inflammation. A definite role of EP4 receptor in OxPAPC-induced anti-inflammatory effects was evident in LPS-induced acute lung injury rodent model where the beneficial properties of OxPAPC observed in wild type mice were abolished in endothelial-specific EP4 knockout mice. These novel finding suggest that OxPAPC also exerts its protective effects via prostaglandin receptor, especially during the advance stage of barrier enhancement, which could be a promising therapeutic target against acute lung injury.

Role of lipoxins in anti-inflammatory effects of OxPAPC

The unique properties of OxPAPC that its single *in vivo* injection can produce long lasting anti-inflammatory and protective effects in lung prompted us to explore other additional mechanisms involved in this process. Recent study by our group revealed that OxPAPC treatment causes significant accumulation of a bioactive lipid mediator lipoxin A4 (LXA4) in HPAEC cultures as well as in the lungs of OxPAPC-treated mice.[75] This lipid mediator is of particular importance, since earlier studies have found that its analogues have anti-inflammatory effects on EC and inhibit VEGF-induced EC permeability and actin stress fiber formation.[76,77] We found that the anti-inflammatory effects of OxPAPC were mimicked by LXA4 against TNF-α induced EC permeability *in vitro* and LPS-induced ALI *in vivo*. In addition, the anti-inflammatory activities of OxPAPC were markedly suppressed in HPAECs following the pharmacological or genetic inhibition of LXA4 formyl-peptide receptor-2 (FPR2/ALX) and also in FPR2/ALX knock out mice.[75] These novel findings warrant a further investigation of such EC barrier and lung protective lipid molecules generated during the oxidation of phospholipids.

Barrier-disruptive phospholipid oxidation products

It is noteworthy to mention that some fragmented oxidation products of phospholipids cause EC barrier disruption (Fig. 2) and even OxPAPC at higher concentrations induces hyperpermeability, actin stress fiber formation and myosin light chain phosphorylation in EC which is mediated by the activation of VEGFR-2.[78,79] In consistence with the role of VEGFR-2 in mediating higher dose of OxPAPC-induced barrier dysfunction, it was found to be a critical regulator during oxidized phospholipids-induced stimulation of EC via the activation of sterol regulatory element-binding protein (SREBP) and Erk 1/2.[68,80] Some studies also reported that oxidized phospholipids-activated VEGFR is responsible for activating transcription factor-4 mediated unfolded protein response in EC.[81,82] The dose-dependent differential effects i.e.; barrier protective at lower concentrations (5–20 μg/mL) and barrier disruptive at higher concentrations of OxPAPC (50–100 μg/mL) is due to the activation of different signaling cascades. The higher concentrations of OxPAPC increases the total cell protein tyrosine phosphorylation followed by the activation of Src kinase and phosphorylation of VE-cadherin limiting its ability to form complex with p120-catenin that results in increased EC permeability.[83] In addition, the fragmented products of PAPC oxidation such as

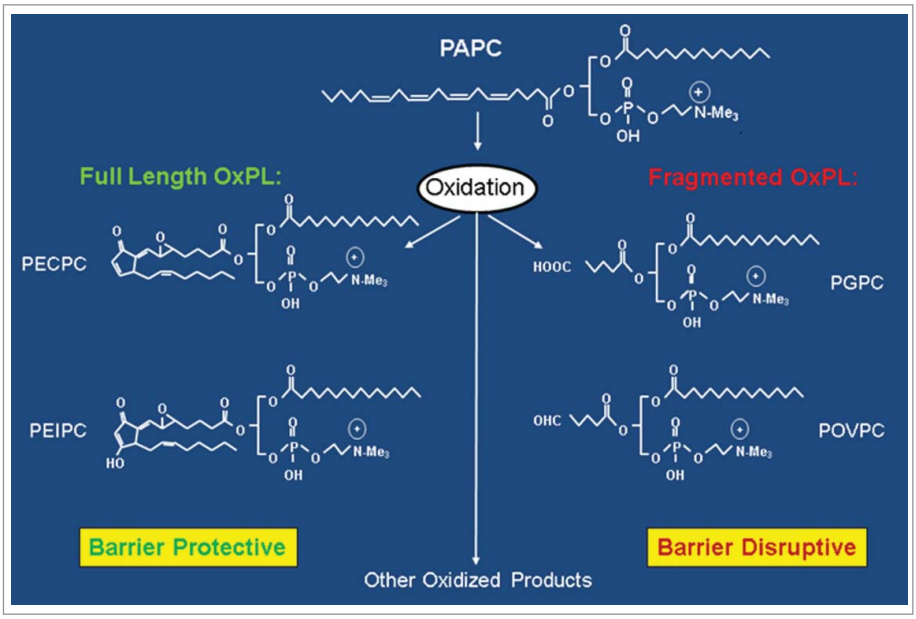

Figure 2. Generation of full length and fragmented oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC).

lysophosphatidyl choline (lyso-PC), and 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-phosphatidylchonine (POVPC) induce EC permeability via reactive oxygen species-dependent activation of Src and VE-cadherin phosphorylation but full length oxidation product 1-palmitoyl-2-(5, 6-epoxyisoprstane E2)-sn-glycero-3-phosphatidyl choline (PEIPC) induces EC barrier enhancement and also reverses barrier disruption caused by former fragmented lipid molecules.[79,83] The fragmented lipids induced the phosphorylation of VE-cadherin at both tyrosine residues 658 and 731 and phosphorylation of VE-cadherin is known to prevent its binding to p120- and β-catenin causing leukocytes transendothelial migration and EC barrier disruption.[84–86] However, a recent *in vivo* study has demonstrated that only phosphorylation at Tyr685 of VE-cadherin is involved in the induction of vascular permeability.[26] A role of oxidized phospholipid in EC barrier disruption was also revealed by a recent study where oxidized cardiolipin, a mitochondrial phospholipid, induced permeability as well as toxicity in cultured bovine pulmonary artery endothelial cells.[87] Based on these findings, it appears that wide varieties of structurally different oxidized phospholipids are generated with varying degree of cellular effects, either barrier protective or destructive functions largely determined by the type of signaling pathways activated.[88,89]

Other lipids in endothelial barrier regulation

Sphingosine-1-phosphate

In addition to oxidized phospholipids, other lipid mediators are also known to enhance endothelial barrier function. Sphingosine-1-phosphate (S1P) represents a major group of biologically active sphingolipids which is produced from the sphingomyelin metabolic pathway.[90–92] (Fig. 3). Platelets are the major source of S1P and earlier studies showed marked endothelial barrier protective effects by platelets-released factors.[93,94] Later, it was confirmed that platelet-derived S1P is responsible for maintaining EC barrier integrity and also protects EC barrier

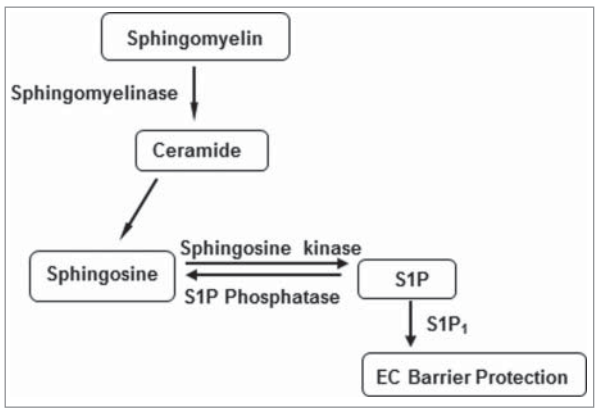

Figure 3. Metabolic pathway of sphingosine 1-phosphate (S1P) biosynthesis.

dysfunction caused by thrombin.[95] S1P binds to five G-protein coupled receptor isoforms $S1P_1$ to $S1P_5$ to exert its effects on EC. The differential binding of S1P to its receptors has contrasting downstream physiological consequences.[96] For example, S1P at higher concentrations binds to $S1P_2$ and $S1P_3$ coupled to $G\alpha_{12/13}$ or $G\alpha_q$ and disrupts EC barrier function through the activation of Rho.[16,97] On the other hand, S1P at lower concentrations binds to $S1P_1$ coupled to $G\alpha_i$ and mediates EC barrier protective effects by the activation of Rac.[95,98] The varying effects of S1P on EC barrier function was also reported by another study which showed that at physiological concentrations S1P bound to $S1P_1$ and preserved the EC barrier function by activating Rac where as excessive S1P bound to $S1P_2$ and induced EC barrier dysfunction by activating RhoA.[99] A number of recent studies have confirmed that $S1P_1$ is involved in strengthening EC barrier, inhibiting the release of inflammatory cytokines and suppressing the expression of EC surface adhesion molecules while $S1P_2$ and $S1P_3$ antagonize these beneficial effects of $S1P_1$.[100–102] Animal models of ALI had also shown the protective effects of S1P on lung function.[103,104] Furthermore, the role of $S1P_1$ in *in vivo* EC barrier protection was evident from a study where the barrier protective and anti-inflammatory effects of $S1P_1$ receptor agonist was reduced in LPS-challenged $S1P_1$ knock out mice.[105] Interestingly, as discussed above, OxPAPC also activates $S1P_1$ to induce EC barrier enhancement. With these promising protective effects and its reduced levels in sepsis patients, S1P might be considered as a potential therapeutic target as well as biomarker for EC barrier dysfunction and sepsis.[106]

The studies have established that activation of Rac and subsequent cytoskeletal remodeling is the major intracellular signaling pathway that mediates the beneficial effects of S1P on pulmonary EC.[95,107,108] The pathways activated by S1P upstream of Rac include PI3K, Src, and elevation of intracellular calcium concentration.[107,109] A synthetic analogue of sphingosine, FTY720 which acts as a potent agonist for $S1P_1$ and $S1P_3$ after getting phosphorylated inside the cells, has shown promising EC barrier protective effects *in vitro* and *in vivo*.[110,111] With its therapeutic potential in reducing ALI in rats with necrotizing pancreatitis and also prolonging the survival of rodents by increasing cytokines production in ischemia-reperfusion model, FTY720 has undergone Phase III clinical trial as an immunosuppressant for the prevention of solid organ transplant rejection.[103,112-114] Several novel derivatives of FTY720 have been developed in the recent years which exhibit preventive effects against EC barrier dysfunction, lung injury and inflammation.[111]

Prostaglandins and other cyclooxygenase metabolites

Prostaglandins (PGs) represent another important group of lipid mediators with potent barrier protective effects in the pulmonary endothelium.[115,116] PGs are the products of cyclooxygenase (COX)-mediated arachidonic acid metabolic pathway and synthesized by many tissues including vascular endothelial cells (Fig. 4). The role of PGs in the regulation of vascular permeability is complex since their cellular effects is largely determined by the receptor types. For instance, PGs may be involved in causing acute lung injury via vasodilatory effects but also exhibit protective effects in the resolution of inflammation as observed with PGI_2 and enhancement of EC barrier function by PGE_2, PGI_2 and PGD_2.[20,117,118] Furthermore, stable PGI_2 analogs beraprost and iloprost which are widely used for the treatment of pulmonary hypertension, exhibit anti-inflammatory and anti-edemagenic effects in various models of acute lung injury.[119,120] A number of studies from our group has shown the barrier protective effects of beraprost and iloprost against various models of ALI including LPS and mechanical ventilation.[121–124] Our studies also established a critical role of cAMP-activated PKA and Epac-Rap1 pathway in mediating the barrier protective effects of PGs and iloprost. Rac is activated by both PKA and Epac-

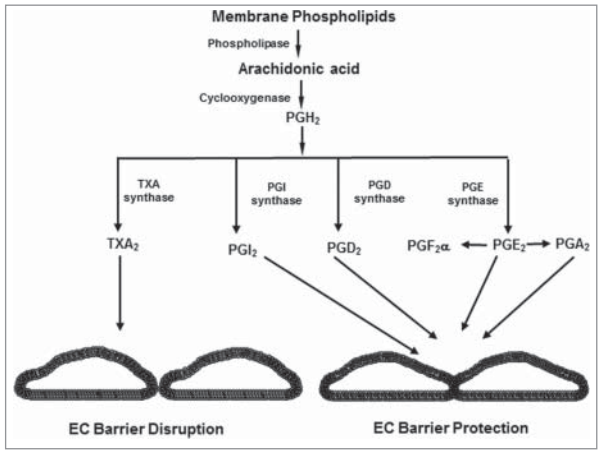

Figure 4. Biosynthesis of prostaglandins and thromboxanes from arachidonic acid.

Rap1, thus both of these signaling axis may converge into a universal pathway of barrier protection via Rac activation which is mediated by Rac-specific GEFs Tiam1 and Vav2.[20,125] In one of our most recent study, we performed a comprehensive analysis of barrier protective and anti-inflammatory activities of various PGs on pulmonary EC *in vitro* and *in vivo* and identify PGE_2, PGI_2 and PGA_2 with most potent beneficial effects.[126] Furthermore, our studies have also identified a critical role of PGs receptor EP4 in PGA2 and OxPAPC-induced prevention of EC permeability and lung inflammation.[74,127]

In addition to PGs, arachidonic acid metabolism by COX also generates other products such as thromboxane A_2 (TXA_2), leukotrienes, and platelet-activating factor (PAF) which mostly act as EC barrier disruptive agents.[128] Among these COX metabolites, TXA_2 is produced by several cell types including platelets, and EC and it causes platelet aggregation and vasoconstriction.[129,130] TXA2 is known to increase EC permeability,[131,132] and more importantly, it is present in the lungs of ALI/ARDS patients where it plays an inflammatory role.[133–135] In consistent with the role of TXA_2 in lung injury, a recent study reported that TXA_2 disrupts EC barrier by the activation of Rho and promotes lung edema formation.[136] Likewise, another study showed that hyperglycemia-activated TXA_2 disrupts the integrity and compromises the function of blood- brain barrier in brain microvascular endothelial cells.[137] Besides TXA_2, the activation of PAF is also known to impair endothelial barrier function.[138,139]

The promising EC barrier protective effects of PGs and oxidized phospholipids *in vitro* and *in vivo* do not easily translate into therapeutics since PGs have very short half-life *in vivo* (~ 40 seconds) and phospholipids are prone to cleavage by intracellular and extracellular phospholipases. To overcome these limitations, we recently developed a novel approach to synthesize a new class of phospholipase cleavage resistant PG-phospholipid compound by incorporating iloprost into phosphatidylcholine (Ilo-PC).[140] Ilo-PC showed more potent and long lasting EC barrier protective and anti-inflammatory effects over free iloprost. These superior effects of Ilo-PC were associated with its ability to induce prolonged activation of Rap1 and Rac GTPases.[140] The enhanced protective effects of Ilo-PC might have been contributed by various factors including its increased stability, ability to better interact with cell lipid layer to engage the receptors more efficiently and involvement of additional signaling pathways to activate Rap1/Rac. Currently, we are investigating all these possible scenarios and, nevertheless the far superior EC barrier protective and anti-inflammatory effects of Ilo-PC suggests that such stable PGs-phospholipid compounds could be potential therapeutics for restoring EC barrier integrity to treat lung injuries.

Future perspectives of therapeutic potential of oxidized phospholipids

The role of oxidized phospholipids in the regulation of EC barrier function has long been appreciated but the later studies have highlighted their barrier protective and anti-inflammatory properties. Among these, our extensive studies provide a substantial evidence that OxPAPC products could be promising prototype molecules for therapeutic treatment of lung injury, acute vascular endothelial inflammation and barrier dysfunction (Fig. 5). Mechanistically, OxPAPC employs multiple barrier protective pathways that makes it a suitable target against wide varieties of insults and injury in the pulmonary endothelium. It also causes a sustained EC barrier enhancement, indicating the very stable and prolonged effects, thus making it therapeutically viable. Further, the ability to produce long lasting protective effects after a single *in vivo* injection makes it an ideal candidate for ALI therapies. The involvement of prostaglandin receptor EP4 in OxPAPC-induced EC barrier function enhancement further strengthens its therapeutic potential since we recently demonstrated that EP4- mediated prostaglandin A2 (PGA2) barrier protective and anti-inflammatory effects were very effective in suppressing thrombin and LPS-induced acute lung injury *in vitro* and *in vivo*.[127] Moreover, our latest findings that OxPAPC treatment generates other important bioactive lipid mediators such as LXA4 with equally effective in anti-inflammatory and barrier protective functions underscores the potential of this compound in therapeutic development against lung injury and inflammation. To further investigate the therapeutic potential of oxidized phospholipids, future studies need to target on identifying more such bioactive, endothelial barrier protective and anti-inflammatory species of phospholipids oxidation products by applying advanced mass spectroscopic analysis and developing antibodies to detect them. In addition, testing the protective efficacy of oxidized phospholipids *in vivo* against various pulmonary and cardiovascular diseases associated with endothelial barrier dysfunction and inflammation will determine the translation of these molecules into clinics. Given that protective effects of phospholipids is mediated by a

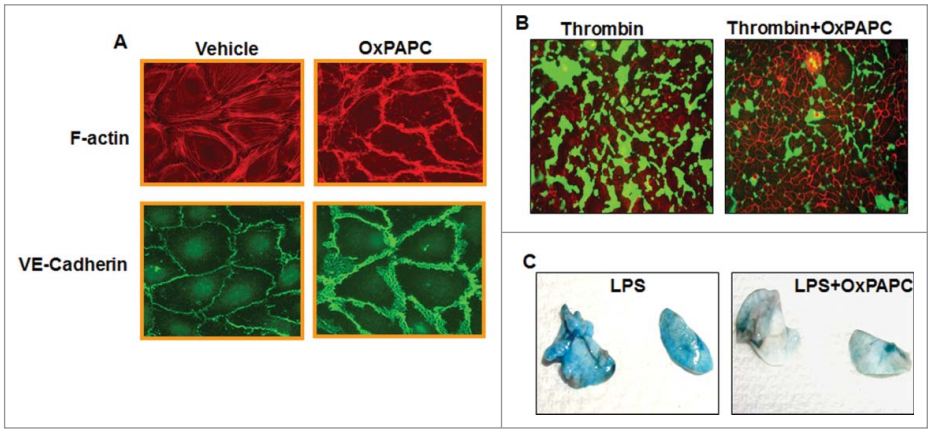

Figure 5. OxPAPC enhances endothelial barrier function and attenuates agonist-induced endothelial barrier dysfunction *in vitro* and *in vivo*. (A) HPAECs grown on coverslips were treated with OxPAPC (15 μg/mL, 30 minutes) and dual immunofluorescence staining with Texas-Red phalloidin and VE-cadherin was performed to monitor actin cytoskeleton and cell junction remodeling. The confocal images illustrated the barrier enhancing effects of OxPAPC as evidenced by increased F-actin and VE-Cadherin staining. (B) HPAECs grown on biotinylated gelatin substrate were pre-treated with OxPAPC (15 μg/mL, 30 minutes) followed by thrombin stimulation (0.5 Units/mL, 10 minutes) and cell permeability was visualized using FITC-avidin as a tracer. OxPAPC treatment protected thrombin-induced permeability as demonstrated by reduced green fluorescence that depicts areas permeable for FITC-avidin. (C) Intravenous injection of (1.5 mg/kg) of OxPAPC after 5 hours of LPS instillation (0.7 mg/kg, intratracheal) in mice protects against LPS-induced lung injury as detected by Evans blue staining of lung.

cumulative and complex cellular events of activation of receptors, intracellular signaling pathways as well as remodeling of cytoskeletal organization, future studies are required to identify the precise molecular and cellular mechanisms of phospholipids-induced endothelial barrier protection *in vivo*. Furthermore, new strategies to get rid of EC barrier disruptive truncated phospholipids should be considered. For this purpose, platelet activating factor acetylhydrolases (PAF-AHs) which specifically target the hydrolysis of truncated phospholipids could be employed. These oxidized phospholipid-specific phospholipases are known to reduce inflammation and apoptosis by selectively removing oxidatively truncated phospholipids.[141,142] Finally, besides EC barrier dysfunction, emerging evidence suggests that oxidized phospholipids play an important role in pathophysiology of other diseases, thus the knowledge on their structure, function, mechanisms of actions could potentially lead to the development of effective therapeutics against these diseases.[143–145] With these diverse effects in multiple diseases, the identification and quantification of various oxidized phospholipids are proposed to be utilized as biomarkers.[146]

Conclusion

Bioactive lipids are critical regulators of endothelial barrier function. Products of polyunsaturated fatty acid (PUFA) enzymatic conversion (Prostaglandins, leukotriens, thromboxanes, lipoxin, resolvins, etc.) and the phospholipids generated during oxidative or nitrosative stress exhibit both pro- and anti-inflammatory effects on EC. Among such oxidized phospholipids species, full length products of phosphocholine and phosphoserine oxidation represent a class of OxPL products with potent EC barrier protective and anti-inflammatory activities *in vitro* and *in vivo*. It appears that cells produce these bioactive lipid molecules in response to injury or inflammation as a recovery or survival mechanism. Since some OxPL products, especially the fragmented oxidation products and also the higher concentrations of full length products, have barrier disruptive and inflammatory effects it is critical to precisely identify the correct species and concentrations to introduce these molecules into clinics. Consideration of employing specific phospholipases such as PAF-AHs which selectively remove truncated oxidized phospholipids might be important for mitigating the deleterious effects caused by these fragmented lipid products. The extensive studies by our and other groups have characterized intracellular signaling pathways and described receptors mediating barrier enhancing functions of OxPAPC. The engagement of multiple EC barrier enhancing and lung injury recovery pathways suggests OxPAPC products as a potential therapeutics of choice, as they show a great potential in suppressing the EC barrier dysfunction and lung injury caused by wide variety of agonists. Moreover, the identification of novel

pathways triggered by other lipid mediators and contributing to their anti-inflammatory and barrier-protective effects could lead to the development of effective therapeutics against EC barrier dysfunction.

Abbreviations

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
EP4	prostaglandin E receptor-4
HPAECs	human pulmonary artery endothelial cells
LPS	lipopolysaccharide
LXA4	lipoxin A4
OxPL	oxidized phospholipids
OxPAPC	oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine
PGs	prostaglandins
S1P	shingosine 1-phosphate
TNF-α	tumor necrosis factor-α

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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