Lysophosphatidic Acid Regulates Endothelial Barrier Integrity

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Abstract The main function of vasculature is to serve as a vessel network for blood circulation between lungs and other organs. The endothelium is a major component of blood vessels, lining the inside of vessels and playing a central role in maintenance of vascular integrity. The endothelial barrier prevents blood component leakage into perivascular tissues. Increases in vascular permeability result in tissue edema, which is a hallmark of acute inflammatory diseases. Lysophosphatidic acid (LPA) is a simple phospholipid that exerts many physiopathological functions in various cell types including endothelial cells (ECs). LPA levels are detectable in plasma. Abnormal changes in LPA levels are correlated to diseases. LPA has been shown to regulate endothelial barrier integrity differently in different types of ECs. This chapter will summarize the current knowledge of the effect of LPA on endothelial barrier function and discuss how different ECs respond to LPA and molecular mechanisms underlying LPA-regulated EC barrier functions.

Keywords Lysophosphatidic acid · Endothelial cells · Vascular permeability · Edema · Signal pathway

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N. L. Parinandi, T. J. Hund (eds.), *Cardiovascular Signaling in Health and Disease*, https://doi.org/10.1007/978-3-031-08309-9_16

Introduction

The blood vessel network circulates blood in the pulmonary and systemic circulatory systems. Oxygenated blood is delivered from the lungs to the left heart, and is then pumped to systemic tissues. Deoxygenated blood is circulated back to the lungs through the right side of heart. Blood vessels exhibit distinct properties and play different physiological roles dependent on their localization in different tissues, if they carry oxygenated or deoxygenated blood, and diameter (reviewed in [1–4]).

Endothelial cells (ECs) form a monolayer and line the interior surface of blood vessels. The major function of ECs is to maintain vessel architecture and prevent blood component leakage into perivessels. The vascular endothelium exhibits a semi-permeable function. In normal physiological conditions, ECs allow solute and certain molecules smaller than 40 kDa to extravasate to surrounding tissues. The barrier formed by the EC monolayer keeps larger molecules and blood cells in circulation in the vessels. During pathological conditions, to a certain extent, EC barrier integrity is disrupted, leading to leakage of blood components including plasma into tissues, causing tissue edema. EC barrier integrity varies between sources of ECs (reviewed in [5–8]). Regulation of EC barrier integrity in blood-air barrier and the blood-brain barrier (BBB) has been well studied (reviewed in [9–15]). ECs express both adherens and tight junctions which control barrier integrity and mediate intracellular signals.

Lysophospholipids belong to a group of bio-active phospholipids that regulates intracellular signals and exerts biological functions through G protein-coupled receptors. Lysophosphatidic acid (LPA) is the simplest glycerolphospholipid, which is considered to be a growth factor in plasma (reviewed in [16–22]). Increase in LPA levels in biological fluids including bronchoalveolar lavage (BAL) has been detected in a variety of diseases including acute lung injury [23–25] and lung fibrosis [25, 26]. Several reports demonstrate the distinct effects of LPA on EC barrier integrity. In this chapter, we will summarize these findings and discuss potential mechanisms by which LPA regulates EC junctions and permeability.

Vascular Endothelial Hyperpermeability in Diseases

Vascular vessels are closed and continuous tubes that carry blood components including blood cells and provide nutrition to tissues. The microvascular EC barrier is a dynamic and complex interface between the blood and the surrounding tissues. Due to its semi-permeability of EC barrier, certain small molecules and solute may pass through the microvascular EC barrier. EC junctions are a major component of the anatomical barrier. In addition to cell–cell junctions, transcellular permeability and specific transporters also control small molecules through EC barrier (reviewed in [6, 7, 9, 10, 13, 14]). The tightness of the microvascular EC barrier is largely

dependent on tissues. The central nervous system (CNS) needs a stabled and controlled microenvironment. The BBB strictly controls influx and efflux of essential substances and promotes the normal physiological functions of the CNS. BBB dysfunction is observed in many CNS diseases, including multiple sclerosis, epilepsy, stroke, and Alzheimer's disease (reviewed [9, 14, 15]). Systemic inflammation caused by sepsis also leads to BBB disruption; in turn, BBB breakdown causes brain tissue edema and neuron inflammation and damage. Another well-studied EC barrier is the pulmonary microvascular EC barrier in the blood-air barrier. The lungs' major function is to facilitate gas exchange between the environment and the bloodstream (reviewed in [10, 11]). The lung epithelial barrier prevents inhaled microbes, allergens, and particulate matters from entering into the bloodstream (reviewed in [27–29]), while the pulmonary microvascular EC barrier limits blood component leakage into alveolar or interstitial tissue to prevent edema. Edema is a condition caused by excess fluid in the lungs due to EC barrier dysfunction (reviewed in [10–12]). The fluid in the alveolar space interferes with air exchange and leads to shortness of breath and death. Local infection by bacterial or virus (such as SARS-Cov2) or systemic inflammation (such as sepsis) causes pulmonary microvascular EC barrier dysfunction, leading to inflammatory cells and protein-rich fluid influx into alveolar spaces (reviewed in [11, 30–32]). Maintaining pulmonary microvascular EC barrier integrity is a novel therapeutic strategy for acute respiratory distress syndrome (ARDS).

EC monolayer barrier integrity was measured by several techniques, including transwell leakage assay, impedance-based cell monitoring, and immunostaining of cell–cell junctions. Measurement of protein levels in BAL and Evans Blue dye leakage in the tissues are common methods to determine vascular barrier integrity in in vivo studies.

EC Cell–Cell Junctions Regulate EC Barrier Integrity

Adherens and tight junctions are two major intercellular junctions that connect neighbor cells together including ECs. Adherens junction has been considered to play a critical role in initiation and stabilization of cell junctions (reviewed in [33–35]). VE-cadherin, also called CDH5 and CD144, is an endothelial-specific adhesion protein located at vascular adherens junction. VE-cadherin belongs to a cadherin family which consists of E-, N-, and P-cadherin. Extracellular domains of cadherins from adjacent cells interact each other in a calcium dependent manner. The intracellular domain of cadherins cross-links with the cytoskeleton. In addition to maintenance of cell–cell junctions, cadherins also mediate intracellular signaling (reviewed in [36–38]). In this chapter, we will focus on discussing the molecular regulation of VE-cadherin and the role of VE-cadherin in maintenance of vascular barrier integrity. VE-cadherin is phosphorylated on several tyrosine (tyr) residues including tyr658 and tyr731 in response to lipopolysaccharide (LPS) and TNFα. These phosphorylations have been reported to modulate endothelial permeability

through regulation of VE-cadherin shedding, internalization, degradation, and dissociation of VE-cadherin with its associated proteins, including p120, α-catenin, and β-catenin [39–41] (reviewed in [36, 37, 42]). Phosphatase SHP-2 and protein-tyrosine phosphatase nonreceptor 14 (PTPN14) negatively regulate VE-cadherin phosphorylation and promote restoration of endothelial integrity [41, 43].

Tight junctions between vascular endothelial cells mostly occur on apical and basolateral junctional complexes. Claudins and occludin are major tight junction transmembrane components. Similar to adherens junctions, claudins and occludin from adjacent cells interact each other and form a strict intercellular seal. Claudins and occluding are four transmembrane proteins, while VE-cadherin is a single transmembrane protein (reviewed in [44–47]). Claudins, including claudin-3, -5, and -12, have been reported in the endothelium (reviewed in [48, 49]). Among them, claudin-5 is well studied (reviewed in [50, 51]). Claudin-5 deficient mice demonstrate an increase in BBB permeability [49]. Knockdown of claudin-5 attenuated simvastatin-induced rescue of lung endothelial barrier integrity [52]. Occludin levels are downregulated in response to endothelial barrier disruption stimuli such as LPS and hypoxia [53–57]. Phosphorylation of occludin by protein kinase Cβ (PKCβ) in response to vascular endothelial growth factor (VEGF) leads to occludin ubiquitination and increase in endothelial permeability [58, 59]. Zonula occludens-1 (ZO-1) is a claudin and occludin adaptor protein. ZO-1 links claudins and occludin with the actin cytoskeleton [60, 61] (reviewed in [48, 62]). ZO-1 depletion reduces tight junctions and leads to stress fiber formation [60]. Angiotensin II is reported to downregulate ZO-1 expression and disrupt endothelial tight junctions [60].

Rho Family of GTPases Regulate EC Barrier Integrity

The Rho family of GTPases belongs to small G protein superfamily. Rho family members are activated after binding to GTP, while the GDP-bound form is in an inactive state. RhoA, Cdc42, and Rac1 are major members of the Rho family (reviewed in [63–65]). The distinct roles of Rho family members in the regulation of EC barrier integrity are dependent on the effects of their activation on reorganization of the actin cytoskeleton (reviewed in [65–67]). RhoA activation leads to myosin light chain (MLC) phosphorylation and promotes stress fiber formation, resulting in cell contraction and EC barrier disruption. RhoA-induced MLC phosphorylation is mediated by Rho-associated kinase (ROCK)/MLC phosphatase [68–70]. In addition to regulation of the rearrangement of the cytoskeleton, RhoA/ROCK pathway promotes downregulation of VE-cadherin, claudins, and occludin; thus RhoA plays a central role in the regulation of EC barrier function through disrupting both adherens and tight junctions and promoting cell contraction (reviewed in [71]). The effect of RhoA on corneal EC barrier restores and repairs after hyperosmotic stress also has been reported [72]. We will discuss the effect of LPA on RhoA activation in ECs in the chapter.

In contrast to RhoA, activation of Rac1 and Cdc42 preserve EC barrier integrity. Rac1 possesses a coordinating antagonism with RhoA [73, 74]. Rac1 is reported to be activated by extracellular adenosine [75], activated protein C [76], and others [77]. Inhibition of Rac1 reduced EC permeability and intercellular gap formation [77–79]. Notably, the role of Rac1 in disruption of lung epithelial cell barrier integrity has been reported. DiPaolo, B.C. et al. demonstrated that Rac1 inhibitor attenuated stretch-induced increases in alveolar epithelial cell permeability [80]. Cdc42 promotes VE-cadherin-mediated adherens junction assembly [81]. Expression of a dominant active mutant of Cdc42 in endothelial cells reduced LPS-induced EC barrier disruption [82].

LPA Production

LPA, naturally presented in plasma and cells, possesses multiple biological functions, including cell growth and proliferation. LPA is a phospholipid derivative that consists of a glycerol backbone, a fatty acid chain, and a phosphate. According to the different fatty acids, LPA exists in different species, such as 16:0, 18:1, and 22:6 LPA (reviewed in [16, 83, 84]). LPA is generated both intracellularly and extracellularly. Intracellular LPA is synthesized from monoacylglycerol by a monoglycerol kinase (MGK) or converted from phosphatidic acid (PA) by phospholipase A2s (PLA2s) (reviewed in [20, 21]). The role of intracellular LPA in the regulation of EC barrier integrity has not been reported. Most studies regarding the effect of LPA on EC barrier integrity are focusing on extracellular LPA that stimulates cells through LPA receptors (LPARs). Extracellular LPA is generated from lysophosphatidylcholine (LPC) by autotaxin (ATX, also called lysoPLD, ENPP2) [85, 86]. LPC is detectable in plasma and bronchoalveolar lavage [87–89]. ATX heterozygous knockout mice show a 50% reduction of plasma LPA levels [90]. Platelets have been shown to release LPA, suggesting that at least part of plasma LPA is from platelets [91, 92]; however, the mechanisms by which activated platelets release LPA have not been reported. LPA also is reported to be generated from phosphoatidylserine-(PS)exposed blood cells by a secretory PLA2 in the pathological conditions [93].

Increases in LPA levels in BAL fluid have been reported in murine models of acute lung injury. Except 18:0LPA, LPA species including 16:0, 16:1, 18:1, 18:2, 20:4, 20:3LPA are increased in murine BAL fluids after intratracheal LPS challenge for 24 h [23]. Mouratis, M-A. et al. examined the time course of LPA generation and found that LPS challenge increased LPA levels in BAL after 12 h and LPA levels remained at similar levels up to 48 h [24]. Increases in ATX activity and protein levels in BAL are correlated with LPA levels. However, bronchial epithelium- or myeloid-specific ATX deletion or inhibition of ATX had minor effects on lung injury [24]; thus, the role of BAL LPA in the pathogenesis of lung injury is unclear. Intratracheal instillation of LPA displays a protective role in LPS-induced lung injury. The protective effect of LPA possibly occurs through enhancing lung

epithelial barrier integrity [94]. Increase in systemic ATX worsened LPS-induced lung injury, suggesting that systemic LPA, not local LPA, contributes to the pathogenesis of lung injury [24]. Increases in ATX and LPA species (18:1, 16:0, 18:0, 20:4, 22:6LPA) in plasma were observed following ischemia and reperfusion (I/R) [95]. Vascular endothelial cells are targets of systemic LPA.

LPARs' Expression in Endothelial Cells

The effects of extracellular LPA on the cellular responses occur through its ligation and activation of a group of G protein-coupled receptors (GPCRs) on the cell surface. LPARs are divided into two groups based on sequence similarity. LPAR1–3 belong to endothelial cell differentiation gene (EDG) family of GPCRs. Other GPCRs, including GPR23/P2Y9/LPAR4, GPR92/LPAR5, P2Y5/LPAR6, and P2Y10, were identified as putative LPARs. LPARs coupled with distinct heterotrimeric G proteins [18, 20, 21, 83]. LPARs are expressed at distinct levels in different endothelial cells. Data from different groups reveal distinct expression patterns of LPARs. For example, Gupte R. et al. reported that LPAR5 is the predominant LPAR in human umbilical vein cells (HUVECs) [96], while Yokiura H. et al. showed that LPAR6 is highly expressed in HUVECs [97]. Other studies revealed the expression of LPAR1 and LPAR3 in HUVECs [98, 99]. The expression of LPA receptors in human pulmonary ECs has been reported. Ren Y. et al. demonstrated that LPAR2 and LPAR6 subtypes are highly expressed in both human pulmonary arterial (HPAEC) and microvascular (HLMVEC) ECs [100]. Cai J. et al. detected LPAR1 protein expression in HLMVECs [101]. Overall, LPARs levels in HLMVECs are much lower compared to their expression in human bronchial epithelial cells (unpublished data). In brain endothelial cells, LPAR6 is determined as the predominant LPAR [102]. On NH. et al. reported expression of LPAR1–3 in human brain capillary ECs and expression of LPAR1–5 in the capillary fraction from mouse brain homogenate [103]. LPAR1, not LPAR3, was detected in cerebral microvessels in rat brain by immunofluorescence staining [104].

LPA in Endothelial Barrier Function in Lungs

Pulmonary microvascular EC barrier integrity is responsible for maintaining the blood-air barrier. Disruption of the blood-air barrier is a hallmark of lung injury caused by inhaled pathogens (such as bacterial and SARS-Cov2) or systemic inflammatory diseases (such as sepsis). EC barrier dysfunction leads to protein-rich fluid influx into alveolar spaces, resulting in reduction of air exchange between the blood stream and atmosphere [10, 12, 30–32]. Brp-LPA, a pan LPA receptor inhibitor, reduced LPS injection-induced endothelial barrier disruption in mouse lungs [105], suggesting that LPA plays a critical role in lung EC barrier dysfunction. The

effects of LPA on HLMVECs and HPAECs have been reported. Ren Y et al. showed that LPA (0.1–30.0 μM) slightly reduced transendothelial electrical resistance (TEER) in HLMVECs using an electric cell-substrate impedance sensing (ECIS) system. The reduction was mild and transwell leakage assay with dextran-FITC did not confirm the phenomenon [100]. In another study, Cai J. et al. showed that LPA (5 μM) rapidly and significantly reduced TEER. The reduction was reversed back to normal levels after 2 h [101], suggesting a role of LPA in HLMVEC barrier disruption occurring in a short time frame. To compare the effect of LPA with lipopolysaccharide (LPS) on HLMVEC barrier disruption, we treated HLMVECs with LPA (1 μM) and LPS (200 ng/ml). Consistent with the study from Cai et al., LPA induced a rapid and significant reduction of TEER, while LPS induced a delayed reduction of TEER. The peak of reduction from LPS occurred after 8 h, and the TEER returned to basal level after 20 h (Y. Zhao, "unpublished data"). LPA is short-lived; 70% of extracellular LPA is degraded by lipid phosphate phosphatase (LPPs) in 2 h [106]. Interestingly, a metabolically stabilized analog of LPA (OMPT) induced a rapid and more severe reduction of TEER. The reduced TEER returned to basal levels after 20 h (Y. Zhao, "unpublished data"). OMPT is also a specific agonist of LPAR3 [107]. The data suggest that activation of LPAR3 results in HLMVEC barrier disruption. Future studies will be focused on examining the effect of LPAR3 in EC barrier dysfunction in murine models of lung injury.

HPAECs are useful cell models for investigating pulmonary EC barrier integrity. The data from different studies are not consistent. It has been reported that LPA treatment increased TEER in bovine pulmonary arterial ECs [108, 109], while Ren Y et al. showed that LPA reduced TEER in a dose-dependent manner in HPAECs [100]. Munoz NM. et al. and our unpublished data showed that LPA (1 μM) had no effect on TEER of HPACEs [110].

As we discussed, the HLMVECs and HPAECs respond to LPA in terms of EC barrier function differently in different studies. Due to the contrasting findings in the data, it is difficult to conclude the effect of LPA on EC barrier function in pulmonary lung ECs. HLMVECs and HPAECs are primary cells, LPARs expression pattern may be distinct from different donors and different passages. Generation of EC-specific LPARs-deficient mice will be helpful to determine the role of LPA/LPARs in EC barrier function in lung disease models.

LPA in Endothelial Barrier Function in BBB

Homeostasis of the brain microenvironment is important for neuronal activity. The BBB functions as a strict and selective barrier between blood stream and brain tissues to remains homeostasis of the brain microenvironment (reviewed in [13, 14, 95]). Using gadolinium diethylenetriaminepentaacetate (Gd-DTPA) contrast-enhanced MRI, the BBB disruption effect of LPA was observed in mice [103]. The conclusion was confirmed in Wistar rats and Sprague-Dawley rats by using fluorescent dye, sulforhodamine B [111] or Evans blue dye for BBB integrity [112]. LPA

treatment of porcine brain capillary ECs in both apical and basolateral side of transwell reduced TEER [113], while incubation of LPA in apical side, not in basolateral side of transwell filter cultured with rat cerebral microvascular ECs, increased transendothelial flux [114]. The localization of LPARs on the basal and basolateral plasma membrane in brain ECs cultured in a transwell chamber in these two cell types was not determined. It is possible that LPARs have different localization patterns in cells from different species. The effects of LPA on increases in permeability have been reported in porcine [115], bovine [103], rat [102], and human brain microvascular EC [100] by independent studies. Recently, Nah, S-Y.'s group demonstrated that gintonin from ginseng reduces BBB through activation of LPAR1/3 [116–118]. All these studies support that LPA increases permeability of brain microvascular ECs. In contrast to this conclusion, LPA increased TEER in corneal ECs isolated from New Zealand White rabbits [119]. Together, these studies indicate that, unlike the controversial conclusions of the effect of LPA on blood-air barrier integrity, BBB is sensitive to LPA. Though the effect of LPA on brain microvascular EC is to increase permeability, the clinical applications of LPA in the brain diseases have not been well studied. Since LPA-increased brain microvascular EC permeability is rapid and transient, it may provide a supplemental therapeutic strategy to increase drugs delivery to brain. Choi S-H. et al. showed that coadministration of gintonin, an LPAR ligand, with donepezil, a potential medicine for Alzheimer disease, increased donepezil concentration in cerebral spinal fluid [118]. The benefit of administration of LPA in delivery of drug to brains needs further evaluation.

LPA in Endothelial Barrier Function in Other Systems

An earlier study demonstrated that LPA decreased permeability in bovine aortic ECs [92]. However, the expression profile of LPA receptors and LPA-mediated signal pathways in bovine aortic ECs have not been determined. HUVECs are used as an EC model for the study of EC functions such as proliferation, cell death, inflammation, and barrier function. The effect of LPA on HUVEC barrier integrity has been studied. LPA treatment of HUVECs increased permeability as evidenced by an increase in leakage of horseradish peroxidase (HRP) [120] and FITC-labeled dextran in the transwell permeability assay [97]. The EC barrier disruption effect of LPA occurred through LPA ligation to LPAR6 [97]. Neidlinger NA et al. showed that LPA treatment of HUVEC monolayers induced cell retraction, increased gaps, and cell detachment, indicating that LPA induces HUVEC barrier disruption [93]. As discussed above, extracellular LPA can be generated by activation of ATX. Incubation of HUVECs with ATX and its substrate LPC increased LPA levels, as well as EC gap formation and permeability. Thus, the data regarding the effect of LPA on HUVEC barrier disruption is consistent in the different independent studies. Interestingly, Hisano Y et al. found that LPA rapidly and transiently increased TEER in LPAR1 overexpressing HUVECs [121], indicating that LPAR isotypes play distinct roles in LPA-altered HUVEC barrier integrity.

Molecular Mechanisms of LPA-Modulated Barrier Function

To investigate the molecular mechanisms by which LPA induces EC barrier disruption, most studies have been focused on the role of LPA in activation of Rho GTPases, cytoskeleton rearrangement, and regulation of cell–cell junctions. Ridley AJ and Hall A found that LPA rapidly induced the formation of focal adhesions and actin stress fibers through activation of Rho in fibroblast cells [122]. This is the initial study that reports LPA activation of Rho and regulation of cytoskeleton rearrangement. Cross MJ et al. were the first to investigate the role of Rho activation in LPA-induced stress fiber formation in ECs. They showed that inhibition of Rho by C3 exotoxin attenuated LPA-induced stress fiber formation in porcine aortic ECs [123]. Further, activation of phospholipase D was identified to activate Rho upon LPA treatment [123]. Though this study did not directly determine the role of LPA/Rho in EC barrier disruption, the data provided indirect evidence that LPA induces EC barrier disruption through PLD/Rho activation. Masago K et al. revealed that Rho regulates LPA-induced BBB dysfunction [102]. The role of Rho in LPA-induced EC barrier disruption has been further confirmed by other groups [112, 116, 120, 124]. MLC phosphorylation and cytoskeleton rearrangement by Rho and Rho kinase upon LPA treatment was shown to play a critical role in the EC barrier disruption [120]. LPA has been reported to regulate Rac1 and Cdc42 in lung epithelial cells (reviewed in [125]); however, the role of Rac1 and Cdc42 in LPA-altered EC barrier function has not been reported.

In addition to regulation of cytoskeleton rearrangement, LPA disrupted tight junctions by altering structural integrity of claudin-5, occludin, and ZO-1 in brain microvascular ECs [102]. LPA also induced phosphorylation of VE-cadherin in HLMVECs [101], which has been shown to be involved in VE-cadherin disruption and internalization, resulting in EC barrier dysfunction. ATP release, and an increase in intracellular calcium have been shown to regulate LPA-induced cytoskeleton rearrangement in HUVECs [126], suggesting that LPA-induced EC barrier disruption may be regulated by ATP release and increase in intracellular calcium.

Summary

LPA is a bioactive lysophospholipid, which induces cellular responses through ligation to a group of LPA receptors on the cell surface. Synthesis and metabolism of LPA occur in both intracellular and extracellular fractions. It has been shown that LPA regulates multiple physiological functions and pathological processes. The effect of LPA on EC barrier integrity has been demonstrated in different types of ECs (Fig. 1.). The EC barrier disruptive effect of LPA has been confirmed in HUVEC model and brain microvascular ECs. In lungs, there are controversial data regarding the role of LPA in lung arterial and microvascular EC barrier integrity. Generation of EC-specific LPA receptor deficient mice will help to understand the

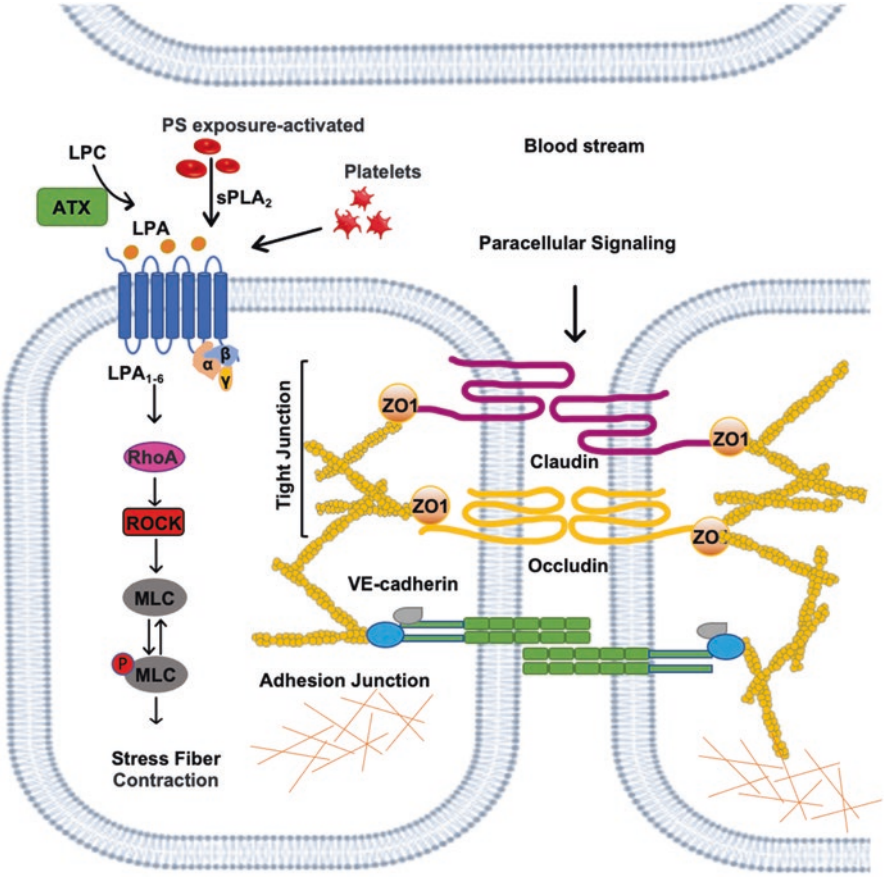

Fig. 1 LPA regulates EC barrier integrity. Extracellular LPA activates intracellular RhoA/ROCK/p-MLC pathway and regulates EC barrier integrity through ligation to LPARs on the cell surface of ECs

role of LPA/LPARs in the regulation of the blood-air barrier. As most studies are focused on extracellular LPA, ATX, and LPA receptors, the role of intracellular LPA in EC barrier integrity has not been investigated. The LPA-derived biolipids such as oxidized LPA need be further focused to investigate their role in EC barrier function. The development of in vivo lipidomic techniques may be useful to determine the local changes of LPA in circulation during disease progress.

Acknowledgement This work was supported by grants from National Institutes of Health (R01 HL131663, R01 HL136294, and R01 HL157164 to Y.Z.; R01 GM115389, R01HL151513 to J.Z.).

Conflict of Interest The authors declare no competing financial interests.

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