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S1P-Mediated Endothelial Barrier Enhancement: Role of Rho Family GTPases and Local Lamellipodia

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S1P-Mediated Endothelial Barrier Enhancement:

Role of Rho Family GTPases and Local Lamellipodia

by

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List of Abbreviations

AJ	Adherens Junctions
APSS	Albumin Physiological Salt Solution
ARDS	Acute Respiratory Distress Syndrome
Arp2/3	Actin Related Protein 2/3
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CA	Constitutive Active
CAMK	Ca2+/calModulin-dependent protein Kinase
cAMP	Cyclic Adenosine Monophosphate
Cdc42	Cell division control protein 42 homolog
CFP	Cyan Fluorescent Protein
DAPI	4',6-diamidino-2-phenylindole
DIP	Dia Interacting Protein
DN	Dominant Negative
EBM	Endothelial Basal Medium
ECIS	Electric Cell-substrate Impedance Sensing
EDTA	EthyleneDiamineTetraacetic Acid
EGM2-MV	Endothelial Growth Medium2-MV
ELISA	Enzyme-Linked ImmunoSorbent Assay
FAK	Focal Adhesion Kinase

FITC	Fluorescein IsoThioCyanate
FRET	Fluorescence Resonance Energy Transfer
GAP	GTPase Activating Protein
GDI	Guanosine nucleotide Dissociation Inhibitor
GDP	Guanosine diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GJ	Gap Junctions
GTP	Guanosine Triphosphate
HDMEC	Human adult Dermal Microvascular Endothelial Cells
HPAEC	Human Pulmonary Artery Endothelial Cells
HRP	Horse Radish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
LPS	LipoPolySaccharides
mDia	Diaphanous-related formin
MLC-2	Myosin regulatory Light Chain-2
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain-associated Phosphatase
PAK	p21-Activating Kinases
PAR	Proteinase-Activated Receptor
PBS	Phosphate Buffered Saline
PECAM	Platelet Endothelial Cell Adhesion Molecule
PKC	Protein Kinase C

Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras Homolog gene family, member A
ROCK	Rho-associated protein kinase
S1P	Sphingosine-1-Phosphate
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
siRNA	Small interfering RNA
TBST	Tris-Buffered Saline and Tween 20
TER	Transendothelial Electric Resistance
TGF-β	Transforming Growth Factor beta
Tiam1	T-Cell Lymphoma Invasion and Metastasis 1
TJ	Tight Junctions
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
YFP	Yellow Fluorescent Protein
ZO	Zonula Occludens

Abstract

The endothelial cells lining the inner surface of the tissue capillaries and post-capillary venules form a semi-permeable barrier between the blood circulation and interstitial compartments. The semi-permeable barrier in these vessels is the major site of blood-tissue exchange. A compromised endothelial barrier contributes to the pathological process such as edema, acute respiratory distress syndrome (ARDS) and tumor metastasis. Sphingosine-1-phosphate (S1P), an endogenous, bioactive lipid present in all cells, is a potential therapeutic agent that can restore compromised endothelial barrier function. On the other hand, S1P also has pleotropic effects and can either increase or decrease arterial tone and tissue perfusion under different conditions.

The detailed mechanisms underlining S1P's endothelial barrier protective effect are still largely unknown, but are suggested to depend on cell spreading termed "lamellipodia". Therefore, to fully take advantage of the beneficial properties of S1P, it is important to first understand how S1P-induced lamellipodia protrusions correlate with its effect on endothelial barrier function. It is also important to know the underlining mechanisms that S1P enhances endothelial barrier function, including intracellular signaling and receptor signaling. To study local lamellipodia activities, we acquired time-lapse images of live endothelial cells expressing GFP-actin, and subsequently analyzed different lamellipodia parameters. Experiments were performed under baseline conditions, and during endothelial barrier disruption or enhancement. The compounds

used in these experiments included thrombin and S1P. Transendothelial electrical resistance (TER) served as an index of endothelial barrier function for *in vitro* studies. Changes of local lamellipodia dynamics and endothelial barrier function within the same time frame were studied. For mechanistic studies, we combined biochemical, immunological and pharmacological approaches. Rho family small GTPase activities were measured with an ELISA pull-down assay. Fluorescence Resonance Energy Transfer (FRET) was also used to study the localization of RhoA activation. Pharmacological compounds targeting intracellular signaling messengers were used to test the involvement of Rac1, RhoA, MLC-2 in endothelial local lamellipodia activity and S1P-mediated endothelial barrier enhancement. Receptor agonists and antagonists were used to study the involvement of S1P receptor signaling. Finally, for cell behavior and cytoskeleton studies, we utilized immunofluorescence labeling that enables direct visualization of changes in cytoskeleton, cell-cell junction and focal adhesions.

We found that S1P increases both local lamellipodia protrusions and TER. The rapid increase in local lamellipodia protrusion frequencies also corresponded to the rapid increase in TER seen within the same time frame. Under the microscope, local lamellipodia protrusions from adjacent cells overlapped with each other and extended beyond junctional cell-cell contacts. Strikingly, S1P-induced lamellipodia protrusions carry VE-cadherin molecules to the cell-cell contact, established junctional adhesions. Combined with our previous published studies on thrombin induced lamellipodia activity changes, we think lamellipodia protrusions are a major component that regulates endothelial barrier function. Combined, our imaging studies revealed the mechanisms on how lamellipodia regulates endothelial barrier function: 1) lamellipodia overlap and

increase the apical to basal diffusion distance, which in turn decreases permeability and upregulates endothelial barrier function. 2) Local lamellipodia protrusions contain VE-cadherin, which is delivered the to the cell-cell contact by the lamellipodia to increase junctional stability.

S1P is effective for rescuing thrombin-induced endothelial barrier dysfunction. The known barrier disruptor thrombin, decreased local lamellipodia protrusions, disrupted VE-cadherin integrity, and caused a drop in TER. S1P increased local lamellipodia protrusions after thrombin challenge, and resulted in faster recovery towards baseline TER compared with vehicle controls. Interestingly, we also found that both thrombin and S1P increased MLC-2 phosphorylation at Thr18/Ser19. We subsequently accessed Rho family GTPase activity after thrombin and S1P. As expected, thrombin rapidly increased GTP-bound RhoA levels, and decreased GTP-bound Rac1 levels. Unexpectedly, S1P not only increased GTP-bound Rac1, but also increased GTP-bound RhoA to a more prominently levels (4-fold).

Since Rac1 has been implicated in promoting lamellipodia protrusions, we tested the role of Rac1 on the local lamellipodia activities first. We found that Wild-Type (WT) Rac1 group had the highest local lamellipodia protrusion frequencies, protrusion distances, withdraw time and highest percentage of protrusions that lasted more than 5 min. WT Rac1 overexpression had greatest protrusion frequencies and lowest monolayer permeability to FITC-albumin compared to GFP and DN-Rac1 overexpression monolayers. These results suggest that Rac1 is important for baseline endothelial barrier function. This is also confirmed by the finding that pharmacological inhibition of Rac1 significantly decreased baseline TER.

Although Rac1 is important for baseline endothelial barrier function, we noticed that it is dispensable in S1P-mediated endothelial barrier enhancement. Rac1 inhibitors, DN-Rac1 overexpression, and Rac1 siRNA knockdown all failed to abolish the S1P-mediated increase in TER. This is partially explained by the findings that S1P-induced Rac1 activation is short-lived and less pronounced in contrast to RhoA activation. We subsequently tested the role of RhoA in S1P-mediated endothelial barrier enhancement, based on our findings that both S1P and thrombin significantly activated RhoA and induced MLC-2 phosphorylation. Significant RhoA activation was found to be mainly at cell periphery and lamellipodia protrusions in HUVEC on FRET, after S1P was given. In addition, RhoA inhibitors significantly decreased the amplitude of S1P-induced MLC-2 phosphorylation, vinculin redistribution and barrier enhancement. The data suggest that the mechanisms involved in S1P-mediated endothelial barrier enhancement depend on RhoA activation and subsequent cytoskeletal rearrangement.

We next investigated which receptor is responsible for the endothelial barrier enhancement of S1P. However, antagonism of S1P1, S1P2 or S1P3 alone with W146, JTE-013 or TY-52156 respectively all failed to attenuate S1P-mediated increase in TER. While agonism of S1P1 with CYM-5442 hydrochloride alone produced significant increase in TER, neither S1P2 nor S1P3 activation (CYM 5520 & CYM 5541) produced any change on TER. Interestingly, S1P1 antagonist failed to block the effect of S1P1 agonist on TER. This could be due to that the S1P1 agonist may not be very selective at concentrations tested. We also identified that S1P4 and S1P5 are present on endothelial cells. Further studies would be necessary to elucidate the roles of newly identified S1P4 or S1P5 alone on endothelial barrier function. It is also worth

investigating in the future if multiple S1P receptors are involved in its endothelial barrier enhancing effect.

In conclusion, we found that lamellipodia protrusions contribute to the endothelial barrier enhancement of S1P. While Rac1 is important for the maintenance of endothelial barrier function, it is dispensable in S1P-mediated endothelial barrier enhancement. On the other hand, RhoA activation appears to be, at least in part, responsible for the endothelial barrier enhancement of S1P. It is currently still unclear if S1P's endothelial barrier enhancing effect is through one single receptor activation or activation of multiple receptors. Future studies are needed to elucidate the receptor signaling that contributes to S1P-mediated endothelial barrier enhancement.

Chapter One:

Introduction

1a. Overview of Endothelial Barrier

The microcirculation is the major site for blood-tissue exchange [1]. It is composed of arterioles, tissue capillaries and post-capillary venules. Blood flow is tightly controlled at the level of arterioles via vasomotor, vasoactive metabolites [2] and hormones [3], to ensure proper perfusion that meets the metabolic demands of tissues. Tissue capillaries and post-capillary venules are the major sites for blood-tissue exchange [4]. In these microvessels, individual endothelial cells anchored on the thin extracellular matrix attach to each other with the help of intercellular adhesion proteins, and form a semi-permeable barrier between the lumens of blood vessels and surrounding interstitial spaces [5]. This semi-permeable barrier selectively allows the exchange of oxygen, fluid, nutrients and cells to, as well as metabolic waste from the surrounding interstitial and tissues. Excess fluid and plasma proteins within the interstitium are drained back into the circulation via the lymphatic system [4].

Appropriate endothelial barrier function is critical for homeostasis as it ensures proper hemodynamics, blood-tissue exchange to support tissue viability [6]. Disruption of the endothelial barrier integrity increases microvascular permeability to macromolecules and fluid. Excessive leakage into the interstitium can result in tissue

edema and impaired organ functions [7], and is seen in multiple pathological conditions such as sepsis [8-10], ischemia-reperfusion injuries [11-13], tumor metastasis [14,15], and exposure to certain toxins [16-18], to name a few. Thus, an effective approach to enhance endothelial barrier function will greatly alleviate the symptoms and even delay the disease progress in patients. Such an approach would also have a wide spectrum of bedside applications given the involvement of microvascular leakage in many diseases. The scheme (Fig. 1) represents two adjacent endothelial cells and the major player in endothelial barrier regulation that are described below.

1b. Junctional Proteins and Endothelial Barrier

Between the bloodstream and interstitium space, solute exchange can occur through both transcellular and paracellular pathways. Transcellular flux is the solute transport through a cell. One example is glucose transport through transporters [19]. On the other hand, paracellular flux is the solute transport between adjacent cells. One example is passage of water and glucose into local tissues [4]. Under pathological conditions, the paracellular route is the major pathway of leakage across the endothelium for solute and macromolecules [20,21]. Cell-cell junctional protein complexes are adhesive structures between endothelial cells that fenestrate protein and solute off the interstitium [12,22]. Three main types of intercellular junctional proteins have been characterized: gap junctions (GJ), tight junctions (TJ) and adherens junctions (AJ). GJ are expressed in almost all tissues within human body [23]. They are composed of connexon channels that enable passage of small signaling molecules and ions between neighboring endothelial cells for communication [24]. To date, the exact roles of GJ in endothelial barrier regulation are still unknown [4]. In contrast to the

elusive role of GJ in endothelial barrier function, TJ and AJ have been well described to limit paracellular permeability to solutes and fluid. The differential distributions of both TJ and AJ proteins provide different levels of permeability based on the requirements of different tissues [25].

TJ express poorly in the peripheral vasculature, but express in high amount in specialized tissues such as blood-brain barrier [26]. Structurally, TJ such as occludins and claudins are composed of transmembrane domains and intracellular molecules. The intracellular domains of TJ proteins interact with intracellular adapter proteins such as zonula occludens (ZO), afadin, and cingulin, to anchor TJ to the actin cytoskeleton [27]. The small pore sizes resulting from TJ bindings between endothelial cells are crucial in restricting solute and fluid movement from the apical side to the basal side. TJ is not the main focus of this dissertation as little TJ are expressed in tissue capillaries and post-capillary venules.

The AJ are mainly composed of a protein named vascular endothelial cadherin (VE-cadherin). Distinct from the limited distribution of TJ, AJ are ubiquitously distributed along the vascular beds [25]. Similar to TJ proteins, VE-cadherin also has extracellular and intracellular domains. The calcium-dependent homotypic binding between the VE-cadherin extracellular domains from adjacent endothelial cells restrict passage of fluid and solutes between bloodstream and interstitial space. The intracellular carboxyl terminals of VE-cadherin bind to β- and γ- catenin, both of which are linked to the actin cytoskeleton by α-catenin [28,29]. The interaction of AJ to the actin cytoskeleton is further supported via adapter proteins such as vinculin and α-actinin [25]. Other adherens junction proteins are also present, such as platelet endothelial cell adhesion

molecule (PECAM), and the nectin-afadin complex, but their roles are less well defined in terms of regulating endothelial barrier function [25]. It is generally believed that the homotypic binding of VE-cadherin extracellular domains is critical for the maintenance of endothelial barrier integrity [21]. For example, disruption of VE-cadherin, via depletion of extracellular Ca^{2+} by either BAPTA [30] or EDTA [31], has been demonstrated to increase paracellular permeability. Vascular endothelial growth factor (VEGF) can induce VE-cadherin phosphorylation at Ser665. Phosphorylated VE-cadherins can subsequently be internalized, result in a loss of junctional integrity and increased paracellular permeability [32]. Notably, Huveneers and colleges recently described that VE-cadherin can serve as a mechanosensor and regulate force dependent remodeling [33]. The mechanosensing property of VE-cadherin can be a unique target to stabilize actin cytoskeleton and endothelial barrier function.

1c. Focal Adhesion and Endothelial Barrier

Similar to VE-cadherin, which stabilizes cell-cell adhesions, focal adhesions are receptors that stabilize the interaction between cells and extracellular matrix. Focal adhesions are critical in regulating a number of physiological and pathological processes such as angiogenesis [34], wound healing [35] and endothelial barrier function [36]. Structurally, the major components of focal adhesions are integrin heterodimers composed of various α and β subunits. Integrin heterodimers recognize and interact within the extracellular matrix proteins, such as fibronectin, laminin, and collagen [37], providing structural support for individual cells. A common feature of the integrins is that they recognize the arg-gly-asp (RGD) sequences of extracellular matrix

proteins [38]. Intracellularly, integrins attach to the actin cytoskeleton through talin, tensin, vinculin, α-actinin, focal adhesion kinase (FAK) and other adapter proteins.

The actin-integrin interaction provides structural support for endothelial cells and is crucial for endothelial barrier integrity. A pioneering study from Wu et al provided direct evidence that interfering with integrin and extracellular matrix interaction with RGD peptide significantly increased baseline permeability of porcine coronary venules to FITC-albumin [39]. The underlying mechanisms by which focal adhesions regulate endothelial barrier integrity are still elusive, however, FAK appears to be important. In cultured pulmonary aorta endothelial cells, a decreased FAK expression potentiated thrombin-induced endothelial barrier dysfunction [40], suggesting a protective role of FAK in endothelial barrier function. In contrast, other studies have demonstrated that FAK played a detrimental role in inflammatory conditions such as increased TGF-β in pulmonary edema [41], neutrophil extravasations [42]. These findings suggest that the role of FAK in endothelial barrier function is dependent on the context. Of note, recent studies reported that vinculin, a focal adhesion complex adapter protein, modulates the responses of focal adhesions to shear stress [43], substrate stiffness [44] to regulate endothelial barrier function [33].

1d. Cytoskeleton and Endothelial Barrier

The cytoskeleton is also critical for the endothelial barrier function. There are three components of the cytoskeleton: microtubules, intermediate filaments and actin microfilaments. Compared with actin microfilaments, the roles of microtubules and intermediate filaments in endothelial barrier function are not as well defined [25]. Recently, a study by Alieva et al has suggested that microtubule depolymerization is

necessary for the initiation of thrombin-induced endothelial barrier disruption [45]. Other studies have also shown that microtubule disruption with vinblastine increased endothelial permeability [46]. However, the mechanism underlying the increase in permeability seen with microtubule disruption is likely to involve actin microfilaments. Another recent study focusing on the intermediate filaments has described that vimentin phosphorylation is responsible for Withaferin A-induced increase in endothelial permeability [47]. A similar finding was also observed in thrombin-induced hyperpermeability in endothelial cells [45]. However, vimentin knockout mice displayed no phenotypic abnormality [48], which bring the *in vitro* findings into question. Overall, the microtubules and intermediate filaments have less well-defined roles in endothelial barrier function.

The role of actin microfilaments in regulating endothelial barrier function is much better defined. Actin filaments are polymers of G-actin. When the intracellular concentration of G-actin is above critical concentration (0.1 μM), a net polymerization is observed [4]. Multiple studies have described that the actin cytoskeleton is critical for the endothelial barrier function. The stabilized cortical actin structures tend to structurally support cell shape, lock junctional proteins in place, prevent excessive passage of fluid and solutes from the paracellular space, and thus decrease permeability [20,49-51]. One example is that the bioactive lipid S1P enhances endothelial barrier function via stabilizing cortical actin structure [52-54]. Another example is that stabilizing actin cytoskeleton with Phallacidin attenuated thrombin induced endothelial barrier dysfunction [55]. The opposite was seen with Cytochalasin D, a drug that disrupts actin filaments, increased endothelial permeability [56]. Importantly,

disrupting actin cytoskeleton with both Cytochalasin D and thrombin resulted in disruption of VE-cadherin, supporting the notion that the actin cytoskeleton provides structural support for the adherens junctions.

Another important aspect of the actin cytoskeleton that regulates endothelial barrier function is the actin-myosin contractile machinery. In endothelial cells, non-smooth muscle myosin interacts with actin filaments, and under certain conditions can perform ATP-dependent power strokes. This is the primary mechanism responsible for the hyperpermeability seen in inflammation [57,58] and burn injuries [59,60]. Actin-myosin contractility is regulated by myosin light chain kinase (MLCK) or myosin light chain associated phosphatase (MLCP). MLCK and MLCP can respectively phosphorylate or dephosphorylate MLC-2 to induce or prevent actin-myosin contraction [4]. The actin-myosin contractile activity can also be inhibited with the pharmacological inhibitor (-) blebbistatin that prevent the myosin head from hydrolyzing ATP, a process required for the power stroke [61]. Several studies have recently demonstrated the effectiveness of (-) blebbistatin in myosin II dependent process [62-64].

Although adherens junctions, focal adhesions and the actin cytoskeleton are independent structures that regulate endothelial barrier function, they are closely interrelated. Changes in one structure often result in changes of the other two. For example, selective preventing actin polymerization with Cytochalasin D disrupts cell-cell adhesions [65] and cell-matrix adhesions [66]. On the other hand, disruption of calcium dependent VE-cadherin hemophilic binding with BAPTA also resulted in actin depolymerization [67]. The mechanism that underlines the crosstalk between junctional

proteins, focal adhesions and cytoskeleton are complex, and are likely to involve activation and inactivation of small GTPases such as RhoA and Rac1.

1e. Rho Family Small GTPases and Endothelial Barrier

Small GTPases are intracellular soluble enzymes that have guanosine triphosphate (GTP) hydrolyzing activities. They switch between the "on" GTP-bound form and the "off" GDP-bound form. The exchange of between GTP and GDP are facilitated by GTPase activating proteins (GAPs) and guanosine nucleotide exchange factors (GEFs) and thus respectively inactivate and activate the GTPases. The guanosine nucleotide dissociation inhibitor (GDI) stabilizes the GTPases in the GDP-bound form and sequesters their activity [20]. Multiple small GTPase families have been shown to regulate endothelial barrier function, but Rho-family GTPases (RhoA, Rac1, Cdc42) are the best characterized.

Rac1 has been shown to be important for baseline endothelial barrier integrity. Rac1 inhibition was demonstrated to result in a loss of VE-cadherin and an increase in hydraulic conductivity both *in vitro* [68,69] and *in vivo* [70,71]. On the other hand, agents that activate Rac1 have been shown to increase VE-cadherin and endothelial barrier function [72,73]. Aside from its effect on junctional proteins, Rac1 has been shown to induce Arp2/3 dependent lamellipodia protrusions [74,75], cortical actin stabilization [76] and new cell-matrix adhesions formation [77,78]. Activation of RhoA and its effector ROCK by inflammatory mediators have been demonstrated to disrupt endothelial barrier integrity via actin-myosin contractility. ROCK can directly phosphorylate MLC-2 or indirectly phosphorylate MLCP to increase actin-myosin contractility [60,79,80]. The

mechanical force generated by the power stroke can pull the cell-cell junctions apart and disrupt the endothelial barrier.

It is generally accepted that Rac1 activation is an enhancer of endothelial barrier function, while RhoA activation causes barrier disruption. Interestingly, while Rac1 and RhoA have opposing roles in regulating endothelial barrier function, they both have been implicated in the formation of lamellipodia. Lamellipodia are sheet like membrane protrusions that are mainly composed of actin microfilaments within the cytoplasm [81-83]. The lamellipodia are vital for numerous physiological and pathological processes, such as angiogenesis, wound healing, and cancer metastasis [84-86]. The mechanisms underlining the formation of lamellipodia are largely unknown, however, several signaling processes have been implicated in their formation. Myosin II has been shown by many studies to be essential for lamellipodia protrusions as both knockdown with siRNA and inhibition with blebbistatin interfered with its formation [87-89]. Rac1 activation, such as seen in S1P and cAMP signaling, was shown to signal through the downstream Arp2/3 and Wave/Wasp complex to promote the formation of lamellipodia as well [20,90,91]. On the other hand, RhoA and its downstream effector Diaphanous-related formin (mDia) activation were recently suggested to also induce the formation of lamellipodia. The RhoA/mDia mediated formation of lamellipodia can be either through a Rac1- and-ARP2/3 dependent mechanism [92-94] or through a Rac1-independent, mDia2- and Dia-interacting protein (DIP) dependent mechanism [95,96]. Some studies have proposed that the lamellipodia protrusions are important for the endothelial barrier integrity [97,98]. This may, at least in part explains why both RhoA and Rac1 activation have been shown to protect endothelial barrier function [20,70,71,99-101]. Other studies

have also challenged the dogma that Rac1 is a barrier enhancer and RhoA is a barrier disruptor. A recent study presented that targeting ROCK with both pharmacological inhibitor and siRNA knockdown impaired endothelial barrier integrity [100]. In addition, Szulcek et al showed that RhoA activation at the cell periphery correlates with barrier integrity, while its activation in the perinuclear area of the endothelial cell contributes to barrier disruption [102]. These observations in part can be explained by the observations that RhoA can stabilize cortical actin through mDia and cofilin [103-105]. Conversely, Rac1 activation, which is generally considered to be endothelial barrier protective, has recently been shown to disrupt endothelial barrier integrity as well. The underlining signaling mechanism is likely to involve p21-activating kinases (PAK) and activation of actin-myosin contractile machinery [20,106]. VEGF has been shown to cause Rac1 dependent VE-cadherin phosphorylation and permeability increase. Rac1 activation has also been shown to cause VE-cadherin phosphorylation and permeability increase seen with VEGF [107,108]. These observations suggest that the effect of Rac1 and RhoA on endothelial barrier function is context dependent.

1f. Thrombin, S1P and Endothelial Barrier

Thrombin is well known for its detrimental effect on the barrier function of endothelial monolayers. It is a serine protease that is produced from prothrombin by prothrombinase complex [109]. Once activated, thrombin can cleave multiple coagulation factors and promote the formation of a clot. In addition to its role in the coagulation pathway, thrombin has also been shown to bind to the G-protein-coupled, proteinase-activated receptor (PAR). Upon interaction of PAR, thrombin cleaves the N-terminal exodomain of the receptor and produces an amino acid sequence that is able

to bind and activate the receptor itself [110,111]. Two PAR receptors (PAR1 and PAR2) have been identified in human endothelial cells and PAR1 is the main one activated by thrombin [111,112]. PAR1 is coupled to multiple G proteins including G_q, $G_{12/13}$, and $G_{i/o}$ [110,113]. PAR1 activation can signal through calcium dependent protein kinase C (PKC), Ca2+/calmodulin-dependent protein kinase (CAMK), and RhoA/ROCK to modulate the balance between MLCK and MLCP [114]. Overall the different couplings result in an accumulation of phosphorylated MLC-2 that generates contractile force within endothelial cells and eventually endothelial barrier disruption.

Sphingosine-1-phosphate (S1P) is a bioactive lipid that is synthesized from platelets and erythrocytes [115]. The plasma concentration of S1P within the circulation roughly ranges from 10 nM to 4 μM [116] depending on the context. Within the circulation, S1P is bound to serum albumin and HDL for distribution due to its lipophilic property [115,117]. The physiologic effect of S1P is generated mainly through its binding to at least five G-protein-coupled receptors. To date, three of the five S1P receptors have been identified on endothelial cells: S1P1, S1P2 and S1P3 [118-120]. Of the three receptors, the pertussis toxin-sensitive S1P1 is thought to be the principal receptor that is responsible for the endothelial barrier protective effect [90,114,121,122]. It is believed that S1P1 dependent, T-Cell Lymphoma Invasion And Metastasis 1 (Tiam1)-mediated Rac1 activation is essential for the endothelial barrier protective effect seen with S1P [53]. Rac1 activation recruits multiple proteins to its proximity and releases ARP2/3, which subsequently induces branched actin network at the periphery and distend the membrane [123-125]. S1P also induces the translocation of VE-cadherin to the periphery in an actin-dependent manner to increase junctional stability

[126]. Interestingly, recent studies have demonstrated that RhoA activation is required for proper VE-cadherin redistribution after S1P. Key findings support the notion is that RhoA inhibition with C3 exoenzyme abolished VE-cadherin translocation to the periphery after S1P [127,128]. While RhoA is generally thought to activate actin-myosin contractile machinery and disrupt endothelial barrier integrity, it seems counterintuitive that RhoA activation also mediates the translocation of VE-cadherin after S1P. One possible explanation is that RhoA-dependent lamellipodia may promote the redistribution of VE-cadherin to the cell periphery after S1P.

1g. Gap in Knowledge and Purpose of The Dissertation

Previously in our lab, we successfully optimized the protocol to expressing GFP-actin in endothelial cells and study cell cytoskeleton. We confirmed that the transfection was 90% efficient and does not alter the natural interaction between actin monomers. Of note, the endothelial monolayer exhibited active membrane protrusions and retractions that were rich in GFP-actin. The endothelial barrier disruptor thrombin strikingly diminished these active protrusions [50]. This raised the question that if the active lamellipodia protrusions are endothelial barrier protective.

S1P is a bioactive lipid that enhances endothelial barrier and ameliorate hyperpermeability. An analog of S1P called fingolimod is currently the treatment for multiple sclerosis. S1P is also being studied to ameliorate hyperpermeability seen in ARDS and sepsis. However, S1P can either increase or decrease the arteriole tone and might not be favored for the shock state seen in sepsis. In addition, S1P can also inhibit the recruitment of immune cells, which is also not beneficial for infectious conditions [118-120]. Therefore, to fully take advantage of S1P's endothelial barrier protective

properties and translate into the practice, it is critical to understand the underlining mechanisms. Of note, it has also been shown that the endothelial barrier protective effect of S1P requires cell spreading [129]. Most of previous studies are based on snapshots of different time points, which provided little information on the relationship of spatial-temporal lamellipodia and endothelial barrier function. Therefore it is important to determine how the lamellipodia protrusions affect junctional integrity in real time, as well as endothelial barrier function.

In this dissertation, we focused on the gap in knowledge and raised the central hypothesis that “local lamellipodia are important for S1P-mediated endothelial barrier function”. We designed our study to provide a foundation of knowledge for future therapeutic development at the molecular level to ameliorate hyperpermeability.

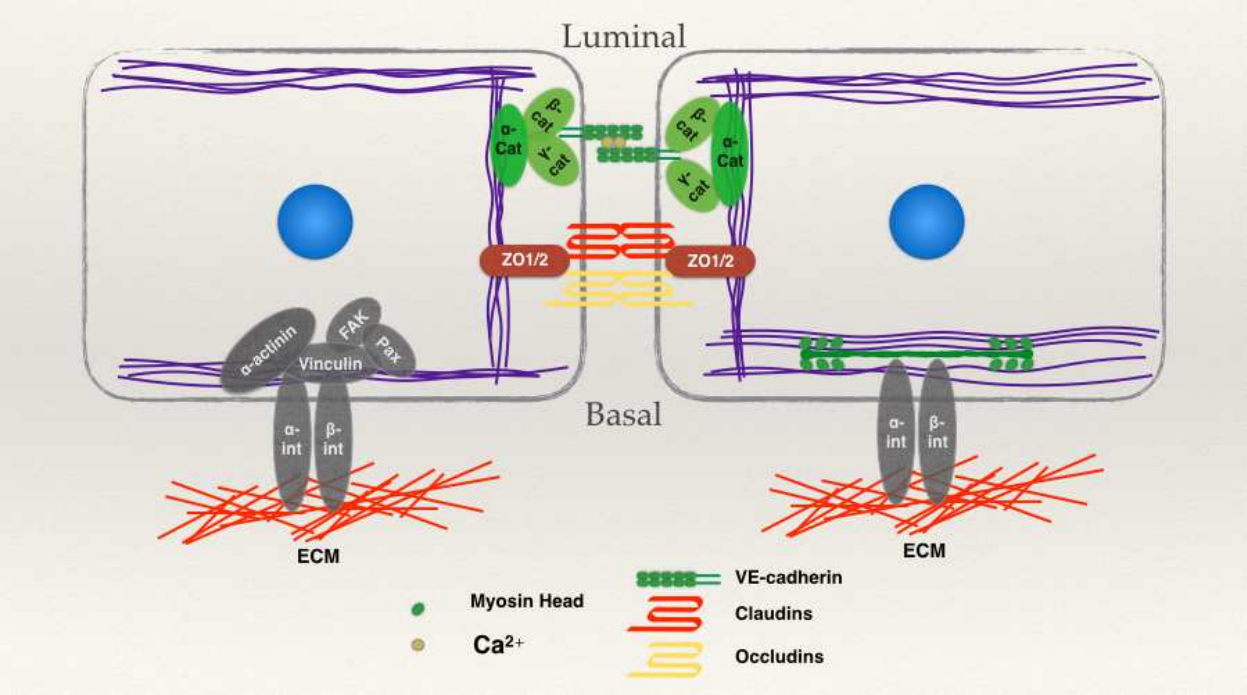

Figure 1 Schematic Graph of Two Adjacent Endothelial Cells

Tight junctions (TJ) and adherens junctions (AJ) lie within the intracellular cleft between two adjacent endothelial cells. TJ composed of claudins and occludins are anchored to actin cytoskeleton via zona occludins (ZO). AJ composed of vascular endothelial cadherins (VE-cadherin) are anchored to actin cytoskeleton via catenins. On the basal side, the endothelial cell cytoskeleton is attached to extracellular matrix via focal adhesion complexes that include focal adhesion kinase (FAK), vinculin (Vin), paxillin (Pax), α-actinin, α- and β- integrins. Intracellularly, actin filaments interact with myosin heads and form the contractile machinery.

Chapter Two:

Materials & Methods

2a. Materials

Human umbilical vein endothelial cells (HUVEC), Human adult dermal microvascular endothelial cells (HDMEC), Endothelial Growth Medium2-MV (EGM2-MV), and Endothelial Basal Medium (EBM) were obtained from Lonza (Basel, Switzerland). The Ingenio® electroporation kit and solution were obtained from Mirus Bio LLC (Madison, WI). The pCMV-GFP-β-actin (herein the protein product is referred to as GFP-actin) plasmid [130,131] vector was a gift from Dr. A. Wayne Orr (Department of Pathology, Louisiana State University Health Sciences Center-Shreveport). The pVE-cadherin-GFP plasmid was generously provided by Dr. Daniel Riveline (Institut de Science et d'Ingénierie Supramoléculaires, Université de Strasbourg, France). The pcDNA3-GFP-Rac1 (wild type; WT) and pcDNA3-GFP-Rac1T17N (dominant negative; DN) plasmids were purchased from Cell Biolabs (San Diego, CA). Rac1 siRNA (Knockdown: UAAGGAGAUUGGUGCUGUA) and control RNA (Non-Targeting: UGGUUUACAUGUCGACUAA) were purchased from Thermo Scientific (Rockford, IL); pTriEx-RhoA FLARE.sc Biosensor WT was a gift from Klaus Hahn (Addgene plasmid #12150). Rac1 Inhibitor NSC23766, Z62954982, (-)blebbistatin, (+)blebbistatin, Mouse anti-GFP (clone 3F8.2), and RhoA inhibitors Y16 and Rhosin, were purchased from

Merck-Millipore (Billerica, MA). Spingosine-1-phosphate (S1P) was purchased from Tocris (Bristol, UK). Goat anti-VE-cadherin (sc-6458) and HRP-conjugated-Mouse anti-β-actin (sc-47778 HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti MLC2-T18/S19 (#3674) was purchased from Cell Signaling Technology (Boston, MA). Mouse anti vinculin (ab18058) was purchased from Abcam (Cambridge, UK). Alexa Fluor 488-donkey anti-rabbit IgG antibody (A21206), 488-donkey anti-mouse IgG antibody (A21202), Alexa Fluor 647-donkey anti goat IgG antibody (A21447), Texas-Red-X Phalloidin (T7471) were purchased from Invitrogen (Carlsbad, CA). All other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2b. Cell Culture and Electroporation

HUVEC and HDMEC were routinely grown in EGM2-MV in 1.5% gelatin-coated culture dishes. For all studies, passage 1-5 cells were used. For transfection, cells grown to 80% confluence were trypsinized and pelleted, and 5 X 10^5 cells were resuspended in 100 µl electroporation master mix containing either 2 µg plasmids or 2 µM siRNA. This mixture was transferred to a cuvette for transfection using a Nucleofector II system (Lonza, Basel, Switzerland) with program A-034 (HUVEC) or M-003 (HDMEC). Warm EGM2-MV (500 µl) was added into the cuvette immediately after electroporation. Cells were later distributed evenly onto gelatin-coated 35-mm dishes for protein extraction, gelatin-coated MatTek 35-mm #1 glass bottom dishes for time-lapse microscopy, or 96W1E ECIS arrays (Applied Biophysics, Troy, NY) for determination of barrier function.

2c. Immunoblotting

Cell protein lysates were obtained as previously described[132]. Protein levels were quantified with the BCA protein assay kit (Thermo Scientific, Rockford, IL). Protein (15 µg) was mixed with NuPAGE® Reducing agent containing NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA), heated at 70 °C for 10 min, and loaded into Novex® 4-12% Bis-Tris precast gels (Invitrogen, Carlsbad, CA) for SDS-PAGE. Proteins were transferred onto 0.45 µm PVDF membrane and blocked with 5% BSA in TBST (20 mM Tris-HCl, PH 7.6, 150mM NaCl, 0.1% Tween 20). Membranes were incubated with primary antibodies at 4 °C overnight, and washed three times in TBST. Afterwards, secondary antibodies were applied at room temperature for 1 h, followed by three washes with TBST. Bands were visualized with Supersignal® HRP substrate (Thermo Scientific, Rockford, IL) and imaged with Bio-Rad Gel Doc™ system (Bio-Rad, Hercules, CA).

2d. Rho-Family GTPase Activity Assay

Colorimetric G-LISA activity assay kits (Cytoskeleton, Inc., Denver, CO, catalog numbers BK124, BK127, and BK128) were used according to the manufacturer's instructions to quantitatively assess GTP-bound RhoA, Rac1, and Cdc42 levels in HUVEC. Cells were grown to confluence in gelatin-coated 100 mm culture dishes, and the medium was changed to serum-free EBM the day before the experiment. After experimental treatments, the cells were washed with ice-cold (4°C) PBS and then lysed in ice-cold lysis buffer. The lysate was clarified at 14000 x g at 4°C for 2 min, a 20 µl aliquot was taken for a protein assay, and the remaining lysate was separated into at least two aliquots, snap frozen in liquid nitrogen, and stored at −70°C until the start of

the ELISA portion of the assay. Protein concentrations were determined using the Precision Red Advanced Protein Assay that came with the kits. Snap frozen lysate was then thawed and the sample protein concentrations were equilibrated using lysis buffer. GTP-bound RhoA, Rac1, or Cdc42 levels were then determined using the RhoA-GTP, Rac1-GTP, Cdc42-GTP binding 96-well plates, including a lysis buffer blank control and GTP-bound recombinant positive controls (80 pg/ml). Absorption of the ELISA wells was determined with a Tecan Infinite 200 Microplate Reader (Tecan, Männedorf, Switzerland). We also routinely verified that the total amount of RhoA, Rac1, and Cdc42 (GTP- and GDP-bound) were equivalent between groups by Western blotting.

2d.i. Data Analysis: Rho Family GTPases Activity Assay

GTPase activity measurements for each time points specified are presented after normalizing to control (mean ± SE). For each measurement, the replicates are greater than three. Significance was determined by one-way ANOVA with Tukey's test for multiple comparisons where appropriate. Significance was accepted when $P<0.05$.

2e. Immunofluorescence Confocal Microscopy

Immunofluorescence labeling and confocal microscopy were performed as previously described[133,134]. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The cells were blocked with 10% donkey serum in PBS, incubated with primary antibodies (1:100 dilution) at 4°C overnight, washed 3X in antibody wash buffer, incubated with secondary antibodies (1:200 dilution) at room temperature for 1 h, and washed 3X again. The cells were then incubated with Texas Red-phalloidin at room temperature for 30 min. The slides were mounted with ProLong Gold antifade reagent containing DAPI to label the nuclei (Life

Technologies, cat. No. P36931). Confocal Images were acquired with Olympus FV1000 microscope system by using a 60X oil immersion objective (Olympus America, Center Valley, PA) in the USF Lisa Muma Weitz Laboratory for Advanced Microscopy and Cell Imaging.

2e.i. Data Analysis: Immunofluorescence Images

After acquiring the confocal images, the images were stacked with Z-projection in Fiji/Image-J in TIFF format. Subsequently, the multi-channel images were split into single emission channels. The brightness and contrast of each image was later adjusted to the same settings for view (this did not alter original pixel data). Finally, the mean intensity of single pictures were measured and presented as raw intensity (mean ± SE). For each time point measured, the replicates are greater than three. Significance was determined by one-way ANOVA with Tukey's test for multiple comparisons where appropriate and was accepted when $P<0.05$.

2f. Live Cell Imaging

For lamellipodia studies, images were captured by an ASI Rapid Automated Modular Microscope that is equipped with a motorized stage and CRISP autofocus system (Applied Scientific Instrumentation, Eugene, OR), a Sutter Instruments Lambda LS 300 W xenon lamp, Lambda 10–3 excitation filter wheel with SmartShutter (Sutter Instruments, Novato, CA) and S492, S572, and D350 filters, a dichroic 2002bs emitter (61002m; Chroma Technology Corporation, Bellows Falls, VT), 40x ELWD and 100X oil objectives (Nikon Instruments), and a Photometrics CoolSNAP HQ2 camera (Photometrics, Tucson, AZ). The cells were transfected with either GFP-actin or VE-cadherin-GFP and grown to form confluent monolayers in MatTek glass bottom dishes.

The cells were then mounted onto the stage and bathed in albumin physiological salt solution (APSS: pH=7.4, NaCl, 120 μM; KCl, 4.7 μM; $CaCl_2 \cdot 2H_2O$, 2 μM; $MgSO_4 \cdot 7H_2O$, 1.2 μM; NaH_2PO_4, 1.2 μM; Na pyruvate, 2 μM; glucose, 5 μM; EDTA, 0.02 μM; MOPS, 3 μM and purified BSA 1 g/100ml). The dish received a continuous in-line infusion of APSS through an in-line heater at a rate of 0.5 ml/min. Both of the stage adaptor and APSS bath temperature were maintained constantly at 37°C with the help of Warner Instruments TC324B temperature controller (Warner Instruments, Hamden, CT). For all imaging collection, MicroManager software [135] was used. First, the fields of view were chosen from areas that have a strong fluorescent emission of either GFP-actin or GPF-VE-cadherin to avoid photobleaching effect over time. After initial equilibration, time-lapse images were collected with exposure time set at 1 s, interval for every 15 s, for up to 2.5 hours. All pharmacological compounds were applied into the bath and infusion solutions.

For RhoA FRET studies, Cells were transfected with the pTriEx-RhoA FLARE.sc biosensor and seeded onto 1.5% gelatin coated MatTek 35 mm #1 glass bottom dishes (MatTek Corp., Ashland, MA), and grown overnight to confluence. The medium was changed to EBM 3 h before the experiment. Each MatTek plate containing cells was transferred to a temperature-controlled (37 °C) imaging chamber. Time-lapse imaging data was acquired with a Leica SP2 confocal microscope with 63X objective at 30-s intervals, using the YFP and CFP channels (Leica Microsystems, Buffalo Grove, IL) in the USF Lisa Muma Weitz Laboratory for Advanced Microscopy and Cell Imaging.

2f.i. Data Analysis: Lamellipodia Dynamics

Time-lapse image stacks were saved in MicroManager as TIF format for storage. The image stacks were exported as TIF files for analysis using Fiji/ImageJ software. Brightness and contrast were adjusted for easier display but the original pixel intensity data were not altered. To assess actin dynamics at the cell periphery, we determined the frequency of lamellipodia formation (protrusion frequency). Filopodia were very infrequent with confluent endothelial cells, and typically formed as the result of the withdrawal of a local lamellipodium and thus were not quantified in this measure. The protrusion frequency was quantified by counting the number of local lamellipodia that formed on the perimeter of the entire cell during a particular time period. The protrusion frequency was normalized to the cell perimeter, which was estimated by drawing lines around the perimeter and measuring them using Fiji. Protrusion frequency is expressed as number/μm perimeter/time. Kymograph analysis of local lamellipodia was used to evaluate their motile dynamics (Fig 2). A single-pixel width line was drawn perpendicular to the edge of a cell (Fig 2 A), and this region was extracted from each image of the time-lapse to generate a montage of the region over time (Fig 2 B). In this panel, the streaks that move rightward and upward represent actin-rich protrusions, while the continuous lines that tend to move rightward and downward represent actin fibers moving toward the center of the cell. To assess protrusion dynamics, a line was drawn on each upward/rightward streak (Fig 2 C). This was easiest when the adjacent cell did not express GFP-actin, but was also achievable when an adjacent cell also expressed GFP-actin by scrolling through time-lapse images to help identify events that were lamellipodia. Using the ImageJ Measure function, bounding rectangle data were

acquired for each line, from which we determined the protrusion time and protrusion distance (Fig 2 D). The protrusion velocity was calculated as the protrusion distance/protrusion time. The withdrawal distance, withdrawal time, and withdrawal velocity were also measured for retracting lamellipodia in the same way with lines drawn along the cell edge (Fig 2 E). For all cells, 6–9 kymographs were generated to produce a representative sample of lamellipodia for study. Measurements of lamellipodia dynamics (protrusion frequencies, protrusion distance, etc.) are presented as mean ± SE. Significance was determined by one-way ANOVA with Tukey's test for multiple comparisons where appropriate, and was accepted when $P<0.05$.

2f.ii. Data Analysis: RhoA FRET

Images were saved in the format of TIFF and were imported to Fiji/imageJ for further analysis. First, cells that have a black background were randomly chosen from the images. Cells of interest were cropped from images, background subtracted and then converted to 32-bit images. Further processing of the cropped images was done by smoothing both CFP and YFP channels, threshold the YFP channel and finally convert the images to ratio images alter we converted (FRET/CFP) by using the analysis method described by Kardash et al [136]. The ratio images were later pseudo-colored in blue-green-red and saved as TIFF for presentation. The raw values of the overall intensity were subsequently normalized to baseline and presented as normalized intensity (mean ± SE). Significance was determined by one-way ANOVA and was accepted when $P<0.05$.

2g. *In Vitro* Endothelial Barrier Function Measurement

Transendothelial electrical resistance (TER), which serves as an index of barrier function of cultured endothelial cell monolayers, was determined with an Electrical Cell-Substrate Impedance Sensor (ECIS) ZΘ System (Applied Biophysics, Troy, NY). Cells were seeded into gelatin-coated wells of ECIS arrays (96W1E) and allowed to attach overnight in EGM2-MV to form a confluent monolayer. The next day, medium was changed to EBM at least 1 h before the experiment. A 1-μA AC signal at 4 kHz was applied. Total impedance was reported by monitoring the voltage across the electrodes and its phase relative to the applied current. The cell-covered electrode unit was treated as an RC circuit, from which impedance data was later converted into monolayer resistance and capacitance, respectively representing barrier function and membrane capacitance [137].

2g.i. Data Analysis: In Vitro Barrier Function Measurement

For each experiment, the "0 min" time point is defined as the time point when the first treatment was applied to the monolayer. Subsequently the TER values were normalized to "0 min" and averaged. Graph plots of mean normalized TER vs. time are presented. The maximum changes of TER (Max ΔTER) in percentage were calculated by dividing the maximal increase or decrease in TER by the baseline TER right before the first treatments were applied and multiplying by 100%. Data was finally presented as Mean ± SE and used for calculation. For comparisons between two groups, student t-test was used; for comparisons between three or more groups, one-way ANOVA with Tukey's test for multiple comparisons were used. Significance was accepted when the P value was less than 0.05.

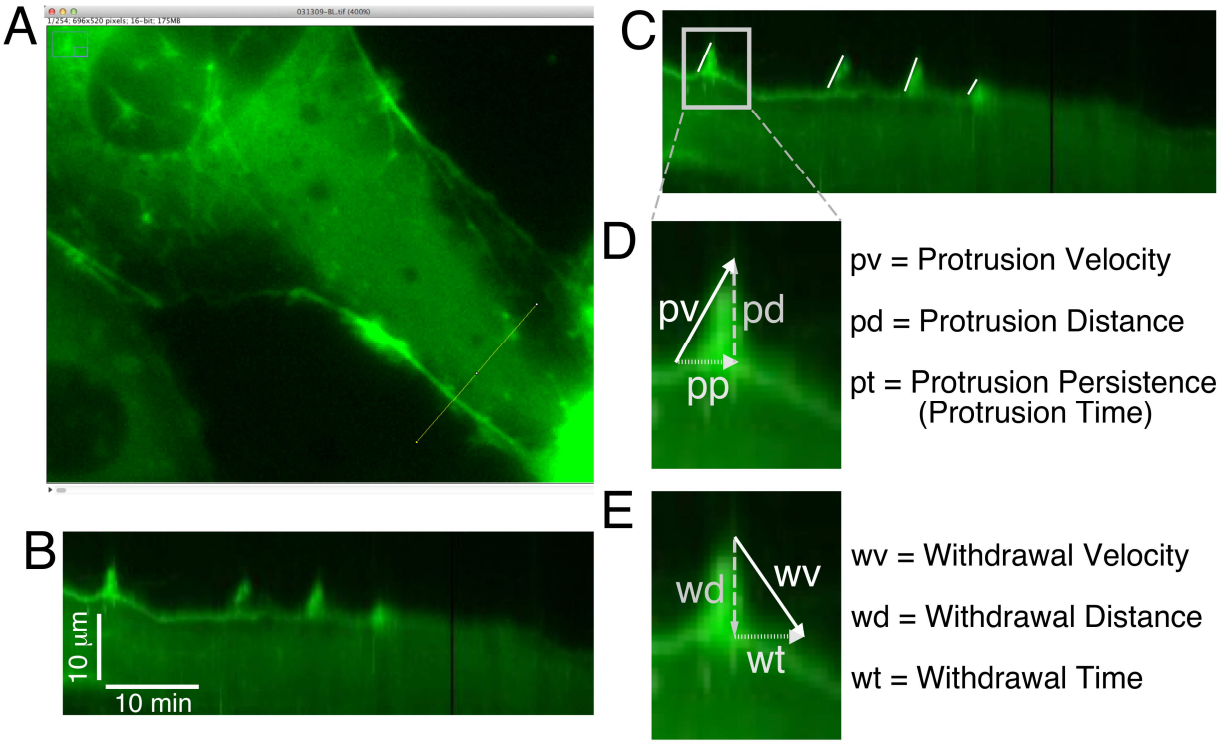

Figure 2 Analysis of Lamellipodia Parameters With Kymograph on GFP-Actin Expressing HUVEC

A. Line was drawn perpendicular to the edge of a cell expressing GFP-actin to generate a kymograph, with the x-axis representing time and the y-axis representing distance. B. Membrane protrusions were then identified in the kymograph and C. lines were drawn from the start point to the finish point for each protrusion. D. Each line was then used to determine the protrusion velocity, protrusion distance, and protrusion persistence. E. A line from the end of the protrusion phase to the point at which the lamellipodium had completely withdrawn was also drawn, and the bounding rectangle data produced the withdrawal distance, velocity, and time.

Chapter Three:

Lamellipodia Activity Correlates with Endothelial Barrier Function Changes

3a. Introduction

The endothelium of capillaries and post-capillary venules forms a semi-permeable barrier that is critical for normal fluid and solute exchange between blood and tissue. The adjacent endothelial cells are held together by the VE-cadherin homophilic interaction. The endothelial barrier disruptor thrombin [4,20,22,138,139] has been indicated to impair the integrity of VE-cadherin and endothelial barrier. Endothelial barrier enhancer S1P has been indicated to increase VE-cadherin between adjacent cells [20,52,90]. These findings highlight the importance of VE-cadherin in endothelial barrier function. In our previous studies, we discovered that endothelial monolayers exhibit active local membrane protrusions and retractions termed “lamellipodia” [140]. Interestingly, thrombin decreased lamellipodia protrusions, induced paracellular pore for formation and disrupted endothelial barrier integrity [134]. On the other hand, S1P and was demonstrated to promote lamellipodia protrusions and enhance barrier function [20,52,67,90,141-143]. These data raised the question as to whether lamellipodia play a role in endothelial barrier function.

It has been suggested that S1P increases VE-cadherin at cell-cell junctions [20,90,114]. It has also been shown that S1P’s endothelial barrier protective effect

requires cell spreading [129]. While previous findings suggest that lamellipodia may help maintain the structural integrity of VE-cadherin [144,145], it is currently unknown if lamellipodia protrusions seen after S1P are important for the increase in VE-cadherin at cell-cell contacts and endothelial barrier function. Many previous studies have presented indirect evidence that lamellipodia protrusions are important for the endothelial barrier function [90,146,147], yet direct analysis linking the two was limited in literature. To date, most of the knowledge that links cytoskeleton changes and endothelial barrier function is from fixed cells, and does not reflect how the spatial dynamic changes in the cytoskeleton impact endothelial barrier function. Our lab has optimized and validated a protocol for cytoskeleton studies in live cells. In this study, we analyzed the lamellipodia activities and endothelial barrier function changes that occurred over time. We tested the hypothesis that S1P-induced lammellipodia protrusions contribute to the increase in endothelial barrier function. The aim of this study was to determine if lamellipodia may be a future therapeutic target for hyperpermeability.

3b. Results

3b.i. Impact of S1P on Local Lamellipodia

Based on our previous finding that thrombin decreases lamellipodia protrusions and endothelial barrier function, we tested if the opposite pattern would be seen with the endothelial barrier enhancer S1P. At baseline, GFP-actin expressing HUVEC exhibited active lamellipodia protrusions and retractions. Immediately after 2 μM S1P treatment, there were rapid, coordinated lamellipodia protrusions along all cell edges that are most prominently seen at 2 min after S1P was added (Fig 3 A, S1P 2 min). Protrusion

frequencies were significantly increased within the first 5 min after S1P was added, however, it rapidly returned to baseline level by 15 min (Fig 3 B). Protrusion persistence, an indicator of the duration of each lamellipodia protrusion, was significantly increased at 10 min (Fig 3 C). The lamellipodia withdrawal time was gradually increased by S1P, and reached significance at 10 min and 20 min (Fig 3 D). No significant changes were observed in protrusion distance and velocity, withdrawal distance and velocity (Fig 4 A-D). In addition, the percentage of lamellipodia that had withdrawal time greater than 5 min was significantly increased after S1P treatment (Fig 4 E). Taken together, these data suggest that 1) the burst in lamellipodia protrusions is likely responsible for the early phase (within 5 min) rise in TER, 2) the decline in TER after the initial rise is likely due to the decreased protrusion frequency, 3) the plateau phase is likely due to the longer lasting lamellipodia that have took longer to withdraw.

3b.ii. Impact of S1P During Thrombin-Induced Endothelial Barrier Dysfunction

Our previous findings indicated thrombin decreased lamellipodia protrusions, disrupted VE-cadherin and endothelial barrier integrity. We tested if S1P is able to improve the recovery from thrombin induced endothelial barrier dysfunction. While thrombin caused a significant decrease in TER, S1P treatment was able to cause an increase in TER and maintain the elevated TER throughout the time course measured (Fig 5 A). The magnitude of the increase is roughly the same as S1P alone treatment without thrombin. It is also worth noting that although S1P elevated TER, it did not significantly alter the recovery course of endothelial monolayer in response to thrombin. We also tested how S1P impacts lamellipodia dynamics in the presence of thrombin. Thrombin alone significantly decreased lamellipodia protrusion frequencies. S1P

treatment immediately caused a spike in lamellipodia protrusions that quickly returned to baseline levels (Fig 5 B). Other lamellipodia parameters were not significantly altered by either thrombin or S1P except the withdrawal time and the percentage of lamellipodia that lasted more than 5 min (Fig 6). Despite the presence of thrombin, S1P impacted TER, lamellipodia protrusion frequencies, withdrawn time, and percentage of lamellipodia that lasted more than 5 min by S1P in a similar fashion as with S1P alone. Overall, these data provided additional evidence supporting the notion that alterations of local lamellipodia protrusion frequencies correlate with endothelial barrier function changes.

3b.iii. Impact of S1P and Thrombin on VE-Cadherin

Adherens junctions play a key role in paracellular permeability [4,20,22]. To assess how S1P and thrombin impact adherens junctions in real-time, we transfected HUVEC with GFP-VE-cadherin. GFP-tagged VE-cadherin was predominantly located between cell-cell contact at cell periphery and in vesicles around the nucleus. A certain amount of GFP-VE-cadherin was also observed within the newly formed lamellipodia that protruded beyond the continuous belt of VE-cadherin (Fig 7 A). After 1 U/ml thrombin treatment, an apparent drop in VE-cadherin containing lamellipodia frequencies were observed, followed by the breakdown of the continuous VE-cadherin belt (Fig 7 B). On the other hand, after 2 μM S1P was added, there was an increase in VE-cadherin containing lamellipodia activity. Of note, the VE-cadherin contained in lamellipodia coalesced and formed a denser VE-cadherin belt between adjacent endothelial cells (Fig 7 C). Similar findings were also observed with immunofluorescence staining: in contrast to the discontinuous VE-cadherin caused by

thrombin (Fig 8 B & C), S1P caused strong and continuous VE-cadherin belt formation (Fig 8 E & F). These findings indicate that lamellipodia protrusions play a significant role in stabilizing VE-cadherin integrity. Combined with the TER data, these findings support the hypothesis that local lamellipodia protrusions contribute to the endothelial barrier function.

3b.iv. Rho-Family GTPase Activity After Thrombin and S1P

Rho-family GTPases are important regulators of actin cytoskeleton and endothelial barrier function. We measured the activity of Rac1, RhoA and CDC42 (Fig 9 A & B) in cultured endothelial monolayer treated with either 1 U/ml thrombin or 2 μM S1P. Both thrombin and S1P significantly increased RhoA activity throughout the time-points measured, which was unexpected. While thrombin decreased Rac1-GTP levels for up to 30 min, S1P caused a rapidly increased Rac1 activity that dissipated by 5 min, giving it a crescendo and decrescendo pattern. Although Cdc42 activity was decreased significantly 1 min after thrombin treatment, no other significant changes were seen with either thrombin or S1P treatments.

3b.v. Impact of Rac1 Overexpression on Lamellipodia Dynamics and Endothelial Barrier Function

Rac1 activity has been shown to be important for both lamellipodia and endothelial barrier function [78]. Based on our finding that Rac1 activity was decreased with thrombin but increased with S1P, we accessed if Rac1 is important for lamellipodia activities and endothelial barrier function. To determine if there is an association between Rac1-mediated lamellipodia protrusions and endothelial barrier function, we employed an overexpression method. HUVEC were transfected with GFP-Rac1 (DN &

WT) or GFP-control plasmids. We assessed the monolayer permeability to FITC-albumin in Rac1 overexpression and GFP- controls groups, and found that WT-Rac1 overexpression group had significant lower baseline permeability to FITC-albumin, compared with GFP-control group (Fig 10 A). In comparison, no significant differences were seen between DN-Rac1 (GFP-Rac1T17N) expressing HUVEC and GFP-control groups (Fig 10 A). We also assessed local lamellipodia formation and its parameters. HUVEC expressing WT-Rac1 had significant greater more protrusion frequencies, withdrawal time and protrusions that lasted greater than 5 min, compared with GFP-control (Fig 10 E-H). In contrast, DN-Rac1 HUVEC group had significant shorter protrusion distances compared with GFP-control groups, but no other significant differences were observed (Fig 10 E-H). WT-Rac1 HUVEC had significant higher protrusion frequencies, longer protrusion distances than DN-Rac1 HUVEC (Fig 10 E-H). Other lamellipodia parameters were not significantly different (Fig 11 A-D).

3b.vi. Impact of Thrombin and S1P on MLC-2 Phosphorylation

Both thrombin and S1P increased RhoA activity. RhoA activation can either directly phosphorylate MLC-2 or indirectly phosphorylate MLCP to cause greater MLC-2 phosphorylation, an important process in leukocytes membrane protrusions seen in transmigration [148]. We tested if the increased MLC-2 phosphorylation can be seen after both thrombin and S1P treatments. Pronounced increased in MLC-2 phosphorylation was seen with both thrombin and S1P treatments. Combined with VE-cadherin studies, it seems that increased MLC-2 phosphorylation can mediate different responses depending upon the context.

3b.vii. Inhibition of Rac1 Effector Arp2/3 and RhoA Effector mDia Formin Both Decreased Endothelial Barrier Function

Both Rac1 and RhoA have been demonstrated to induce lamellipodia formation [149,150]. Rac1 activates its downstream effectors and promote Arp2/3-mediated lamellipodia formation [151,152]. In contrast, RhoA activates its downstream effector mDia, which stimulates the formation of Arp2/3-independent lamellipodia formation [105,153,154]. As S1P activates both Rac1 and RhoA, we tested whether inhibition of their effectors and lamellipodia activity can impair endothelial barrier integrity. Inhibition of both Arp2/3 and mDia formin caused a significant decrease in TER. These data suggest that lamellipodia play an important role in endothelial barrier integrity under baseline conditions.

3c. Discussion

In this series of experiments and resulting data, we presented evidence that local lamellipodia activity changes correlate with S1P-mediated endothelial barrier enhancement. Under baseline conditions, endothelial cells display active lamellipodia protrusions. After S1P treatment, there was a burst in the lamellipodia protrusion frequencies. The significantly increased lamellipodia protrusion frequencies occurred within the same time frame when the rapid rise in TER was observed. Later, as the lamellipodia protrusion frequencies dropped towards baseline levels, we observed an increased in both lamellipodia protrusion persistence and prolonged withdrawal time. These increased lamellipodia parameters were also observed within the same time frame when the plateau TER phase was seen. Combined, these data suggest that the lamellipodia protrusion frequencies may contribute to the rapid increase in TER early

after S1P, while the later plateau TER phase may be due to increased lamellipodia withdrawal time. Many factors contribute to the increased paracellular perpeability. One important factor that deserves discussion here is the paracellular diffusion distance. The paracellular permeability is inversely related to the paracellular diffusion distance [155]. Our live-cell imaging data showed that lamellipodia protrusions overlap beyond the adjacent contacts, these overlapping membrane protrusions can potentially increase the solute diffusion distances from apical side to the basal side and thus decrease permeability. When endothelial cells were stressed by thrombin treatment, S1P was still able to partially rescue the TER. On thrombin-pretreated endothelial cells, S1P also caused a significant “burst” in lamellipodia protrusions, followed by significantly increased lamellipodia withdrawal time. The latter are likely to explain the higher TER seen after S1P rescue. Coupled with our previous work that thrombin decreased lamellipodia protrusions and endothelial barrier function [50,134], we think that lamellipodia activities are closely related to the endothelial barrier function.

Paracellular flux is the major route for solute and fluid passage in response to inflammatory stimuli [141,156]. Proper junctional integrity between adjacent endothelial cells is crucial for optimal endothelial barrier function. Unlike brain endothelium, VE-cadherin is the major junctional protein throughout the vascular bed. The adhesion strength between the VE-cadherin molecules limits the paracellular cleft size and restricts solute passage [157]. In our experiments, we noticed that VE-cadherin was the most concentrated at cell periphery and in vesicles. Under baseline conditions, there was constant VE-cadherin remodeling and lots of VE-cadherin containing lamellipodia extended beyond the continuous VE-cadherin belt. S1P has been described to

redistribute and increase VE-cadherin strength between adjacent endothelial cells [52,90,141,143,158]. Soon after S1P was added, lamellipodia protrusions were increased, these VE-cadherin containing lamellipodia protrusions appear to deposit the VE-cadherin at cellular junctions, resulting in denser VE-cadherin belt between cells in a similar fashion as seen at the baseline. The same findings were also observed with immunofluorescence staining. On the other end of the spectrum, thrombin treatments lead to decreased lamellipodia protrusions frequencies and disrupted VE-cadherin integrity. Combined with the lamellipodia dynamics data, we speculate that lamellipodia protrusions after S1P carry the VE-cadherin to the cell periphery and beyond, promote the deposition of free cytosolic VE-cadherin to form a denser VE-cadherin belt. Functionally, increased VE-cadherin at interendothelial cleft restricts solute passage and decrease paracellular permeability. Interestingly, while thrombin did decrease lamellipodia protrusions, there was a transient increase in VE-cadherin containing lamellipodia immediately after thrombin was added to the bath, suggesting that lamellipodia activities may also serve as a compensation mechanism in response to stress to maintain an adequate level of barrier function.

One unexpected finding was that S1P significantly increased both Rac1-GTP and RhoA-GTP levels. While RhoA activation was prolonged and pronounced, Rac1 activation was short-lived and less remarkable. Rac1 has largely been described as an endothelial barrier enhancer [20,68,90], although some data did suggest it activates production of reactive oxygen species and disrupt endothelial barrier integrity [107,108]. On the other hand, RhoA has been chiefly described as an endothelial barrier disruptor, yet some have suggested that RhoA can stabilize cortical actin [103-105] and promote

recovery of endothelial barrier after thrombin [102]. While many studies have reported that Rac1 activation promote the lamellipodia protrusions [74,90,145,151], it was not certain if the impact of Rac1 on lamellipodia activity in endothelium had an impact on endothelial barrier function, we tested for this by using Rac1 overexpression technique. Not only did WT-Rac1 overexpression significantly increase lamellipodia protrusion frequencies, but it also resulted in significantly greater protrusion distance, compared with DN-Rac1 overexpressing groups. Likewise, DN-Rac1 overexpression groups had significantly shorter protrusion distances, compared with both WT-Rac1 and control groups. The mean withdrawal time, as well as the percentage of lamellipodia that lasted greater than 5 min, were both significantly greater in WT-Rac1 overexpression groups. The WT-Rac1 expressing monolayer had significantly lower permeability to albumin than GFP control group. The WT-Rac1 expressing monolayer is only slightly less permeable compared with DN-Rac1 expressing group, with a P value of 0.08. Coupled with our previous findings that pharmacological Rac1 inhibition decreased lamellipodia protrusion frequencies as well as endothelial barrier function [134], we think that Rac1 contributes to the endothelial barrier function. In addition, Rac1 can promote local lamellipodia protrusions, a process that can be modulated by either thrombin or S1P.

In our experiments, RhoA activity was also increased by S1P, which is somehow counterintuitive, as RhoA activation under inflammatory conditions commonly result in hyperpermeability, such as seen with thrombin [20,159]. A couple of reports described that through activation of ROCK or mDia, RhoA is able to either destabilize [20,160,161] or stabilize [153,154] interendothelial cohesions respectively. It is proposed that on the endothelial monolayer, actin-myosin bundles are arranged in a parallel way that has

minimal contractile impact on intercellular adhesions [162]. The crosstalk between ROCK and mDia also contribute to the stabilized junctions [163,164]. We have observed an increase in RhoA activity after S1P, this raised the question if the organized parallel actin-myosin bundles are seen with S1P treatment similar to those described by previous studies. Immunofluorescence staining revealed that S1P caused organized MLC-2 phosphorylation, and is less pronounced than thrombin. We think the MLC-2 phosphorylation may provide structure support for the Rac1-WAVE-Arp2/3 or RhoA-mDia dependent lammellipodia activities. We subsequently questioned ourselves if pharmacological inhibition of lamellipodia activities with Arp2/3 or mDia inhibitors can disrupt endothelial barrier. Both Arp2/3 and formin mDia inhibitors caused a significant drop in TER, suggesting both Rac1 and RhoA effectors are important in maintaining baseline endothelial barrier function.

3d. Conclusions

The exact mechanisms underlying lamellipodia protrusions are still being determined, and we were limited in the methodology that was available for our study. Based on the current work and our studies accomplished in the past [50,134], we think the results strongly suggest that lamellipodia activities correlate with endothelial barrier function changes.

In the current study, we presented novel data that increased lamellipodia activities after S1P correlate with the endothelial barrier function enhancement. We proposed two possible mechanisms that help explain the observation: 1) lamellipodia extend beyond cell-cell contacts and overlap with each other to increase the mean paracellular diffusion distances; 2) lamellipodia protrusions carry and deposit cytosolic

VE-cadherin to the cell-cell junction and promote junctional stability. While both Rac1 and RhoA are activated by S1P, and both of them can promote lamellipodia activities, it is yet not clear which GTPase plays the dominant role in the process. Further investigation utilizing siRNA and overexpression techniques may help to figure out the roles of Rac1 and RhoA in S1P-mediated endothelial barrier enhancement.

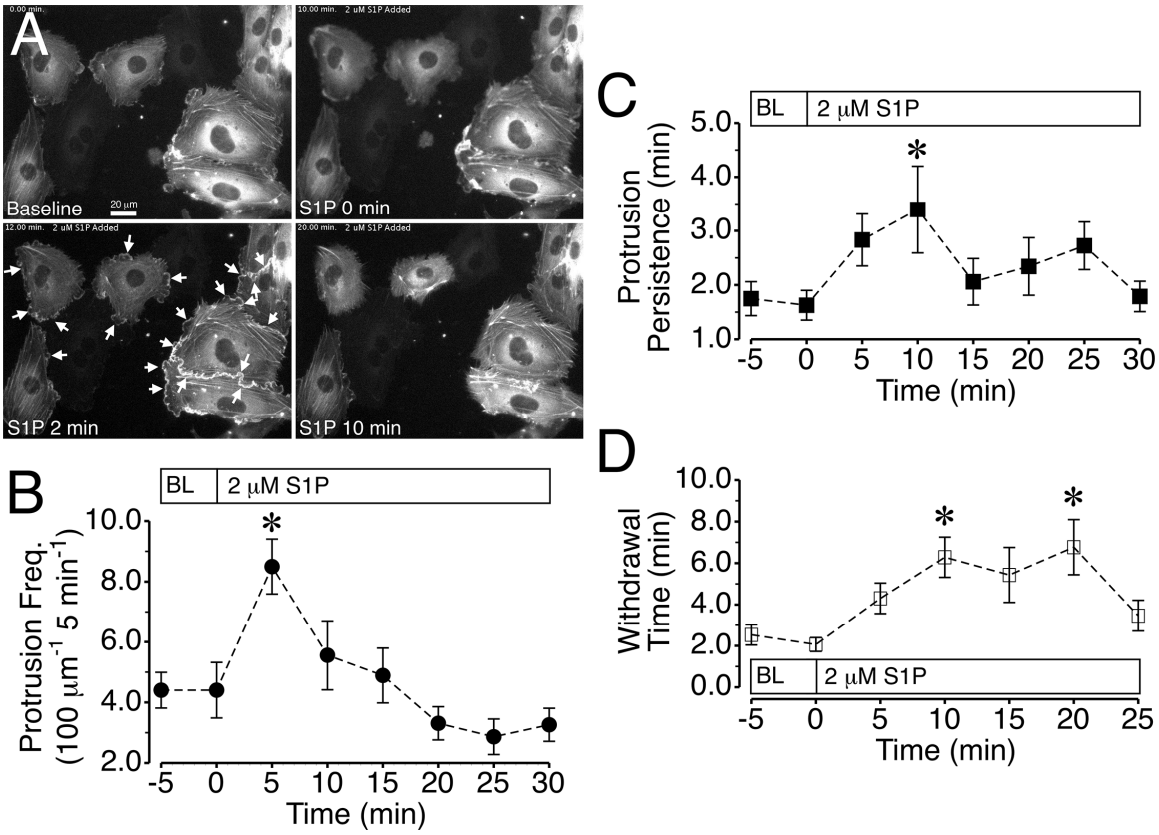

Figure 3 S1P Causes Lamellipodia Protrusions

A. HUVEC expressing GFP-actin displayed frequent protrusion and withdrawal of lamellipodia, (baseline-0 min S1P) and addition of 2 μM S1P caused a coordinated increase in protrusion of lamellipodia (2 min, arrows). Within 10 min, the initial lamellipodia that had formed after S1P was added typically had withdrawn. B. S1P caused a brief, significant increase in protrusion frequency. C. Protrusion persistence also increased significantly at 10 min after S1P was added D. Withdrawal time was significantly sustained for at 10 and 20 min after S1P was added. *P<0.05 versus time 0 min (baseline). For the imaging experiments, N = 9 cells were studied.

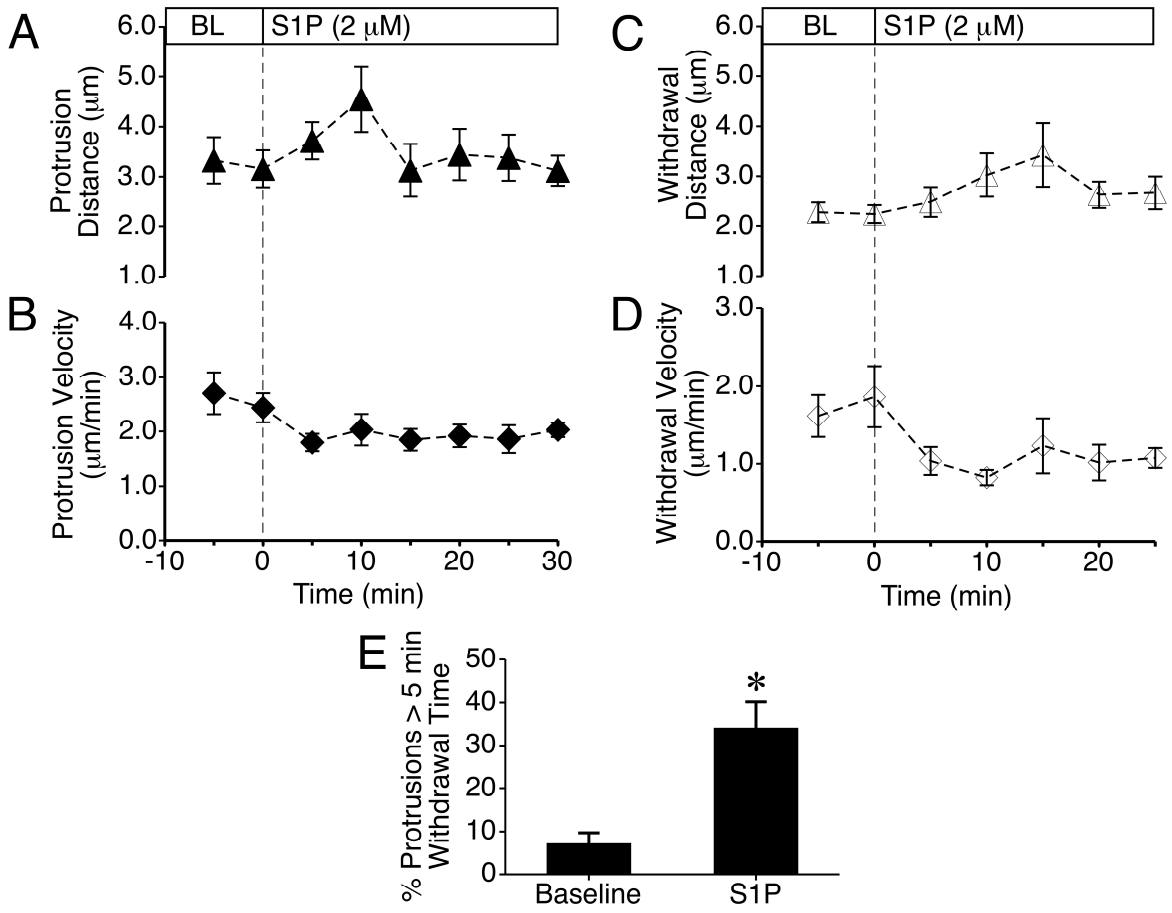

Figure 4 Lamellipodia Parameters of HUVEC Monolayer Before and After S1P Treatment

A. Protrusion distance. B. Protrusion velocity. C. Withdrawal distance. D. Withdrawal velocity. E. Number of protrusions (% of total) that had a withdrawal time lasting 5 minutes or more. *P<0.05, baseline vs. S1P. N = 9 cells studied.

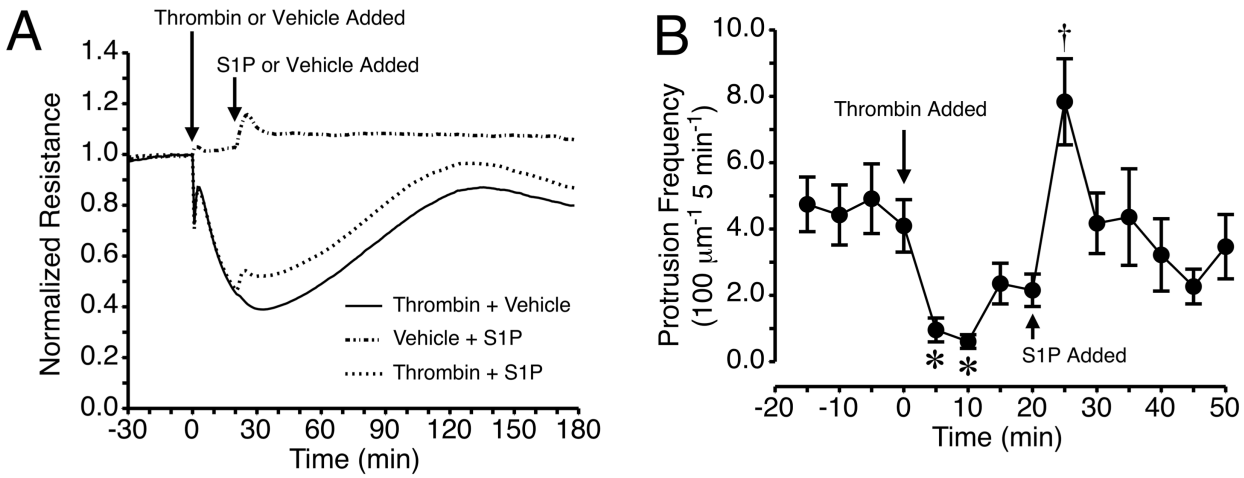

Figure 5 Impact of S1P During Thrombin-Induced Endothelial Barrier Dysfunction.

A. Time course of changes in TER of HUVEC monolayers treated with 1 U/ml thrombin or vehicle at the indicated time point, followed by addition of 2 μM S1P or vehicle 20 min. later. The TER tracings are an average for N = 8 electrode wells in each group. B. Protrusion frequency of HUVEC expressing GFP-actin treated with 1 U/ml thrombin, followed by 2 μM S1P 20 min later. *P<0.05 versus the 0 min time point when thrombin was added. †P<0.05 versus the 20 min time point when S1P was added. N = 9 cells studied.

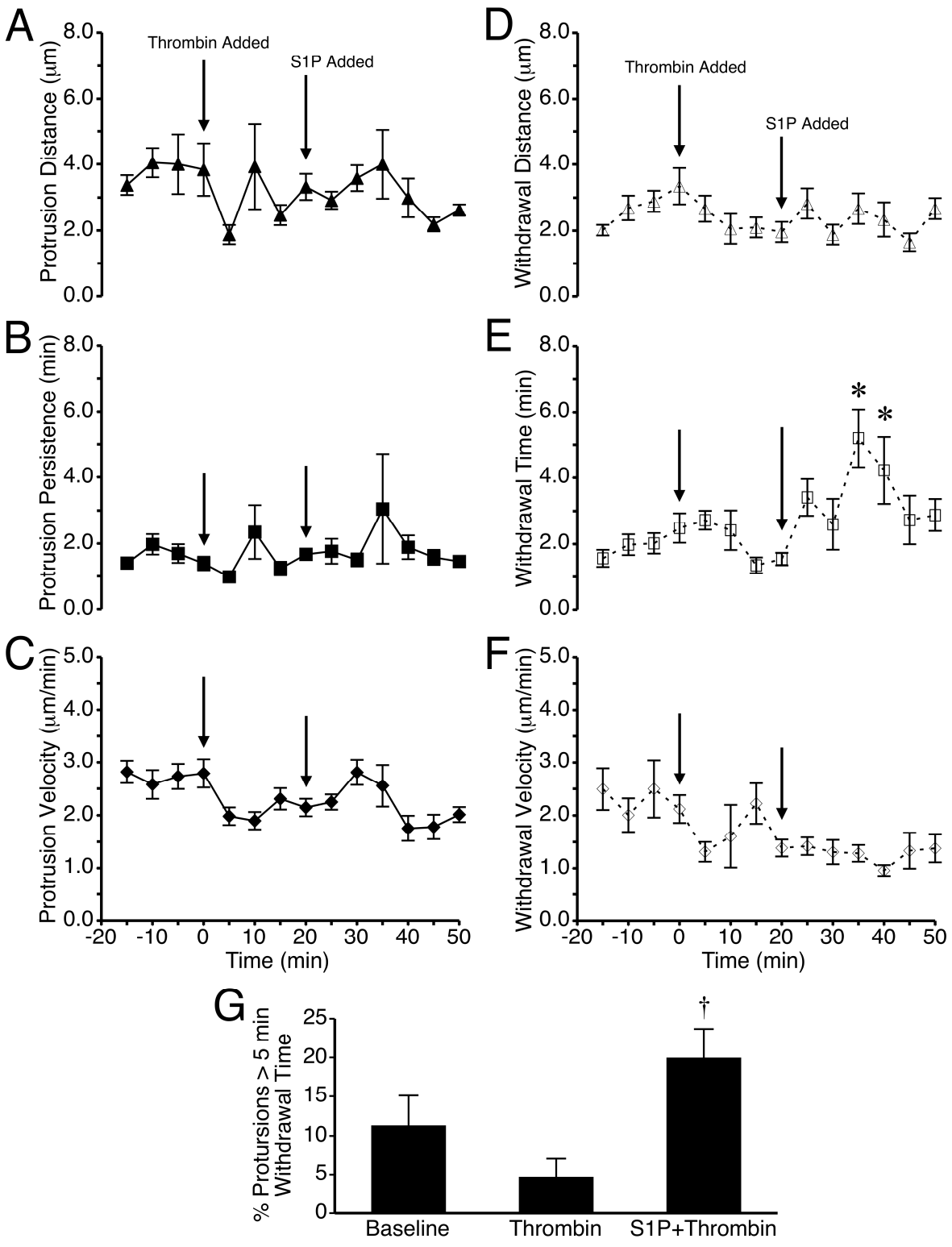

Figure 6 Impact of S1P on Lamellipodia Parameters During Thrombin Treatment

A. Protrusion distance. B. Protrusion persistence. C. Protrusion velocity. D. Withdrawal distance. E. Withdrawal time. F. Withdrawal velocity. G. Percentage of protrusions with a withdrawal time lasting 5 minutes or more. *P<0.05 versus the 20 min time point (when S1P was added). †P<0.05, S1P+thrombin vs. thrombin alone. N = 9 cells studied.

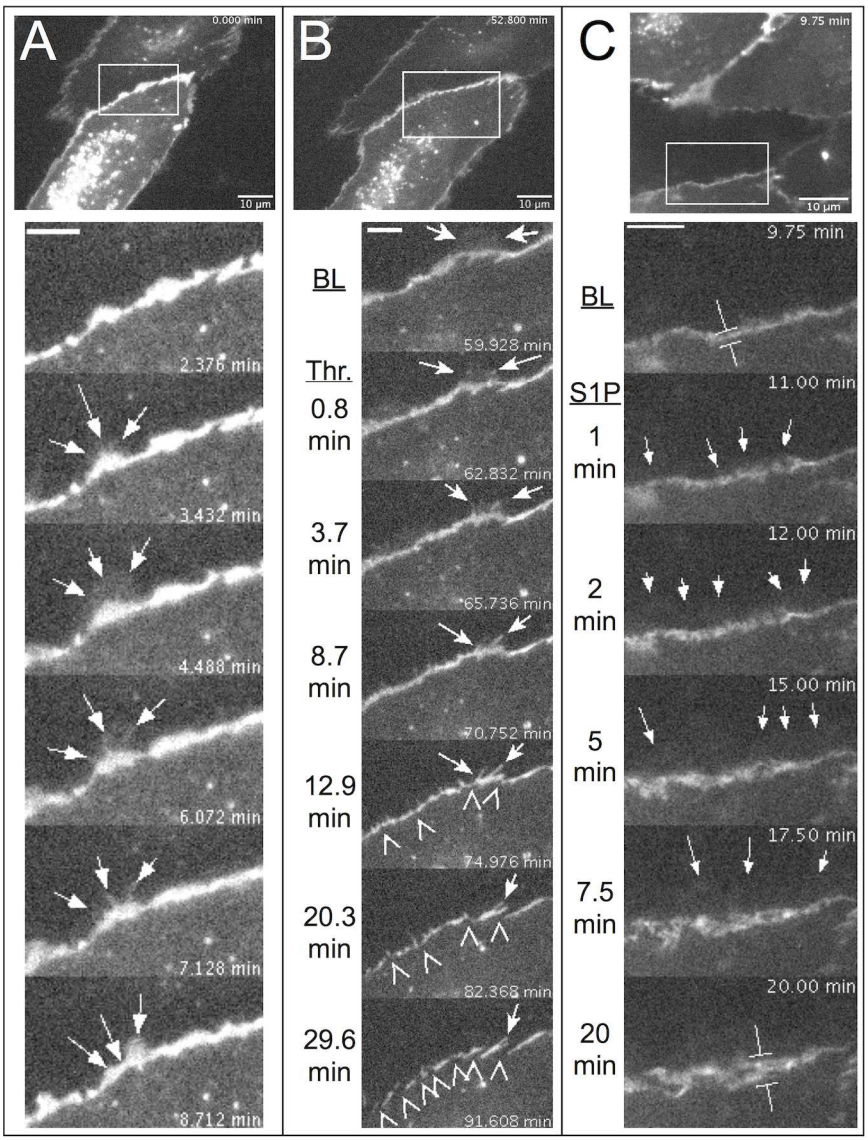

Figure 7 Local Lamellipodia Protruded Beyond Endothelial Adherens Junctions Containing VE-Cadherin-GFP and Were Associated with Junction Stability

At the top of all three panels, an image of HUVEC expressing VE-cadherin-GFP is shown. The bounding box in each top image shows the area studied in the time-lapse montages below. Confluent monolayers were used for all experiments, but not all cells expressed detectable levels of VE-cadherin-GFP. A. Time-lapse imaging revealed that VE-cadherin-GFP was most intense at intercellular junctions and in vesicles around the nucleus. Select time-lapse images of the area in the box from top panel show the protrusion and withdrawal of a local lamellipodium (arrows) that spread toward the cell

in the top of the image from the belt of VE-cadherin-GFP located between two cells. B. The same cells were tracked just before and during 1 U/ml thrombin treatment. Selected time-lapse images from the bounding box in the top panel show how the withdrawal of a local lamellipodium that had protruded prior to thrombin treatment yielded filopodia-like structures containing VE-cadherin (arrows). Subsequently, as fewer lamellipodia protruded from the cell edge, breaks in the continuous belt of VE-cadherin emerged (arrowheads). C. Time-lapse studies before and after treatment with 2 μM S1P show that lamellipodia spread beyond the VE-cadherin-GFP-rich junctions (arrows). In addition, over time the junctional areas containing VE-cadherin-GFP appeared wider than during baseline (compare the calipers at BL and 20 min). Images are representative of observations from at least three different experiments each with thrombin and S1P

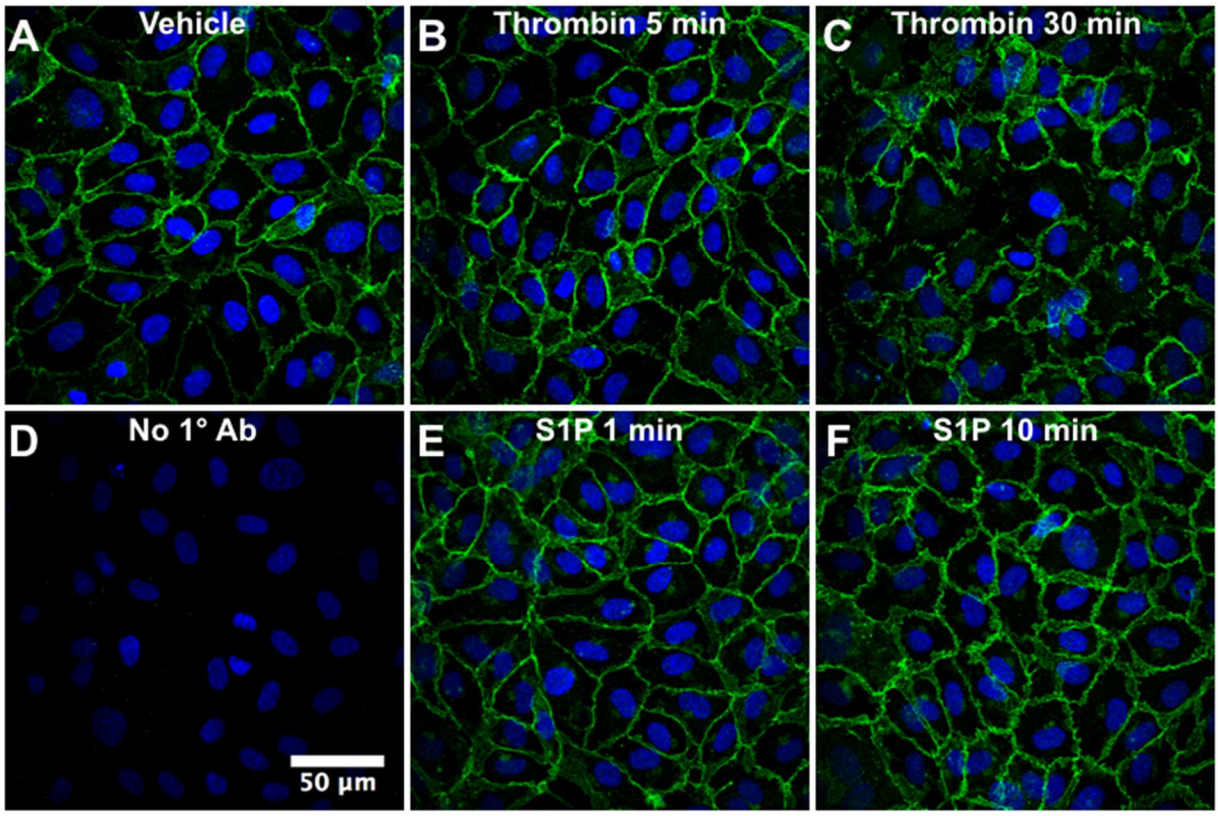

Figure 8 Impact of Thrombin and S1P on VE-Cadherin

A. VE-cadherin of vehicle treated control. B & C. VE-cadherin of monolayer treated with 1U/ml thrombin for 5 min and 30 min respectively. D. No primary antibody control. E & F. VE-cadherin of monolayer treated with 2 µM S1P for1 min and 10 min, respectively. Representive of three separate experiments.

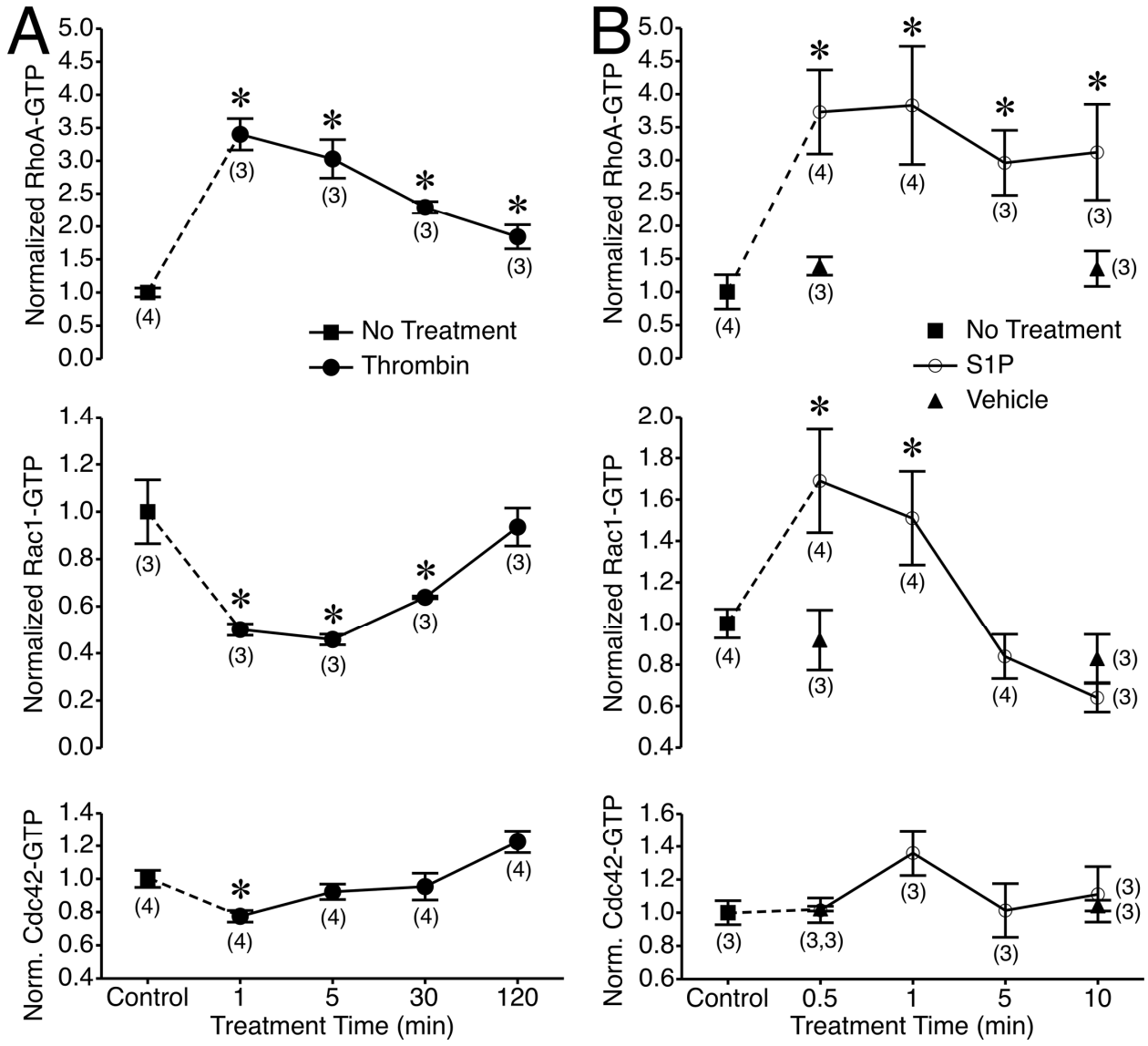

Figure 9 GTPase Activities of RhoA, Rac1 and Cdc42 In Response to Thrombin and S1P

A. Impact of thrombin on GTP-bound RhoA, Rac1, and Cdc42 in cultured HUVEC. Untreated cells served as control. B. Impact of S1P on GTP-bound RhoA, Rac1, and Cdc42 in cultured HUVEC. Untreated cells served as control, and vehicle controls were also tested at the 0.5-min and 10-min time points. The numbers in parentheses indicate the number of replicates for each group. *P<0.05 compared to no treatment control (Note: This data was obtained by former lab technician Peter Hickman).

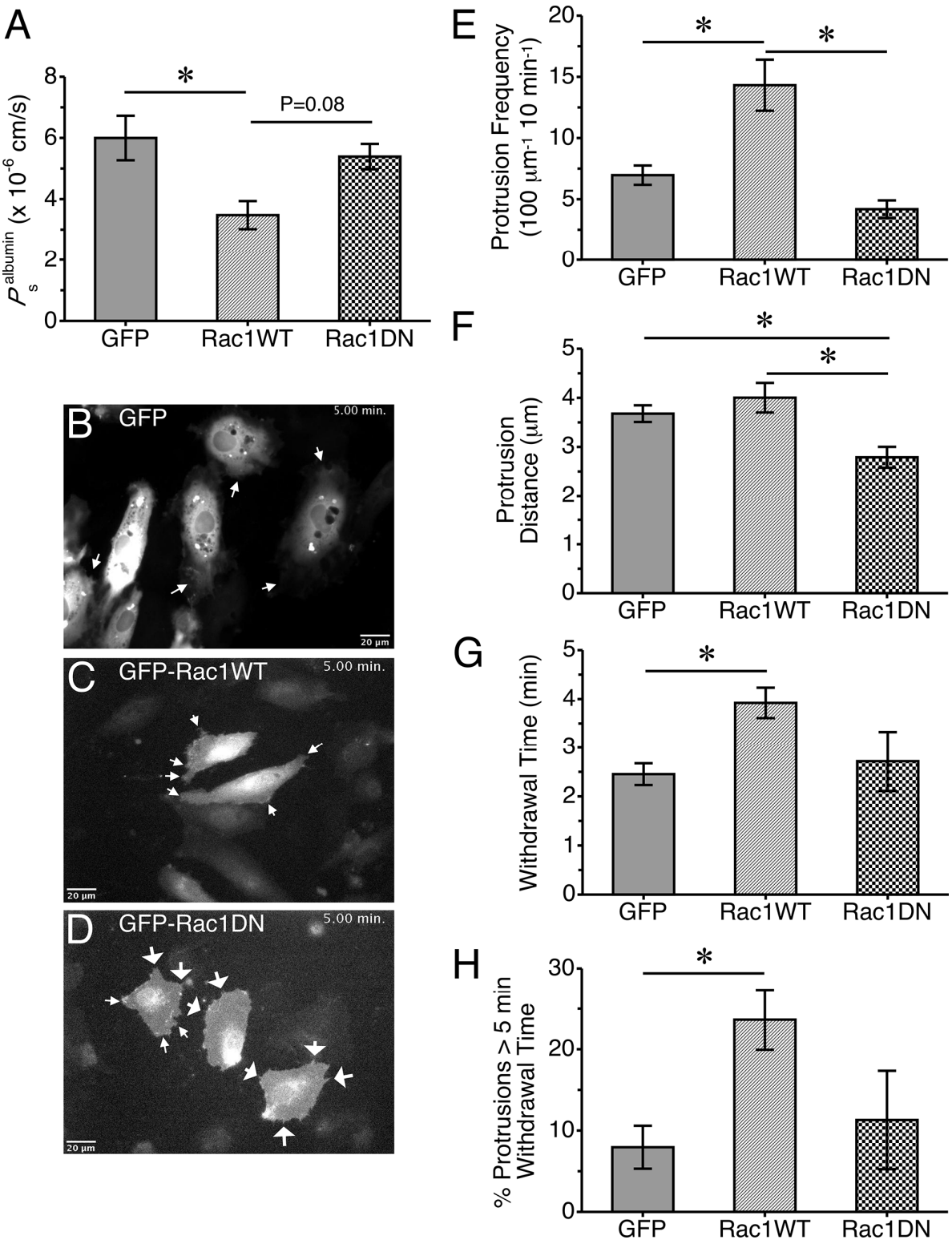

Figure 10 Impact of Overexpression of Wild-Type (WT) or Dominant-Negative (DN) Rac1 on Endothelial Barrier Function and Local Lamellipodia Dynamics

A. $P_s^{albumin}$ of HUVEC monolayers expressing GFP, GFP-Rac1-WT, or GFP-Rac1-DN (N = 4 for each group) ~16 h after transfection. Panels B, C, and D show expression of each construct. These images were obtained ~16 h after transfection. The small arrows indicate lamellipodia, while the arrows with wider arrowheads show filopodia that were prevalent in cells expressing GFP-Rac1-DN. Lamellipodia parameters were also evaluated over a 10-min period: E. Protrusion frequency, F. Protrusion distance, G. Withdrawal Time, H. %Protrusions with a withdrawal time > 5 min. *P<0.05 between the indicated groups. N = 9 cells studied in each group.

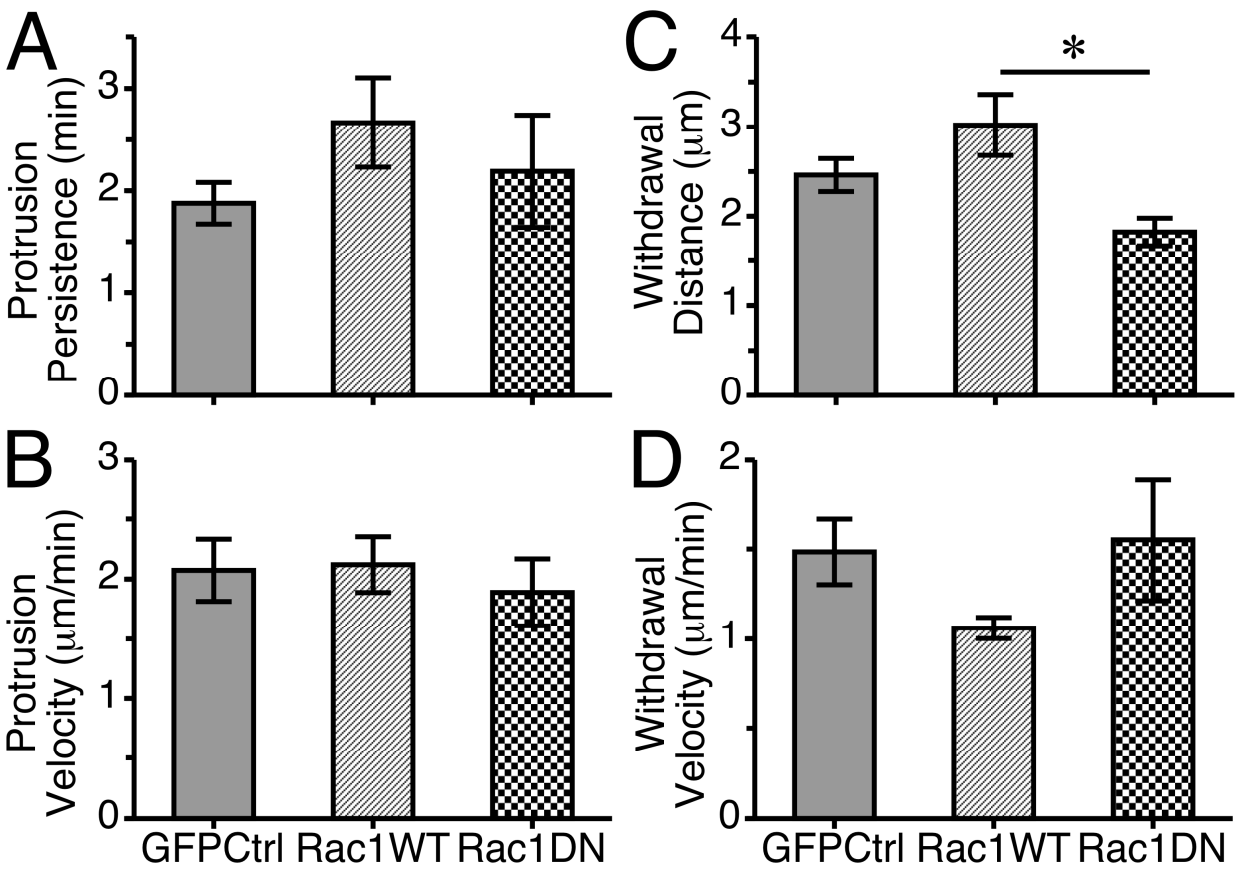

Figure 11 Impact of Overexpression of Wild-Type (WT) or Dominant-Negative (DN) Rac1 on Local Lamellipodia Dynamics

Expression of GFP served as control. A. Protrusion persistence. B. Protrusion velocity. C. Withdrawal distance. D. Withdrawal velocity. *P<0.05 between the indicated groups N = 9 cells studied for each group.

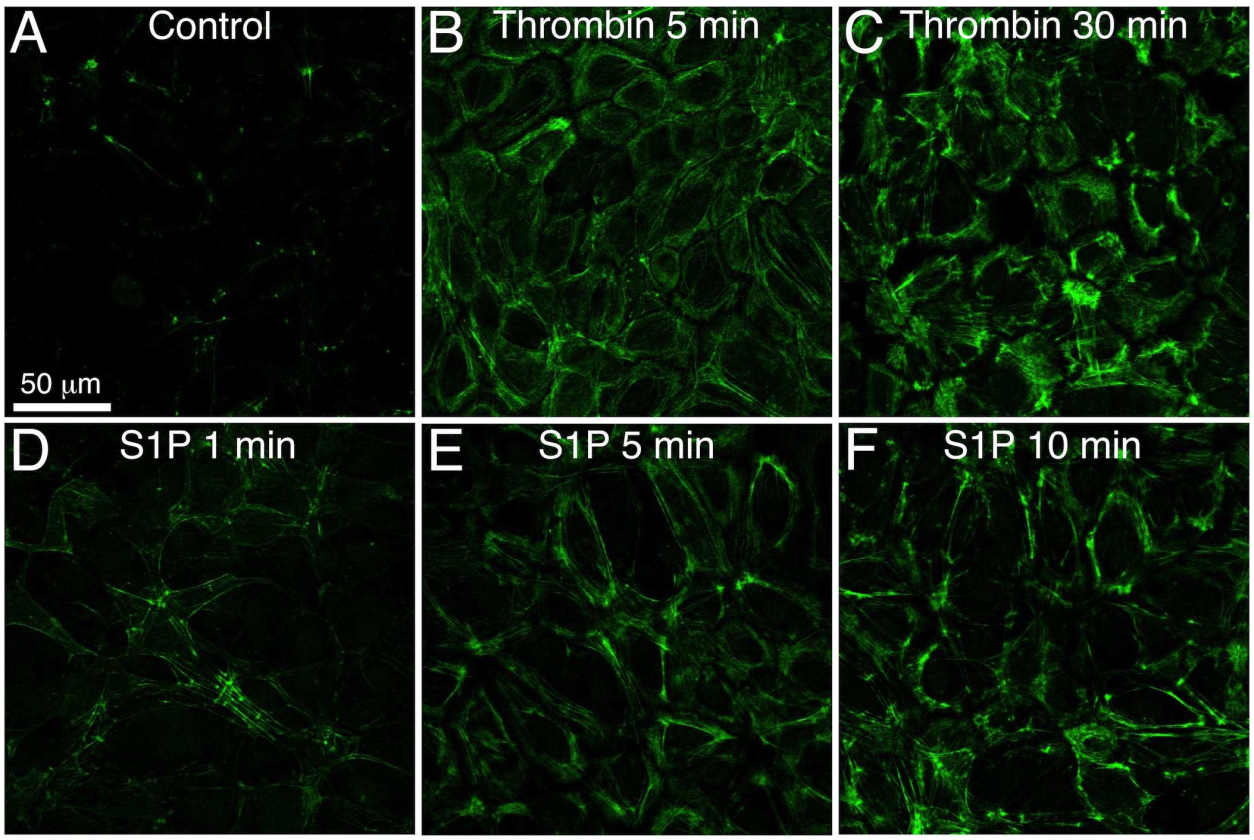

Figure 12 Both Thrombin and S1P Increased MLC-2 Phosphorylation at Thr-18/Ser-19

A. Untreated controls B & C. MLC-2 phosphorylation at Thr-18/Ser-19 after 5 min or 30 min respectively of 1U/ml thrombin treatment. D, E, F. MLC-2 phosphorylation at Thr-18/Ser-19 after 1 min, 5 min and 10 min of 2 μM S1P treatment. Scale bar = 50 μm. Representative of three separate experiments.

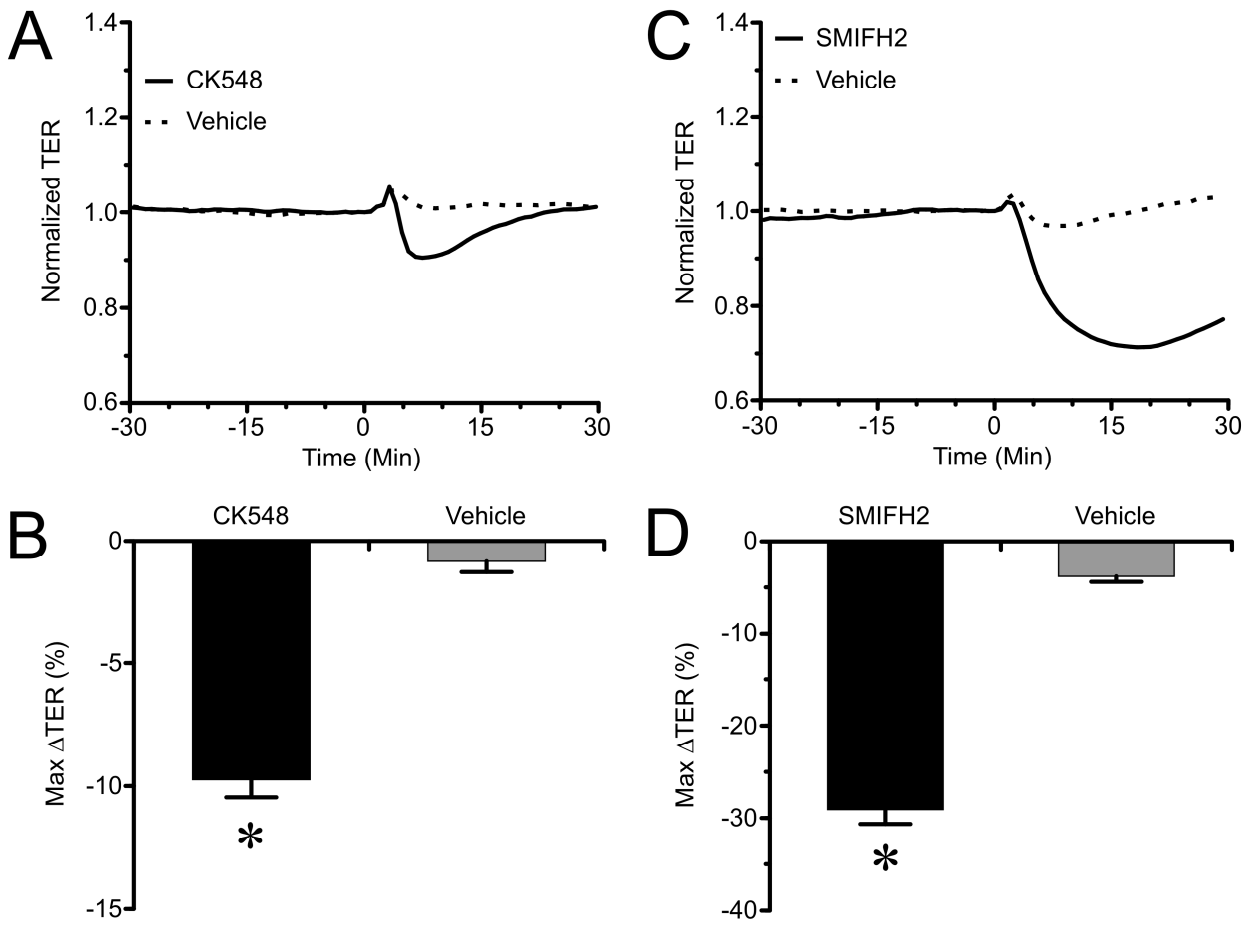

Figure 13 Impact of The Arp2/3 Inhibitor CK548 and Formin Inhibitor SMIFH2 on Baseline Barrier Integrity

A & C. Time courses of TER changes when treated with Arp2/3 inhibitor CK548 (A) and formin mDia inhibitor (C), N = 8 for each group. B & D. Mean maximal changes in TER (%) after CK548 (B) or SMIFH2 (D). *P<0.05, inhibitors vs. vehicle treated group.

Chapter Four:

Activation of RhoA, But Not Rac1, Mediates Early Stages of S1P-Induced Endothelial Barrier Enhancement

4a. Introduction

S1P is an endogenously released, bioactive lipid that has potent endothelial barrier enhancing effect at its physiological concentration. After binding to its receptors, S1P induces dynamic cytoskeletal, junctional and adhesion changes and increases endothelial barrier function [20,52,90,142,143,158]. It is thought that Rac1 activation is responsible for S1P-mediated endothelial barrier enhancement. Several studies have shown that activation of Rac1 contributes to the endothelial barrier enhancement [4,122,156,165]. In contrast, RhoA activation usually results in an increased permeability on the endothelial monolayer [4,99,166,167]. Collectively these data have led to the general notion that Rac1 activation enhances endothelial barrier enhancement, while RhoA activation disrupts integrity of the endothelium. Some recent data have challenged this paradigm, however an elegant study by Szulcek and colleagues demonstrated that RhoA activation at the cell periphery correlates with barrier integrity, while its activation in the perinuclear area of the endothelial cell contributes to barrier disruption [102]. Another observation challenging this paradigm is our recent finding that S1P elicits a strong increase in the GTP-bound, activated forms of both RhoA and Rac1 [134]. In addition, Xu and colleagues previously observed that

inhibition of RhoA effector Rho-associated protein kinase (ROCK), attenuates S1P-induced endothelial barrier enhancement [52,129]. These findings raise the question on the relative contribution of RhoA and Rac1 in S1P-induced endothelial barrier enhancement, and whether spatiotemporal activation of RhoA is a key factor.

In this part of the study, we investigated the roles of Rac1 and RhoA in S1P-induced endothelial barrier enhancement, the spatiotemporal activation of RhoA in response to S1P, and the potential roles of MLC2 phosphorylation, actin fiber formation, and redistribution of the focal adhesion protein vinculin.

4b. Results

4b.i. S1P Rapidly Increased Endothelial Barrier Function in A Concentration-Dependent Manner

S1P has a well-characterized ability to enhance endothelial barrier function at its physiological concentration. S1P, at higher than its physiological concentration, can disrupt endothelial barrier integrity [168]. To gain knowledge on how different concentrations of S1P affect endothelial barrier function, we performed a concentration-response study on HUVEC monolayers (Fig 14). When examining the maximum increase in TER that could be elicited by each concentration of S1P, we observed that treatment with S1P significantly increased TER compared to vehicle control in a concentration-dependent manner (Fig 14 A). However, it is worth noting that while all concentrations tested caused a rapid, significant, initial rise in TER, with higher concentrations of S1P this elevation in TER was typically not sustained, and sometimes fell below the baseline TER within 30 min (Fig 14 B).

4b.ii. Rac1 Is Important for Baseline Barrier Integrity.

We previously observed that overexpression of Rac1 can reduce permeability of endothelial monolayers. Here, we assessed whether Rac1 is critical for maintaining baseline barrier integrity in both HUVEC and HDMEC monolayers. Consistent with our previous finding, treatment with the Rac1 inhibitor Z62954982 caused a concentration-dependent decrease in TER in both HDMEC (Fig 15 A & B) and HUVEC (Fig 15 C & D). We also used a second approach, which was to knockdown Rac1 expression using siRNA. Significant Rac1 knockdown was achieved in both HUVEC and HDMEC as assessed by Western blot (Fig 15 E). Rac1 knockdown rendered a significantly lower baseline TER at 72 h compared to controls (Fig 15 F). Taken together, our data indicate that Rac1 has a critical role in the maintenance of baseline endothelial barrier integrity.

4b.iii. Inhibition of Rac1 Failed to Block S1P-Induced Endothelial Barrier Enhancement.

Rac1 has been previously reported to mediate the barrier protective effect of S1P [114]. We tested the extent to which the selective Rac1 inhibitor Z62954982 would attenuate S1P-induced endothelial barrier enhancement. We applied the inhibitor at a concentration of 50 μM (based on our results in Fig 14) to either HUVEC or HDMEC (Fig 16 A & C). To our surprise, after pretreatment with Z62954982, S1P still significantly increased TER in a similar manner as with HUVEC that did not receive pretreatment with the inhibitors (Fig 16 B & D). These data suggest that the endothelial barrier enhancement elicited by S1P may not require Rac1 activation.

4b.iv. Overexpression of WT Rac1 or DN Rac1 Did Not Affect S1P-Induced Endothelial Barrier Enhancement.

In addition to the pharmacological inhibitor data, we tested the role of Rac1 by transfecting HUVEC or HMDEC with WT and DN Rac1 plasmids (Fig 17), a method that has been described by our lab not to significantly alter baseline endothelial barrier function. Again to our surprise, S1P treatment significantly increased TER in the cells transfected with DN Rac1 in a similar manner to WT Rac1 (Fig 17 B & D). These data provided additional evidence that S1P is able to enhance endothelial barrier function independently of Rac1 activation.

4b.v. Knockdown of Rac1 with siRNA Did Not Affect S1P-Induced Endothelial Barrier Enhancement.

A third approach we used was to test the extent to which siRNA-induced Rac1 knockdown would inhibit S1P-induced endothelial barrier enhancement (Fig 18). Despite the significant reduction in Rac1 expression and reduction in TER (Fig 15 F), no inhibitions of S1P-induced increases in TER were apparent (Fig 18 A & C). To our surprise, S1P-induced elevation in TER was significantly greater in the Rac1 knockdown HUVEC and HDMEC, compared to transfection with the scrambled siRNA (Fig 18 B & D), probably because the baseline TER is slightly lower after Rac1 knockdown (see Fig 15 F; The TER data in Fig 18 are normalized to the time point just prior to addition of S1P). These data demonstrate that reduction of Rac1 expression does not impair the ability of S1P to enhance endothelial barrier function.

4b.vi. Inhibition of RhoA Attenuated S1P-Induced endothelial Barrier Enhancement.

We previously reported that that S1P causes a strong and sustained activation of RhoA in HUVEC (Fig 9 B). To test whether inhibition of RhoA impacts S1P-induced increase in TER, we utilized the specific RhoA inhibitors Rhosin (25 μM) and Y16 (25 μM) on both HUVEC and HDMEC. As the two drugs can work synergistically to keep RhoA in its inactive, GDP-bound form[169], we also utilized their combination (5 μM each). In HUVEC, pretreatment with either Rhosin or Y16 alone significantly reduced S1P-induced increase in TER (Fig. 21), yet each drug alone did not cause a significant inhibition in HDMEC (Fig 20). In both HUVEC and HDMEC, pretreatment with the combination of Rhosin and Y16 caused a significant attenuation of the S1P-induced increase in TER (Fig 19). These data suggest that RhoA activation partially mediates S1P-induced endothelial barrier enhancement, although to different extents in HUVEC and HDMEC.

4b.vii. S1P-Induced RhoA Activation Occurred Primarily at The Endothelial Cell Periphery.

As the RhoA inhibitor produced its maximum inhibitory effect on HUVEC, we focused on HUVEC for further studies of the mechanism. The recent report of differential localized RhoA activation in endothelial cells during endothelial barrier maintenance and disruption [102] prompted us to investigate the localization of RhoA activation after stimulation with S1P. We transfected HUVEC with a RhoA FRET biosensor to measure RhoA activation over time in individual cells. During baseline, RhoA activation at any given point in the cell was low and oscillatory in nature, primarily

located in the outer peri-nuclear region. S1P treatment significantly increased RhoA activity, shifting the maximal activity primarily near cell borders (Fig 22) The data suggest that S1P elicits a specific spatiotemporal activation of RhoA near the borders in order to promote enhanced endothelial barrier function.

4b.viii. Inhibition of RhoA Attenuated S1P-Induced Myosin Light Chain 2 (MLC-2) Phosphorylation.

Because S1P elicited increased phosphorylation of MLC-2 in the vicinity of cortical actin near the cell junctions is thought to confer stronger endothelial barrier function [170], we evaluated the extent to which inhibition of RhoA with combined Rhosin and Y16 (both at 5 μM) can block S1P-induced MLC-2 phosphorylation on its Ser18/Thr19 activation site (Fig 23 A). The results show that S1P rapidly increased MLC-2 phosphorylation within 1 min, demonstrating a peak at 10 min that returned to baseline level by 30 min. Inhibition of RhoA completely blocked this S1P-induced phosphorylation of MLC-2 (Fig 23 B). These data suggest that RhoA is a critical mediator for S1P-induced phosphorylation of MLC-2.

4b. ix. Inhibition of RhoA Attenuated S1P-Induced Vinculin Mobilization to The Cell Periphery.

Both RhoA activation and MLC-2 phosphorylation has been implicated in the reorganization of the actin cytoskeleton and formation of focal adhesions. We tested whether the role of RhoA in the formation of actin fibers and vinculin-containing focal adhesions near intercellular junctions [171]. Pretreatment with Rhosin and Y16 abrogated S1P-induced F-actin and vinculin recruitment to the cell periphery (Fig 24).

These data suggest that S1P-induced F-actin and vinculin assembly at the cell periphery involves RhoA activation.

4c. Discussion

This study presents new evidence that Rac1 activation is not required for S1P to enhance endothelial barrier function in both HUVEC and HDMEC monolayers. We were initially surprised by these results based on the well-established role of Rac1 in maintaining baseline endothelial barrier function [141,165,172] and reports that have suggested its role in S1P-induced enhancement of the endothelial barrier [90,142,143]. However, closer investigation of the literature revealed that the data directly supporting the role of Rac1 in S1P-induced endothelial barrier enhancement were quite limited. To our knowledge there have been no previous investigations that rigorously coupled siRNA knockdown of Rac1, DN Rac1 expression, and pharmacologic strategies all in one model to test the role of Rac1 in S1P-induced endothelial barrier enhancement.

To understand discrepancies between our study and reports in the literature, it is important to discuss the time course of the endothelial barrier response to S1P. Our current results indicate that all concentrations of S1P tested (0.05–10 μM) initially increase barrier function of HUVEC monolayers (Fig 14 A), but with higher concentrations of S1P (10 μM), this increase is often not sustained (Fig 14 B). Previously it was reported that physiological concentrations (10 nM– 2 μM) of S1P enhance endothelial barrier function, but that high concentrations of S1P (≥ 5 μM) disrupt endothelial monolayer integrity, however detail of the time-course of changes in barrier function were limited. Adamson et al reported that 1 μM S1P decreased hydraulic conductivity of single-perfused microvessels at 30 min but not 60 min, while

higher concentrations (5 and 10 μM) increased hydraulic conductivity at 60 min but not 30 min [173]. Other groups have reported similar results [168,174,175]. In our previous study, we showed that 2 μM S1P caused initial, rapid spreading and subsequent contraction of endothelial cells, and that the S1P-induced initial increase in TER directly correlated with increased protrusion of local lamellipodia at endothelial junctions. It is clear that different S1P concentrations have distinct impacts on whether the initial endothelial barrier enhancement is sustained. While this study focused mainly on the mechanism responsible for the early stage endothelial barrier enhancement by S1P, a potential limitation is that higher concentrations of S1P may activate additional receptor subtypes and differentially affect Rho family small GTPases, or stimulate additional signals, resulting in a less sustained response and eventual reduction in barrier function, explaining the findings reported in other studies [129,168,173].

We confirmed that Rac1 is critical for baseline barrier integrity, as shown by other investigators [20,141]. However, we found no evidence that inhibition of Rac1 activation, either by pharmacologic agents, overexpression of dominant-negative Rac1, or depletion of Rac1 with siRNA, could inhibit S1P-induced barrier enhancement. One previous report indicated that Rac1 depletion with siRNA ablated TER enhancement following 1 μM S1P treatment in HPAEC, but this data was limited to a single time point at 60 min [176]. Studies utilizing expression of a dominant-negative Rac1 in HPAEC showed reduction of the peak S1P-induced increase in TER detected within the first 15 min of treatment [147], and that DN Rac1 expression attenuated S1P-induced increase in TER from baseline in HPAEC [177]. Possible explanations for the differences between the current study and previous reports are the use of different endothelial cell

types and experimental conditions. In HUVEC, pretreatment with 10 μM NSC23766 reportedly reduced the increase in barrier function elicited by 0.5 μM S1P [168]. In that study, normalized data were presented, less frequent time points were obtained, and a different detection system (Ussing chamber method) was used to determine TER, which is less sensitive than ECIS. With the less frequent time points measured, it is possible that the initial peak increase in TER caused by S1P was missed. It is also notable that because the IC_{50} of NSC23766 is 50 μM that perhaps only a 20% inhibition of Rac1 activity occurred. An additional study, which nicely showed that release caged S1P loaded intracellularly can also increase TER, application of 50 μM NSC23766 could significantly attenuate S1P-induced barrier enhancement [178]. However, it is difficult to compare these results with ours as only one single time point was shown and it is unclear whether NSC23766 had any impact on the baseline barrier function. It is worth noting that we have observed that 50 μM NSC23766 can reduce barrier function of dermal lymphatic endothelial cells and increase permeability of intact, isolated rat mesenteric venules [134]. In current study, we elected to use the Rac1 inhibitor Z62954982 (IC_{50} = 12 μM) that is 4 times more effective than NSC23766 (IC50 = 50 μM) [179], which significantly reduced TER, in a concentration-dependent manner. These data indicate reductions in barrier function elicited by inhibition of Rac1 must be taken into consideration in the overall data analysis. Based on the current data, we think that the rapid, S1P-induced, early rise in TER occurs independently of Rac1 activation. The apparent discrepancy of our data from reports in the literature may be summarized by differences in the time points of data collected, whether baseline changes are reported, and perhaps to some extent the endothelial cell types or other experimental conditions.

A current limitation is the lack of studies with Rac1 deletion or specific inhibition in intact postcapillary venules, which represents a future step that will help resolve this issue.

Previously we showed that in addition to a transient Rac1 activation, RhoA activity was greatly increased and sustained for at least 10 min upon S1P treatment [134]. In the current study, we employed a FRET biosensor to monitor RhoA activity over time in individual cells. We observed that RhoA activity during our baseline measurements oscillates in the outer peri-nuclear regions. We also observed an overall increase in RhoA activation after S1P treatment, with high levels of RhoA-GTP near endothelial cell borders than in the central areas of cells (Fig 23 A). Our results are in agreement with data presented by Szulcek and colleagues, who demonstrated RhoA activation localized near intercellular gaps during their closure [102]. In their study they also demonstrated that RhoA activation in the central area of the cells is barrier disruptive while peripheral RhoA activation is barrier protective. With the concept in mind, it is not surprising that we observed that pretreatment of the endothelial monolayers with RhoA inhibitors attenuated the S1P-induced barrier enhancement that begins almost immediately after S1P is added to the bath. There is some variation of how the Rho inhibitors affect the ability of HUVEC and HDMEC to respond to S1P, which could be due to a variety of reasons including vessel source, donor source, and how well each type of cell thrives in culture. Still, this data suggests that RhoA is involved in the initial rise in TER elicited by S1P, and is in agreement with data from other groups that have shown that inhibition of the RhoA effector, ROCK, attenuates S1P-induced barrier enhancement[52,102,129]. Combined, these data indicate that the RhoA/ROCK pathway contributes, at least in part, to S1P-induced endothelial barrier

enhancement. It is also worth noting that in some studies, inhibition of RhoA or ROCK has caused a decrease in the baseline TER [100]. Such data supports that the peripheral activation of RhoA indicated by our FRET probe study and that of Szulcek and colleagues contributes to endothelial barrier maintenance [102].

Several reports have indicated that RhoA- or ROCK-mediated increases in phosphorylation of MLC-2 is endothelial barrier disruptive, particularly with inflammatory stimuli, such as LPS, signals from activated neutrophils, or VEGF [100,129,133,180-182]. However, Garcia and colleagues characterized that S1P increases cortical MLC-2 phosphorylation and suggested that this contributes to the S1P-induced barrier-protective effect [52]. Moreover, Dudek and colleagues revealed that myosin light chain kinase (MLCK) activation by Abl tyrosine kinase is important for S1P-induced barrier enhancement [170]. Such findings suggested that the role of MLC-2 and the actin cytoskeleton have a general role in mediating either increases or decreases in endothelial barrier function. Concordantly, we observed that S1P significantly increases the phosphorylation of MLC-2 on Ser18/Thr19. Garcia and colleagues also observed that inhibition of MLCK failed to block the ability of S1P to increase TER [52]. With the knowledge that ROCK can increase MLC-2 phosphorylation by inhibiting the MLC-2 phosphatase by phosphorylating the targeting subunit MYPT-1 [60], we studied this alternative pathway. We found that inhibition of RhoA abrogated the S1P-induced phosphorylation of MLC-2 at its regulatory sites. MLC-2 phosphorylation at the cell cortex is thought to stabilize the cortical actin cytoskeleton [122,183]. In addition, myosin activation has been suggested to promote lamellipodia formation, and several reports have suggested that local lamellipodia formation at intercellular junctions

contribute to endothelial barrier integrity [170,184,185]. We recently showed that S1P increased local lamellipodia at cell borders in association with increased TER, and that blockade of the myosin II ATPase, which selectively reduced local lamellipodia without affecting other actin-containing structures like stress fibers or cortical actin cables, decreased TER. In addition, it is interesting that RhoA inhibition also blocked vinculin mobilization to the cell periphery. Previous studies have suggested that S1P-induced endothelial barrier enhancement can be VE-cadherin independent [129]. Combined with the data in current study, S1P's barrier protective effect appears to be complex, as few inhibitors completely blunt its barrier protective effects. Based on our data and those of others [52,170], we think it is reasonable to state that phosphorylation and MLC-2 and actin cytoskeleton activation have general roles in the control of endothelial barrier function, and are likely guided by other factors or by location of action within cells. We speculate that increased MLC-2 phosphorylation at the cell periphery may stabilize cortical actin, promote lamellipodia protrusions that anneal cellular gaps, induce focal adhesion complex assembly and mobilization to the periphery to maintain or enhance endothelial barrier function.

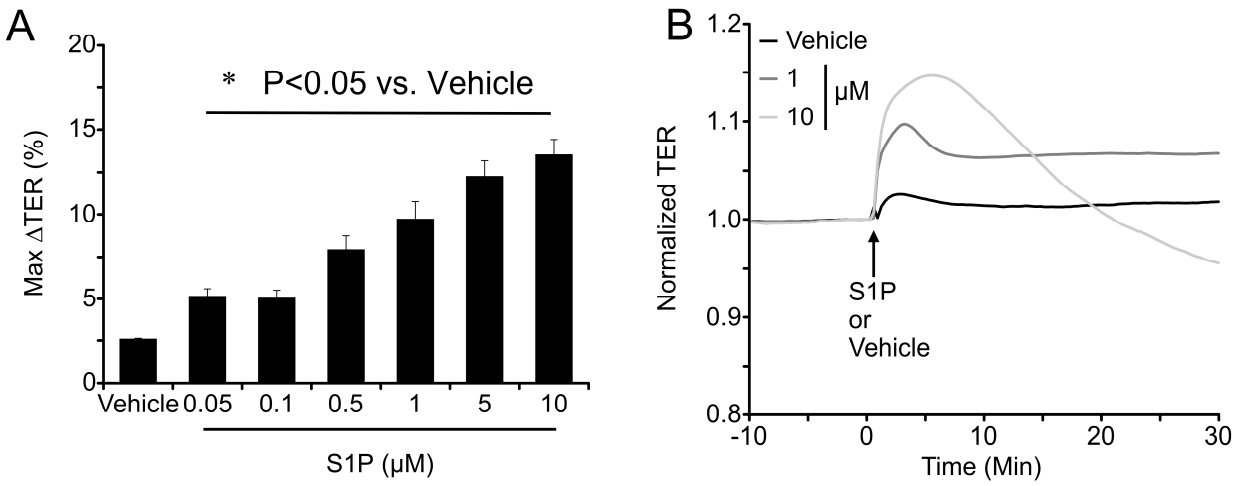

Figure 14 S1P Caused Initial Endothelial Barrier Enhancement Is Concentration-Dependent

A. Maximal change in TER (%) from baseline within the initial 10-min time window after addition of the indicated concentrations of S1P. *P<0.05 versus vehicle treated group. B. Comparison of representative tracings of TER of HUVEC monolayers treated with 1 μM or 10 μM S1P. After the initial increase in TER, with higher concentrations of S1P (10 μM shown here), this elevated TER is typically short-lived and often decreases to a level below the initial baseline.

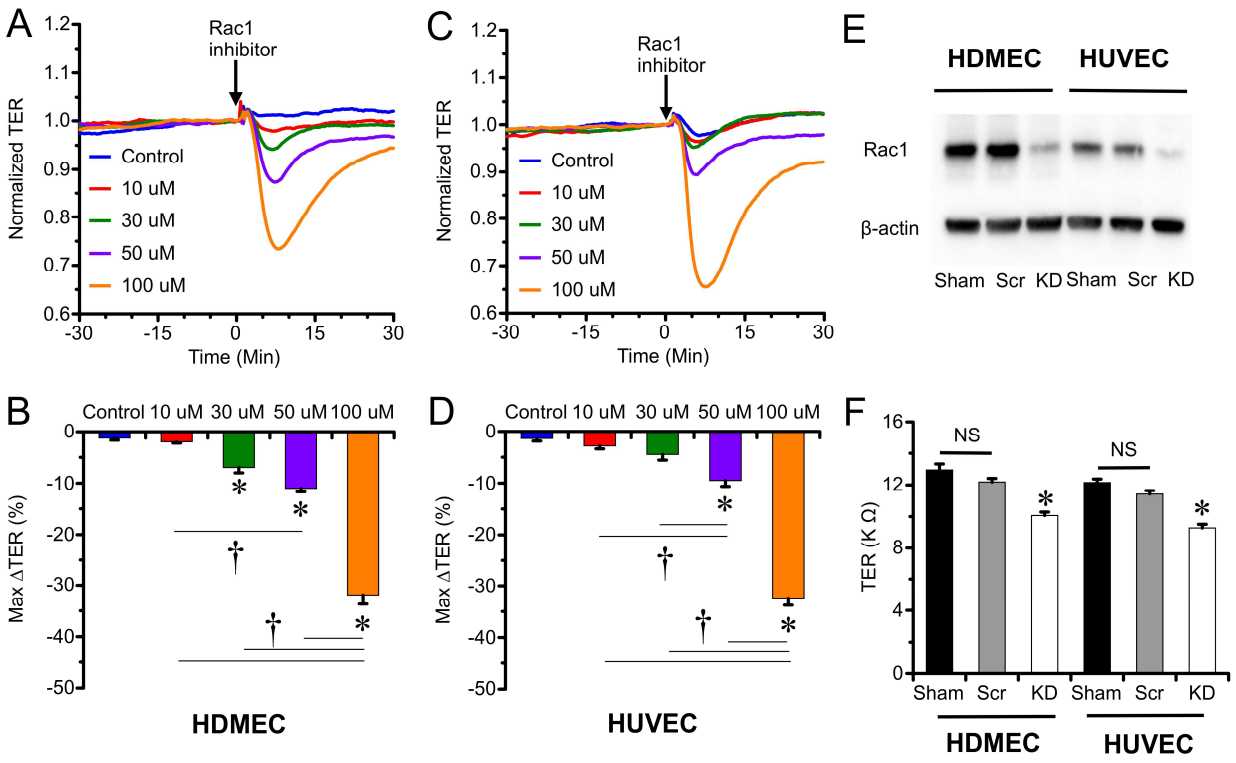

Figure 15 Pharmacological Inhibition or siRNA-Mediated Knockdown of Rac1 Impaired Baseline Endothelial Barrier Integrity

A. Treatment with the selective Rac1 inhibitor Z62954982 reduces TER in a concentration-dependent manner in HDMEC monolayers. B. Comparison of the mean maximum decreases in TER in the 30-min time window for each concentration of Z62954982 in HDMEC monolayers. Panels C & D show that Z62954982 produces a similar concentration-dependent TER in HUVEC monolayers. E. Western blot confirming knockdown (KD) with Rac1-specific siRNA, compared to sham and scrambled RNA (Scr) control groups. Bands for β-actin from re-probed blots confirmed equivalent loading of protein for each lane. F. Mean baseline TER values of HDMEC and HUVEC monolayers in Rac1 knockdown, scrambled control, and sham-transfected groups. *P<0.05 versus vehicle treated group. †P<0.05 versus other concentrations.

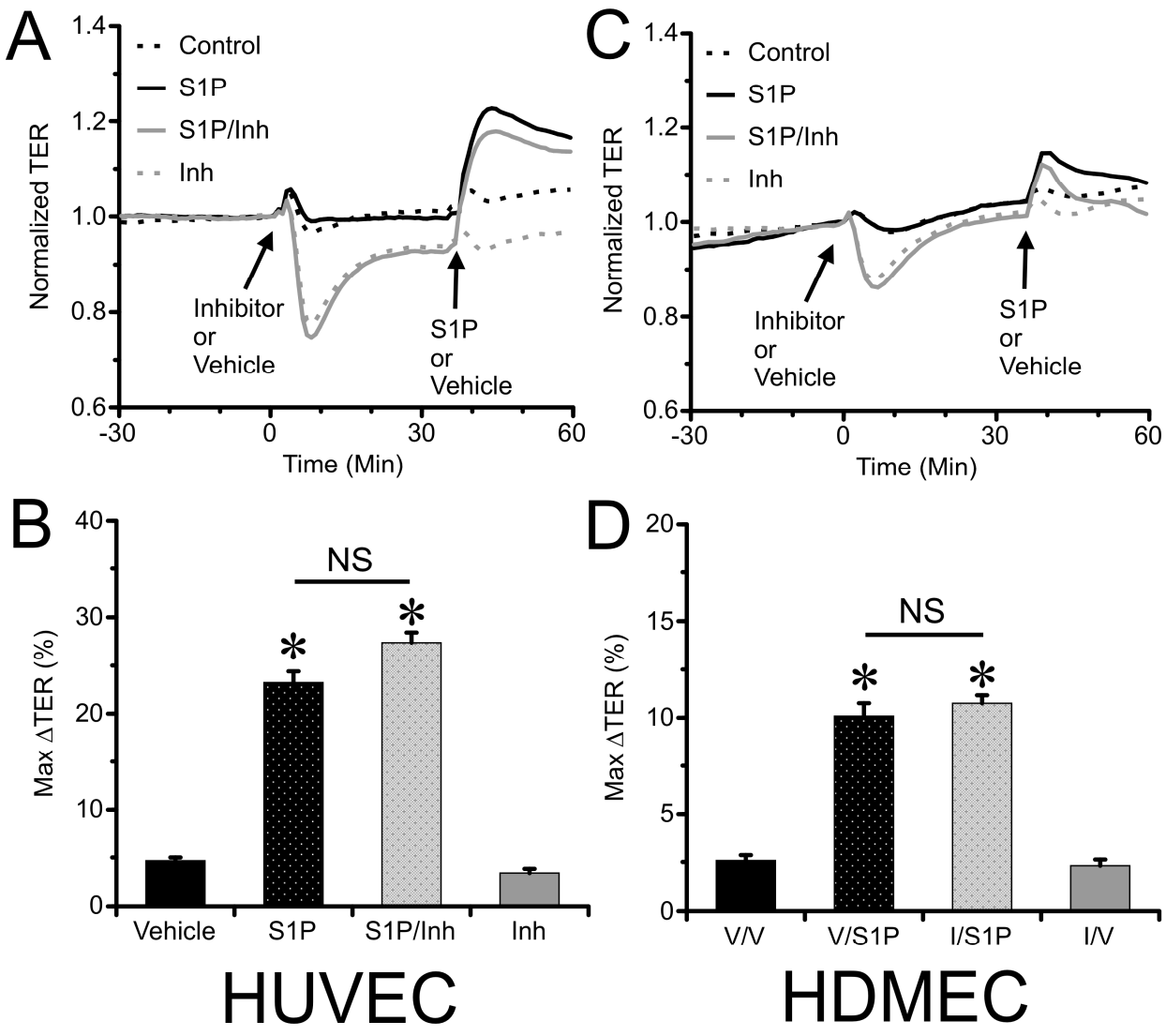

Figure 16 Pharmacologic Inhibition of Rac1 Failed to Block S1P-Induced Endothelial Barrier Enhancement in HUVEC and HDMEC Monolayers

A. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with Rac1 inhibitor Z62954982 or vehicle control, followed by treatment with 2 μM S1P are shown (N = 8 for each group). B. Mean maximal change in TER (%) of HUVEC monolayers after S1P treatment within the first 10-min window. Panels C & D show corresponding results for HDMEC monolayers (N = 8 each group). *P<0.05 vs. S1P Vehicle pretreated groups.

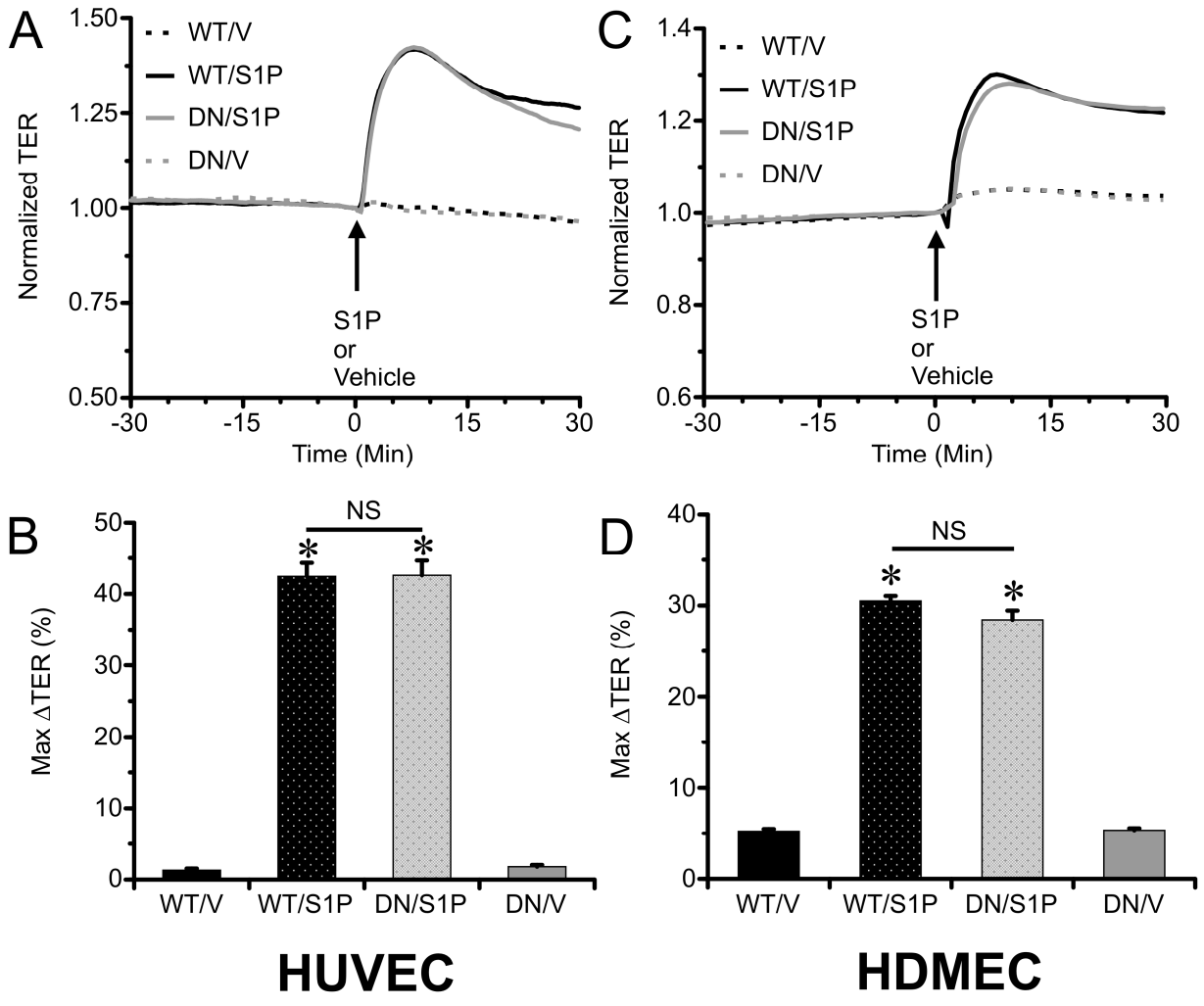

Figure 17 Overexpression of WT or DN Rac1 in HUVEC or HDMEC Did Not Alter S1P-Induced Endothelial Barrier Enhancement

A. Time course of TER of HUVEC monolayers transfected with WT and DN Rac1 plasmids, treated with either 2 μM S1P or vehicle (N = 8 each group). The TER is normalized to the time point just prior to addition of S1P for a more direct comparison of the magnitude of the response. B. The mean maximal change in TER (%) of HUVEC monolayers in the 10-min window immediately following S1P treatment. The corresponding data for HDMEC monolayers are shown in panels C & D (N = 8 each group). *P<0.05 vs. Vehicle treated groups

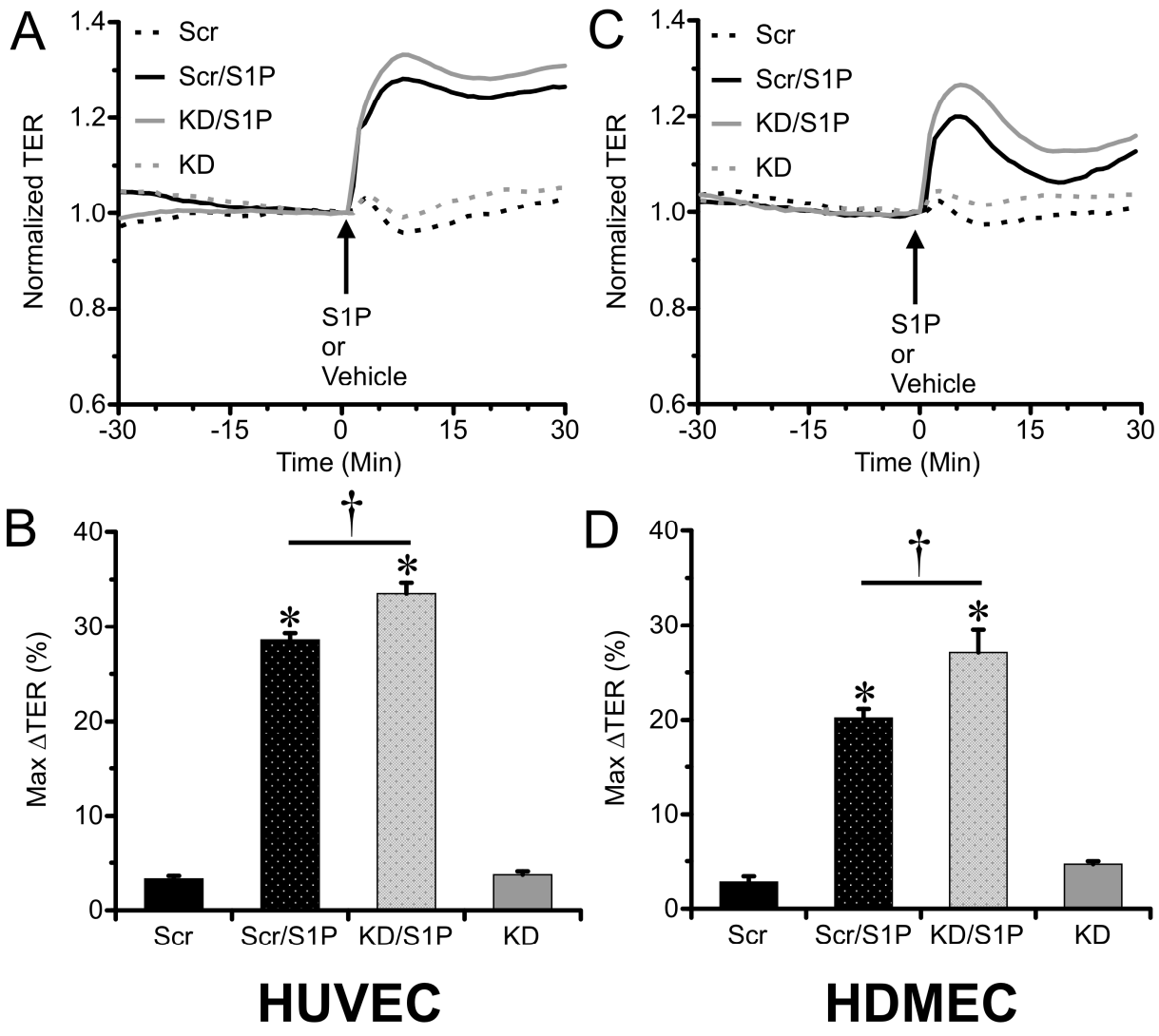

Figure 18 Knockdown of Rac1 Expression With siRNAs Did Not Diminish S1P-Induced Barrier Enhancement on Both HUVEC or HDMEC Monolayers

A. Time course of changes in TER of HUVEC monolayers before and after treatment with 2 μM S1P or vehicle control, for the Rac1 knockdown and scrambled RNA transfected groups (N = 8 each group). The TER is normalized to the time point just prior to the addition of S1P, for more direct comparisons of the responses to S1P between the groups. B. The mean maximal change in TER of HUVEC monolayers (%) during the first 10 min after S1P was added. The corresponding results for HDMEC

monolayers are shown in panels C & D (N = 8 each group). *P<0.05 vs. vehicle control groups; †P<0.05 vs. scrambled RNA sequence group.

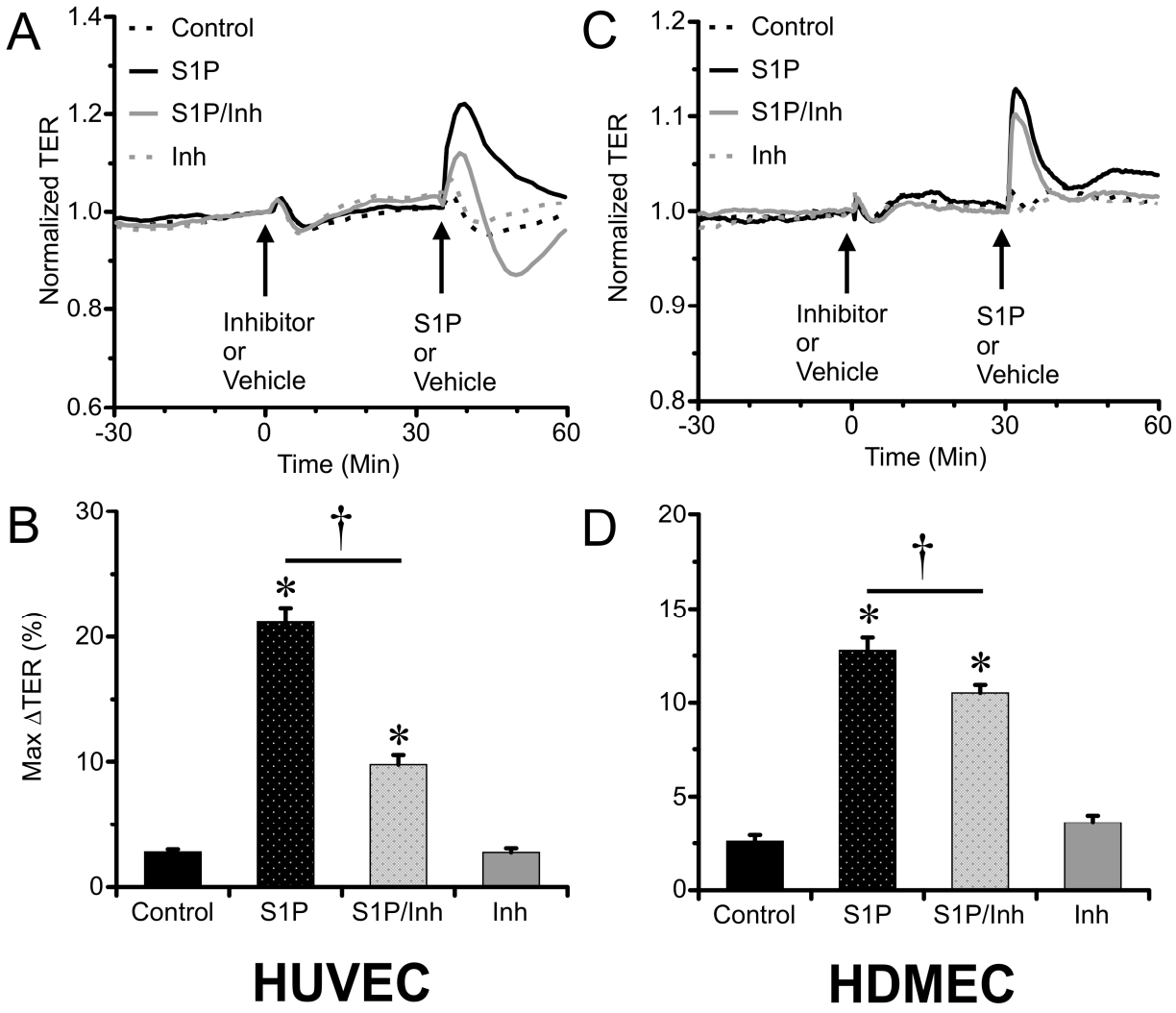

Figure 19 Inhibition of RhoA Attenuated S1P-Induced Barrier Enhancement on HUVEC Monolayers

A. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with the combination of Rhosin and Y16 (5 μM of each) or vehicle control, followed by treatment with 2 μM S1P or vehicle (N = 8 for each group). B. Comparison of the mean maximal changes in TER of HUVEC monolayers (%) within the first 10 min after S1P or vehicle. The corresponding results for HDMEC monolayers are shown in panels C & D (N = 8 each group). *P<0.05, S1P vs. vehicle treated group. †P<0.05, inhibitor vs. vehicle pretreatments.

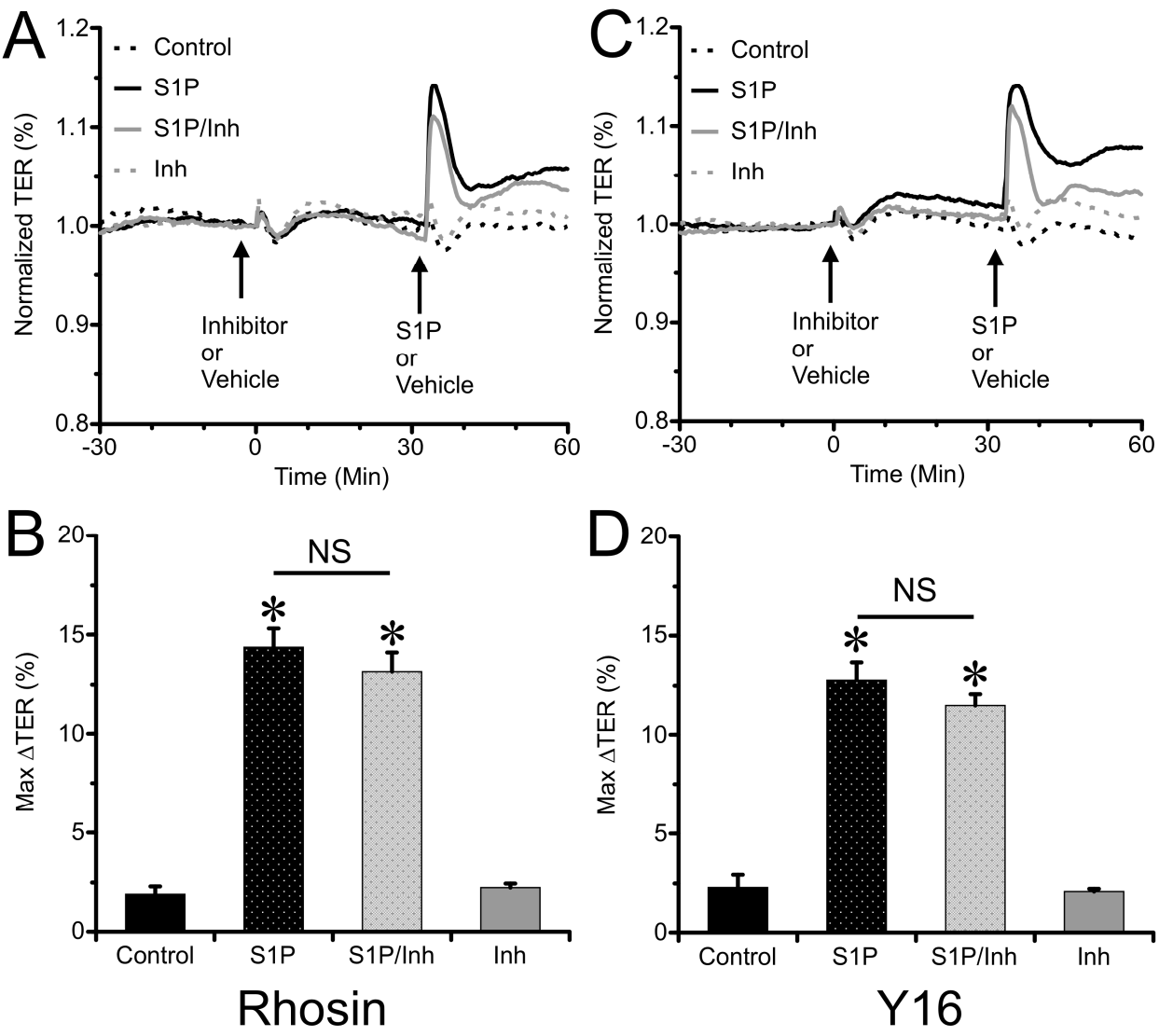

Figure 20 Use of The Individual RhoA Inhibitors Did Not Attenuate S1P-Induced Barrier Enhancement on HDMEC Monolayers

A & C. Time courses of TER changes during pretreatment with 25 μM Rhosin (A) or 25 μM Y16 (C), and subsequent treatment with 2 μM S1P or vehicle (N = 8 for each group). B & D. Mean maximal changes in TER (%) within the first 10 min after S1P. *P<0.05, S1P vs. vehicle treated group, same color bar. †P<0.05, inhibitor vs. vehicle pretreatments.

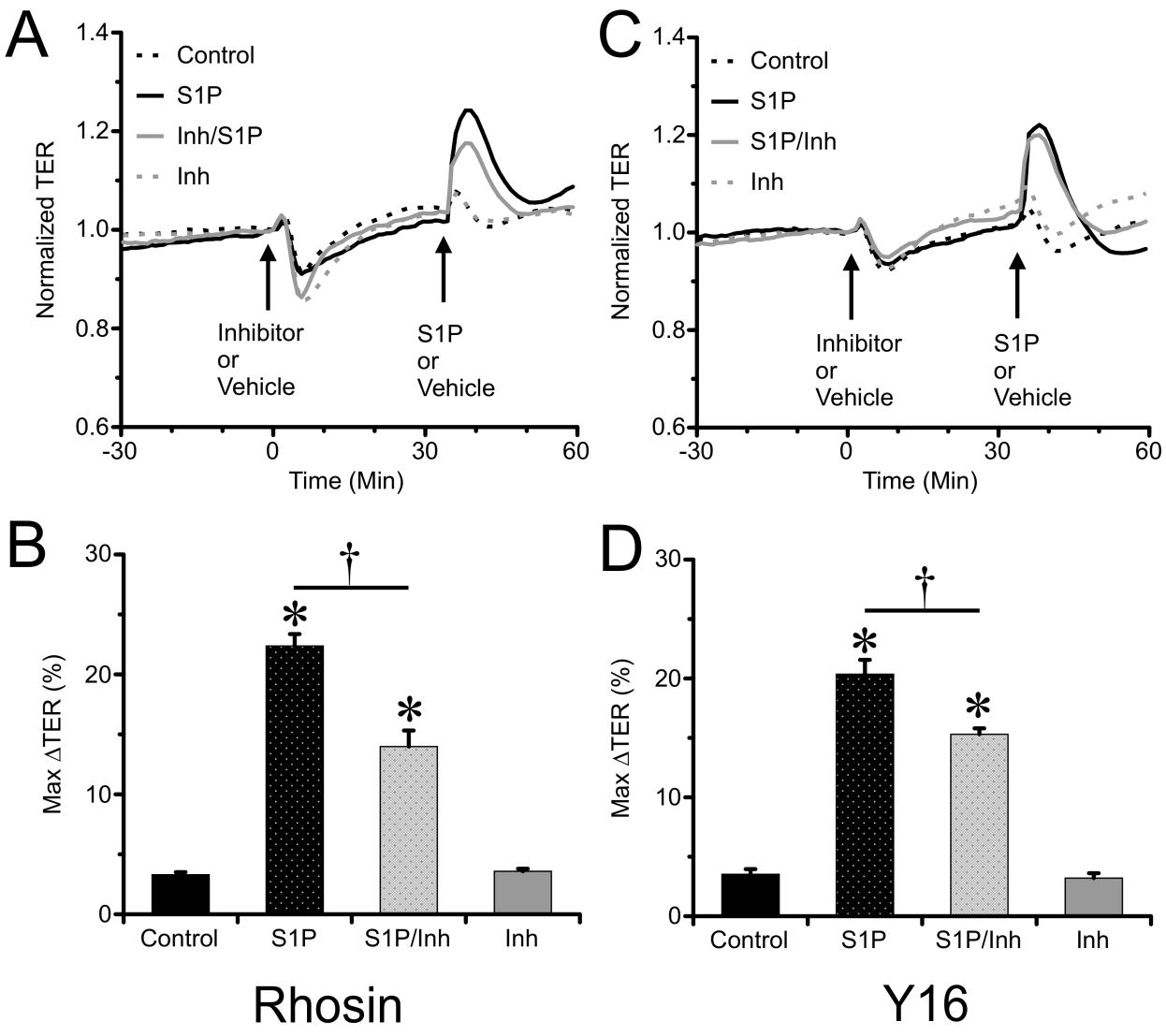

Figure 21 Inhibition of RhoA Attenuated S1P-Induced Barrier Enhancement on HUVEC Monolayers

A. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with the combination of Rhosin and Y16 (5 μM of each) or vehicle control, followed by treatment with 2 μM S1P or vehicle (N = 8 for each group). B. Comparison of the mean maximal changes in TER of HUVEC monolayers (%) within the first 10 min after S1P or vehicle. The corresponding results for HDMEC monolayers are shown in panels

C & D (N = 8 each group). *P<0.05, S1P vs. vehicle treated group. †P<0.05, inhibitor vs. vehicle pretreatments.

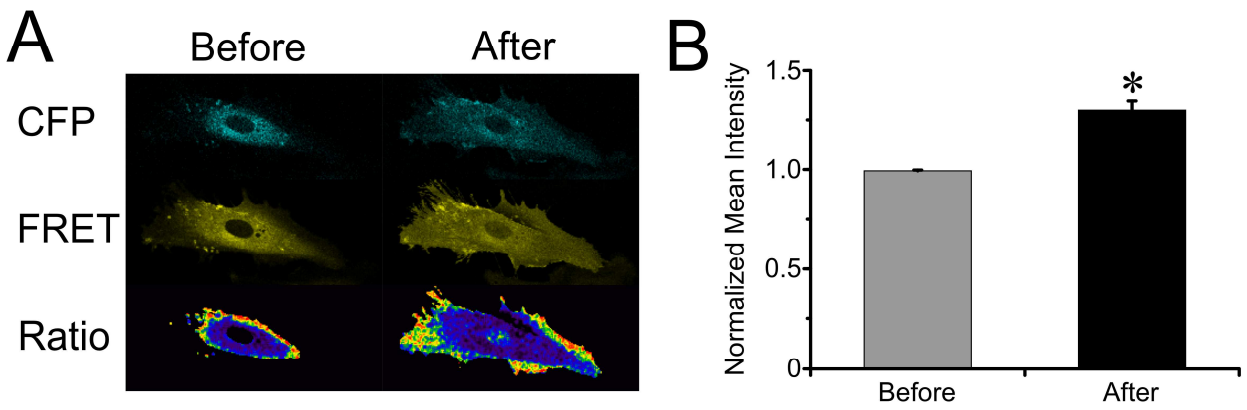

Figure 22 S1P Activated RhoA Primarily at Cell Periphery

A. Representative images of HUVEC expressing the pTriEx-RhoA FLARE.sc Biosensor, showing the CFP and YFP channels, and the ratio (FRET) indicating RhoA activation, during baseline and after the treatment of 2 μM S1P. The entire time course can be viewed in S1 Movie. B. Normalized mean intensity of the whole cell before and after S1P treatment (N = 5 cells studied). *P<0.05, before vs. after S1P treatment.

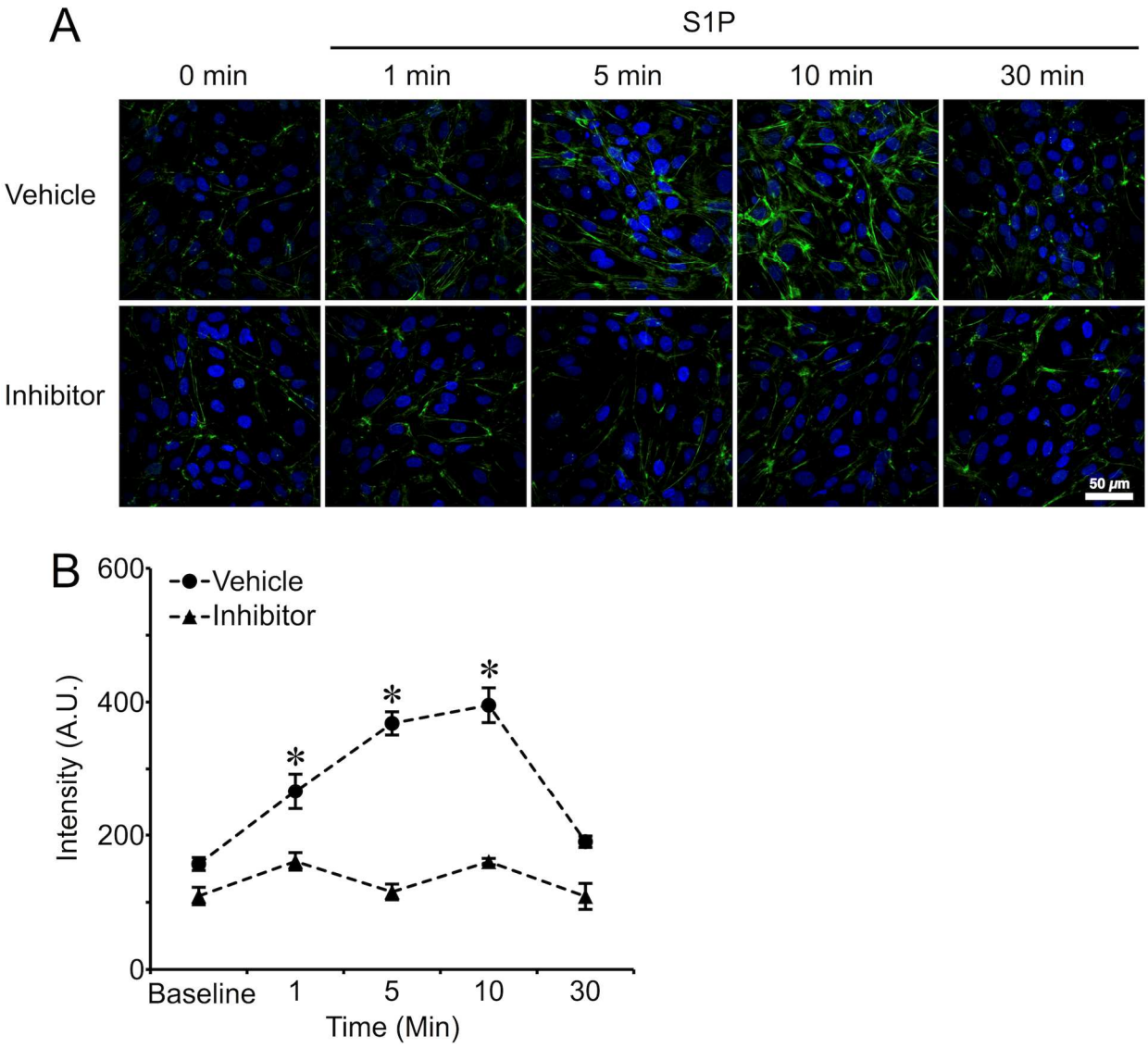

Figure 23 Inhibition of RhoA Abrogated S1P-Induced Phosphorylation of MLC-2 at Thr-18/Ser-19

A. Z-projection confocal immunofluorescence microscopy images of phosphorylated MLC-2 on HUVEC monolayers are shown. Each image represents three replicates for each time point. S1P was applied at 2 μM. Combined Rhosin and Y16 pretreatment was for 30 min, at 5 μM each. B. Quantification of phosphorylated MLC-2 intensity for each time point. *P<0.05, S1P treatment compared with baseline.

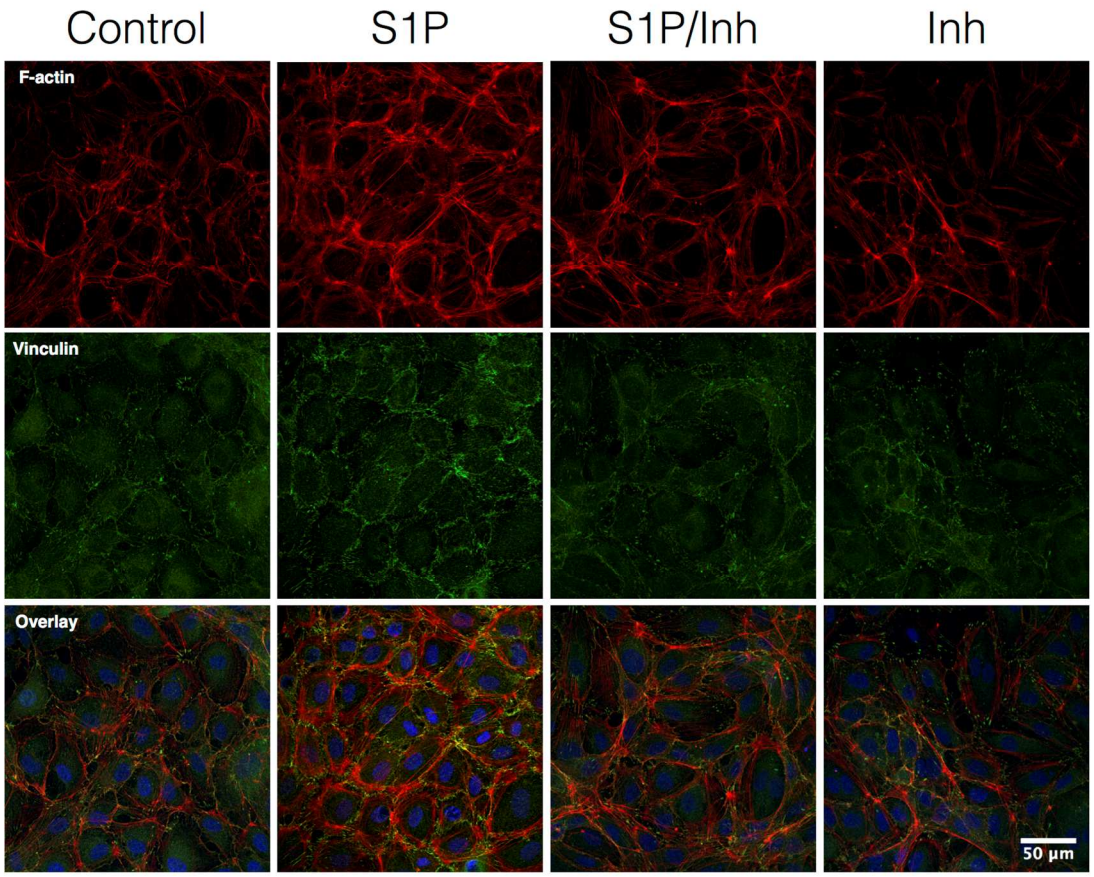

Figure 24 RhoA Inhibition Abrogated S1P-Induced F-Actin Formation and Recruitment of Vinculin Near The Cell Periphery

The results showed that S1P increases F-actin and vinculin labeling in the peripheral areas of cells (10 min after the addition of S1P). This was inhibited after pretreatment with combined Rhosin and Y-16 (Inh; 5 µM each, 30 min). The inhibitors alone had no impact. All images are representative of 3 separate experiments.

Chapter Five:

S1P Receptors and Endothelial Barrier Function

5a. Introduction

S1P is a bioactive lipid that can regulates various biological functions both intracellularly [186] and extracellularly [187]. While the intracellular signaling is poorly understood [121], the extracellular effects of S1P are mainly through binding to its receptors [118,188]. Currently, three G-protein coupled S1P receptors (S1P1, S1P2, S1P3) have been identified on endothelial cells [188]. S1P1 on endothelial cells is thought to enhance endothelial barrier function through G_i coupled signaling pathways [52,90,121,122]. S1P2 activation was suggested to promote endothelial hyperpermeability [189,190], but discrete report also suggested knockdown of S1P2 attenuated S1P-mediated endothelial barrier enhancement [191]. Some researchers have also suggested that the balance between S1P1 and S1P2 is critical for endothelial barrier integrity [192]. On the other hand, S1P3 knockdown has been reported to potentiate S1P-mediated endothelial barrier enhancement [191]. Interestingly, while S1P analog fingolimod can induce S1P receptor internalization on immune cells, inhibit lymphocytes trafficking, and treat relapsing MS, discrete findings are seen in endothelial cells. A couple of studies showed that S1P1 agonists also causes receptor internalization in endothelial cells [193-195], but others also argue that the same

findings does not occur as it is in lymphocytes [196]. Currently, it is believed that S1P1 and Rac1 activation are important in its endothelial barrier enhancing effect. However, as noted in the previous chapter, our findings revealed that S1P can enhance endothelial barrier independently of Rac1, but are partially dependent on RhoA. Combined with other findings, we think it is necessary to study S1P receptors and investigate which S1P receptors are important for its endothelial barrier protective effect.

5b. Results

5b.i. S1P1-S1P3 Receptors Expression in Endothelial Cells.

S1P receptors are differentially expressed in tissues, it is currently accepted that S1P1, S1P2 and S1P3 are expressed on endothelial cells [118,121]. While S1P1 has been demonstrated to be important for the endothelial barrier protective effect of S1P, S1P2 has been demonstrated to compromise endothelial barrier in a cerebral stroke model [197]. Recently, studies have reported that S1P3 knockdown could potentiate S1P's endothelial barrier protective effect [191]. We confirmed the expression of S1P1-S1P3 on three different endothelial cell types with both immunoblotting and immunofluorescence labeling (Fig 25). In HUVEC, all of the three receptors are mainly expressed in the cytoplasm, except that S1P1 is also expressed at high levels in the nucleus. Similarly, all three receptors are primarily expressed in the cytoplasm in HCMEC and HDMEC, besides the cytoplasm, S1P3 is expressed at high levels on the membrane in HCMEC, observed with confocal microscopy.

5b.ii. Effects of S1P1 Agonist and Antagonist on Endothelial Barrier Function.

S1P1 is thought to be the main receptor that is responsible for S1P's endothelial barrier protective effect [53]. We tested the effect of S1P1 antagonist W146 on S1P-mediated endothelial barrier enhancement. W146 pretreatment decreased baseline TER, suggesting that S1P1 is important for baseline barrier function. However, S1P was able to increase TER in the presence of W146 (Fig 26 A & B). We next tested if S1P1 agonist CYM5442 is able to enhance endothelial barrier function. As expected, CYM5442 increased TER in a concentration dependent manner (Fig 27 A & B) in a similar fashion as S1P. We next tested if W146, an S1P1 specific antagonist is effective in blocking CYM5442-mediated endothelial barrier enhancement. While W146 pretreatment alone caused a significant decrease in the basal TER (Fig 27 C), W146 did not block CYM5542-induced increased in TER (Fig 27 D). The data suggest that at 10 μM, CYM5442 may signal through other S1P receptors besides S1P1 to enhance barrier function.

5b.iii. S1P2, S1P3 Agonists and Antagonists on Endothelial Barrier Function.

We previously observed that inhibition of RhoA attenuated S1P-mediated increase in TER. Activation of both S1P2 [198] and S1P3 [199] have been demonstrated to activate RhoA. We tested if activation of S1P2 and S1P3 are sufficient to impact endothelial barrier function. We used S1P2 and S1P3 allosteric agonists CYM5520 and CYM5541, respectively, because there were no specific agonists available. Neither CYM5520 nor CYM5541 had a significant impact on endothelial barrier function (data not shown). We also tested if the S1P2 antagonist JTE-013 or S1P3 antagonist TY-52156 is effective in blunting S1P-induced increase in TER.

Neither 1 μM JTE-013 (Fig 28 A & B) or 10 μM TY52156 (Fig 28 C & D) was able to block S1P-induced increase in TER, nor did they cause any significant drop in baseline TER.

5b.iv. Expression of S1P4, S1P5 on Endothelial Cells

Because antagonism of S1P1, S1P2 or S1P3 alone did not affect S1P-mediated increase in TER, we tested if other S1P receptors are present on endothelial cells. We found that both S1P4 and S1P5 are present in all three cell types tested (HUVEC, HCMEC, HDMEC). Both receptors are expressed in high amount in the cytoplasm across three cell types, and lesser amounts were observed on the cell surface.

5c. Discussion

In this chapter, we studied the effect of S1P receptors with different agonists and antagonists. It is generally thought that the endothelial barrier enhancement effect of S1P is through S1P1. However, while we observed that RhoA inhibition attenuated S1P-mediated increase in TER, we think it is important to study S1P receptors.

Interestingly, while the expected increase in TER was observed with S1P1 agonist CYM5442, the effect on endothelial barrier by CYM5442 is much weaker compared to S1P at the same concentration. These results suggest that other S1P receptors might be involved in the optimal S1P-mediated endothelial barrier enhancement. We subsequently found out that S1P1 specific antagonist W146 failed in abrogating S1P-induced increase in TER. This result further supports the notion that the optimal endothelial barrier enhancing effect of S1P might involve more than just S1P1. It is worth noting that while W146 did not block S1P-induced increase in TER, itself alone caused a significantly drop on baseline TER. It is possible that endothelial cells are

releasing a basal level of S1P in an autocrine fashion to maintain baseline barrier function and viability. To our surprise, W146 did not block 10 μM CYM5542-induced increased in TER, this might be due to the fact that higher concentration of CYM-5442 started to bind to other S1P receptors non-specifically. Taken together, these results suggest that S1P1 is important for the maintenance of baseline barrier integrity, through an autocrine manner. Our studies also opened new questions about S1P receptors and their roles in S1P-mediated endothelial barrier enhancement.

We evaluated if S1P2 or S1P3 are important for endothelial barrier function. However, neither S1P2 agonist CYM5520 nor S1P3 agonist CYM5541 had any impact on endothelial barrier function up to 10 μM (data not shown). There are two possible explanations for the observation: first, activation of S1P2 and S1P3 has no effect on endothelial barrier function; or the allosteric agonists are not as effective as S1P. We further tested that if antagonism of either S1P2 or S1P3 alone is effective to blunt the increase in TER seen after S1P treatment. Neither 1 μM JTE-013 or 1 μM TY-52156 significantly affected baseline TER, nor did they block S1P-mediated endothelial barrier enhancement. These results suggest that S1P2 or S1P3 receptors alone are not a significant determinant of endothelial barrier integrity under baseline conditions, neither are they alone responsible for S1P-mediated endothelial barrier enhancement. Interestingly, while 1 μM JTE-013 did not affect baseline barrier function, 10 μM JTE-013 caused a significant drop on baseline TER. It is worth noting that at 10 μM, JTE-013 was documented to have a weak inhibitory effect (4.2%) towards S1P3 in addition to S1P2 [200]. These results suggest that multiple S1P receptors are responsible for baseline barrier integrity regulation as well.

In our studies, we identified S1P4 and S1P5 on endothelial cells. A logical next step is to test the role of S1P4 and S1P5 each alone on endothelial barrier function, as well as their role on S1P-induced increase in TER. We will also test to target different S1P receptors at the same time and asscess if they are effective in attenuating S1P-mediated endothelial barrier enhancement. We did utilize siRNA to knockdown S1P1 in our studies, however, we could not achieve a desired knockdown effect (data not shown). A workaround method would be to use genetic knockout models such as an inducible, endothelial-specific, S1P1 knockout mouse, which is currently in the development. Finally, it is possible that S1P-mediated endothelial barrier enhancement is through both receptor-dependent and receptor-independent mechanisms. In that situation, the genetic knockout animal models would help to unravel the mystery.

In summary, our data suggest that S1P1 is critical for maintaining basal level endothelial barrier function, likely through the S1P release from endothelia themselves in an autocrine fashion. Our studies also raised new questions if there is S1P receptor redundancy for regulating endothelial barrier function. Future studies are needed to rigorously assess these possibilities.

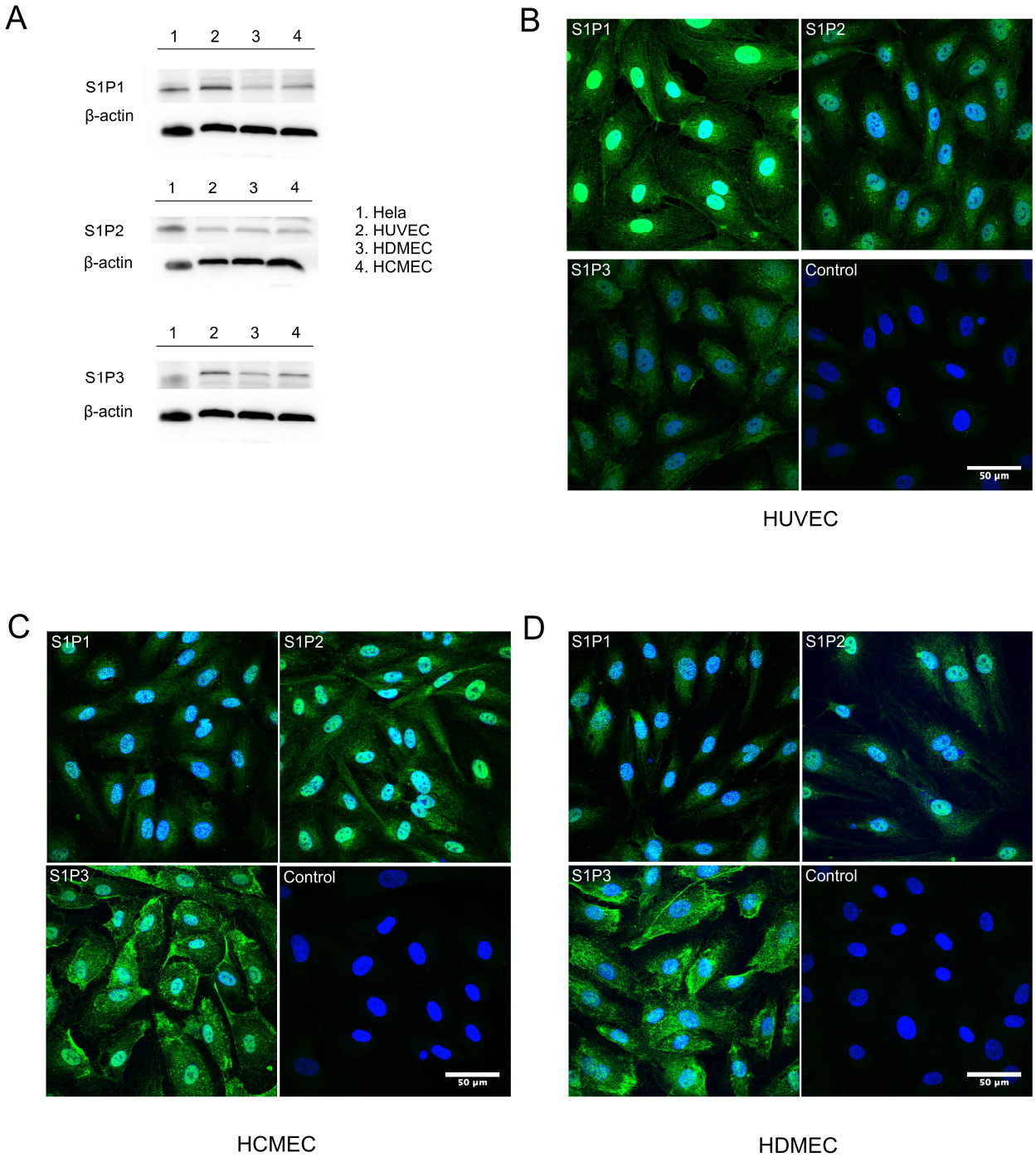

Figure 25 S1P1, S1P2 And S1P3 Expression in HUVEC, HCMEC and HDMEC

A. Immunoblotting showing that S1P1-S1P3 receptors are expressed in all three cell types tested (HUVEC, HCMEC and HDMEC). B, C and D. Confocal immunofluorescence microscopy pictures showing S1P1-S1P3 expression in HUVEC (Panel B), HCMEC (Panel C) and HDMEC (Panel D).

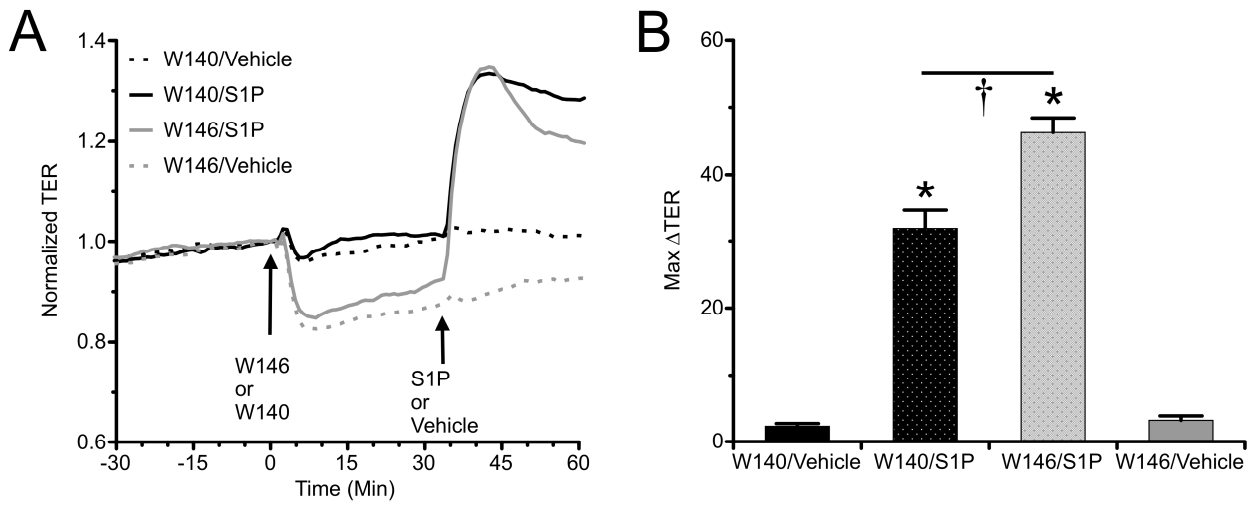

Figure 26 S1P1 Antagonist W146 Failed to Attenuate S1P-Mediated Endothelial Barrier Enhancement

A. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with either S1P1 antagonist W146 or its inactive control W140 (1 μM of each), followed by treatment with 1 μM S1P or vehicle (N = 8 for each group). B. Comparison of the mean maximal changes in TER of HUVEC monolayers (%) within the first 10 min after S1P or vehicle. (N = 8 each group). *P<0.05, S1P vs. vehicle treated group. †P<0.05, W146 vs. W140 pretreatments.

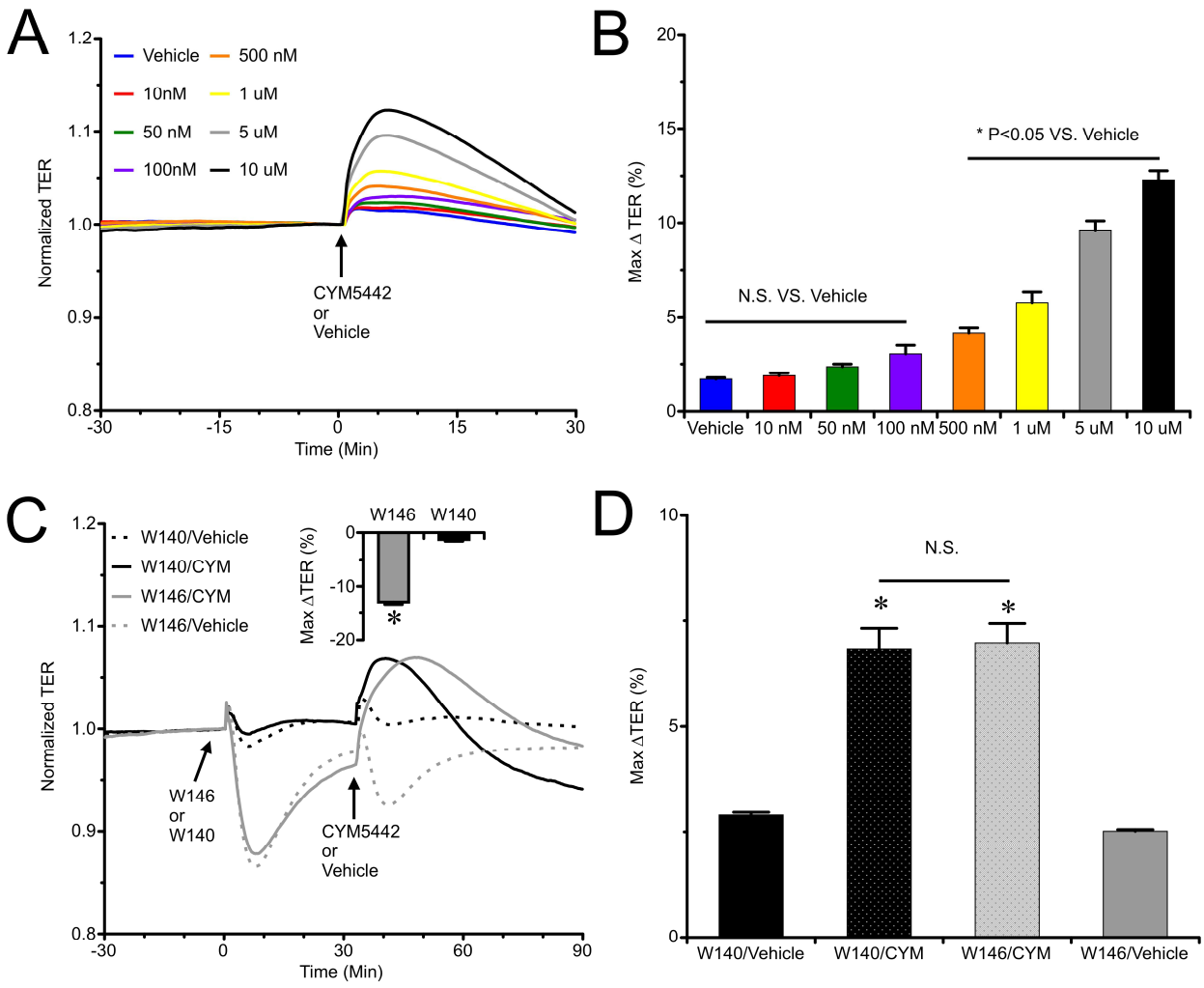

Figure 27 Effect of S1P1 Agonist and Antagonist on Endothelial Barrier Function

A. Tracing of TER of HUVEC monolayers treated with different concentrations of CYM5442. B. Maximal change in TER (%) from baseline within the initial 15 min time window after addition of the indicated concentrations of CYM5442. C. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with either S1P1 antagonist W146 or its inactive control W140 (1 μM of each), followed by treatment with 10 μM S1P1 agonist CYM5442 or vehicle. D. Comparison of the mean maximal changes in TER of HUVEC monolayers (%) within the first 15 min after CYM5442 or vehicle. *P<0.05 versus vehicle treated group.

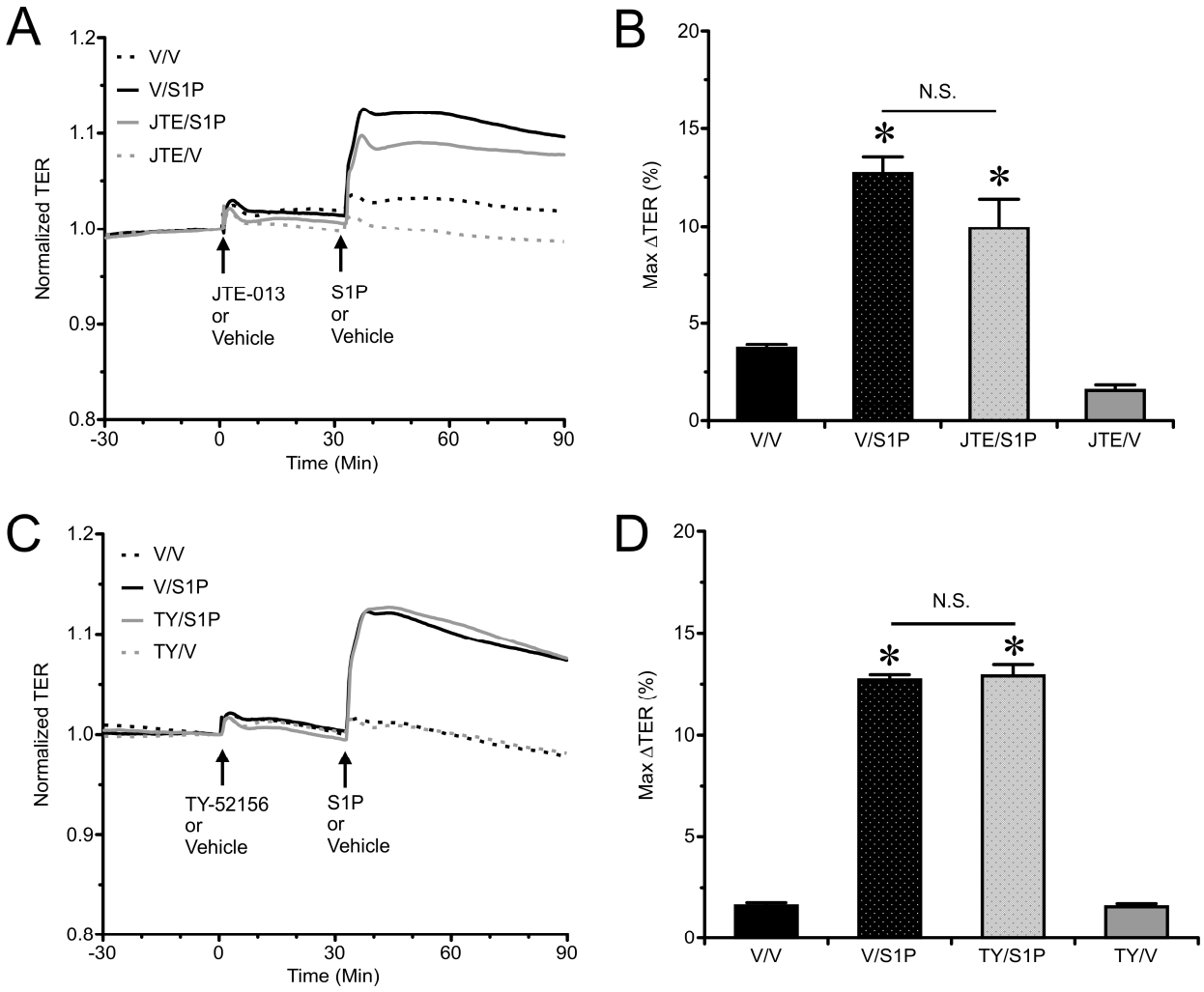

Figure 28 Effect of S1P2 and S1P3 Antagonists on S1P-Mediated Endothelial Barrier Enhancement

A. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with either 1 μM S1P2 antagonist JTE-013 or control, followed by treatment with 1 μM S1P or vehicle. B. Maximal change in TER (%) from baseline within the initial 15 min time window after addition of 1 μM of S1P. C. The time course of changes in of TER of HUVEC monolayers pretreated with the 30 min with either 1 μM S1P3 antagonist TY52156 or control, followed by treatment with 1 μM S1P or vehicle. D. Maximal change in TER (%) from baseline within the initial 15 min time window after addition of 1 μM S1P. *P<0.05 versus vehicle treated group.

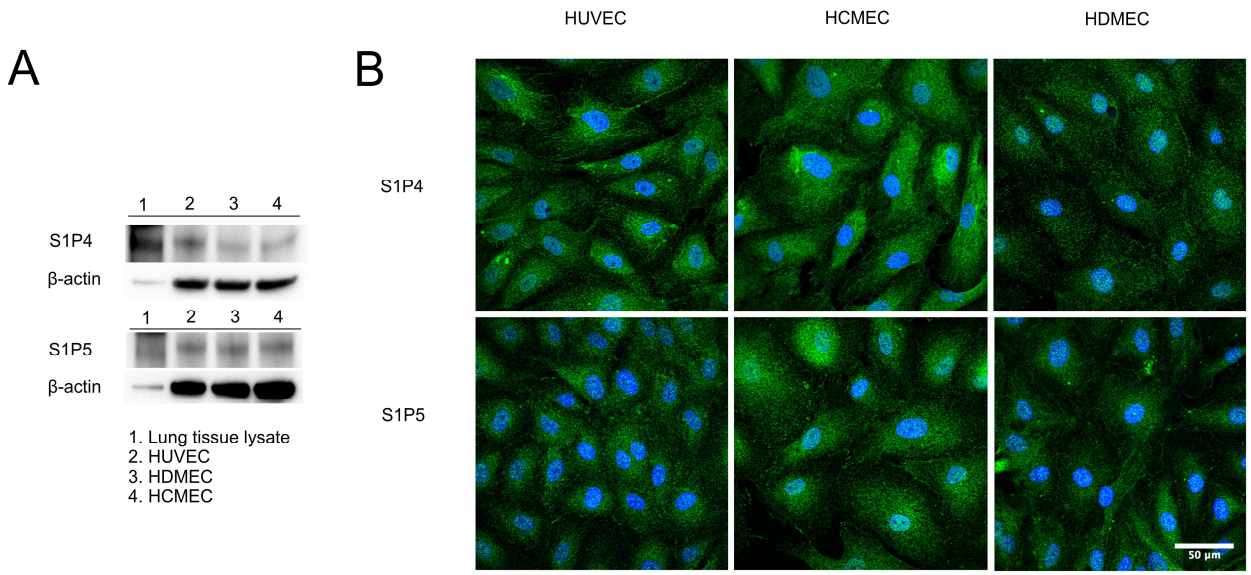

Figure 29 Expression of S1P4 and S1P5 on Different Types of Endothelial Cells

A. Immunoblotting showing that S1P4 and S1P5 receptors are expressed in all three cell types tested (HUVEC, HCMEC and HDMEC). B. Confocal immunofluorescence microscopy pictures showing S1P4 and S1P5 expression in HUVEC, HCMEC and HDMEC.

Chapter Six

Overall Conclusions

6a. Overall Objectives of The Dissertation

The semi-permeable endothelial barrier in tissue capillaries and post-capillary venules are critical for normal tissue viability. A compromised endothelial monolayer can result in excessive and uncontrolled microvascular leakage, which is a hallmark of many inflammatory diseases. The excessive fluid accumulated within the interstitium results in tissue edema and impairs homeostasis. S1P is a bioactive lipid that has endothelial barrier protective effect and has the therapeutic potential towards controlling endothelial barrier dysfunction and hyperpermeability seen in different pathological processes. It was described that S1P enhances endothelial barrier function independently of VE-cadherin, but requires cell spreading [129], an actin polymerization dependent structure also termed as lamellipodia. In the microcirculation field, it is well known that stable junctions are critical for endothelial barrier integrity. It is also well known that cytoskeleton rearrangements can alter junctional stability and regulate endothelial barrier function. However, it is yet unclear how the dynamic changes in cytoskeleton after S1P affect junctional integrity in real time, as well as endothelial barrier function.

Most of the existing lamellipodia studies focused on wound healing and cancer metastasis, very little is known on how the lamellipodia activities affect endothelial

barrier function. Of those that studied the involvement of lamellipodia, most came from fixed cells and provided little information on spatial–temporal dynamics [201-203]. To our knowledge, no groups yet have systemically analyzed the lamellipodia and endothelial barrier function change at the same time. A couple of impressive studies demonstrated that lamellipodia is important for structural integrity of VE-cadherins [144,145], however, they mainly focused on VE-Cadherin structure changes and did not provide insight on how the dynamic change in lamellipodia affect endothelial barrier function. The lamellipodia protrusions have also been shown to close leukocyte transmigration pores, and micro-wounds between endothelial cells [204,205]. Further, different studies have demonstrated that lamellipodia protrusions contribute to the S1P-mediated barrier enhancement [52,158,206]. Despite the strong evidence, most of the observations were from fixed cells that only represent a snapshot, and do not reflect the dynamic relationships between lamellipodia activity and endothelial barrier function.

To fully take the therapeutic advantages of S1P, we must fill in the gap of knowledge and first determine if the dynamic changes in lamellipodia activities caused by S1P play a role in endothelial barrier function. We also have to elucidate how lamellipodia contribute to S1P's endothelial barrier protective effect. This dissertation focused on the gap in knowledge mentioned above and proposed the overall hypothesis that "S1P-induced lamellipodia protrusions protect endothelial barrier function". We also investigated the signaling mechanisms that are responsible for S1P-mediated endothelial barrier enhancement. Finally, we examined the receptors that are responsible for S1P-mediated endothelial barrier enhancement. We expect the

foundational knowledge gained in this dissertation can be utilized for the development of future therapeutic strategies.i

6b. Overall Findings in The Dissertation

We presented that increased lamellipodia protrusions after S1P correlate with increased endothelial barrier function. Out of all the parameters, changes in lamellipodia protrusion frequencies correlate with endothelial barrier function changes the best, suggesting lamellipodia protrusion frequencies is a good indicator of endothelial barrier function. S1P is also effective in rescuing thrombin caused hyperpermeability, indicating that S1P and its downstream effectors may have their therapeutic uses in the proinflammatory conditions triggered by thrombin.

The mechanisms that underline S1P's endothelial barrier enhancement are likely to involve lamellipodia and VE-cadherin. Unlike thrombin, which decreases lamellipodia protrusions, leads to VE-cadherin belt breakdown and paracellular gaps formation, S1P does the exact opposite. It increases the lamellipodia protrusions, which helps to redistribute the cytosolic VE-cadherin to the cell-cell contacts. The end result is stronger cell-cell adhesion and enhanced endothelial barrier function.

It is worth noting that while Rac1 and its downstream effectors are important for the baseline lamellipodia protrusions and endothelial barrier function, they appear dispensable in S1P-mediated endothelial barrier enhancement. On the other hand, increased RhoA activity, mainly at the periphery, appears to play an important role in S1P-mediated endothelial barrier enhancement. The exact mechanisms are currently unknown, but are likely to involve RhoA-MLC-2 dependent lamellipodia activities and nascent focal adhesions.

There appears to be an autocrine mechanism of S1P in endothelial cells because antagonism of S1P1 significantly impaired baseline endothelial barrier function. It is very likely that the optimal S1P's endothelial barrier enhancement effect depend on multiple receptors, as none of the S1P receptors alone is sufficient to abrogate its endothelial barrier enhancement effect. It is also possible that S1P acts on multiple receptors to achieve its maximum endothelial barrier enhancement effect, or there could be an unidentified receptor-independent mechanism. Future studies are needed to elucidate the underlining mechanism.

In summary, there is a strong correlation relationship between lamellipodia protrusions and endothelial barrier function. Based on our finding, we proposed that lamellipodia protrusions help to strengthen cell-cell adhesions and increase paracellular diffusion distance, both of which help to enhance endothelial barrier function. The endothelial barrier enhancement effect of S1P depends on RhoA activation but not Rac1 activation. Finally, S1P1 is important for the maintenance of baseline barrier integrity, but the detailed receptor signaling that mediates S1P's endothelial barrier protective effect still require further investigation.

6c. Limitations & Future Directions

In the dissertation, we utilized live cell imaging to study lamellipodia dynamics. One limitation with our study is that there are currently no pharmacological inhibitors that specifically inhibit the lamellipodia activities as the exact mechanisms underlining initiation, protrusion and retraction of lamellipodia are still elusive [207]. We utilized a workaround method, which is to analyze lamellipodia parameters. This is not a direct measurement of how lamellipodia influence endothelial barrier, but provides insights

into how lamellipodia activities correlate with endothelial barrier function. Combined with our studies on the baseline and thrombin treated groups [134], we think it is safe to draw the conclusion that lamellipodia protrusions contribute to endothelial barrier integrity.

To date, the detailed mechanisms that control lamellipodia protrusion, halt, and retraction are still elusive. Investigations focusing on the topic are also restrained with methods that could be used. Some studies have demonstrated that Rac1 activation is required for lamellipodia protrusions [151,208,209], while others have shown that RhoA activation is sufficient to induce the protrusion [93,95,210]. In our own studies, high RhoA activities were observed at the site of protrusions. Interestingly, RhoA and Rac1 were shown to inhibit each other's activity by competing for the GEFs [211]. In the future, one way that may help to elucidate the mechanism regulating lamellipodia activities is to utilize endothelial cells expressing both Rac1 and RhoA FRET proteins. This would enable us to analyze how the spatial activation of Rac1 and RhoA regulate protrusions and advance the field. In addition, it would also be interesting to compare and contrast how Rac1 effector Arp2/3 and RhoA effector mDia differentially regulate lamellipodia activities afterwards, because both proteins have been implicated in lamellipodia protrusions as well as endothelial barrier function. Another prospective that is supplemental to the aforementioned is to determine the role of RhoA in Rac1 knockdown endothelial cells. This would enable us to determine if a certain amount of interplay between Rac1 and RhoA is required for S1P-mediated endothelial barrier enhancement.

Our studies also demonstrated that Rac1 is dispensable in S1P-mediated endothelial barrier enhancement *in vitro* by coupling Rac1 pharmacological inhibitors, overexpression and siRNA knockdown techniques. Although we tried our best to minimize the impact of Rac1 overexpression and knockdown on baseline endothelial barrier function, one common pitfall with the overexpression and knockdown techniques is that they tend to alter the natural physiology of cell behaviors. An alternative method would be to deliver Rac1 inhibitory peptide W56 into both endothelial cells and isolated intact mesenteric venules with the methods previously described by our lab [212]. Another way to overcome this limitation would be to employ Rac1 genetic deletion animals in the future. We can couple both *ex vivo* isolated mesenteric venules permeability assay and *in vivo* intravital microscopy for permeability studies to determine if Rac1 activation is essential in S1P induced endothelial barrier enhancement. Due to the fact that RhoA inhibition only attenuated S1P's effects on the macrovascular cell type HUVEC, but not HDMEC, it is also important to test if our *in vitro* findings also occur in *ex vivo* and *in vivo* setting in the microvasculature.

Our results suggest that there is some autocrine mechanism of S1P maintaining baseline endothelial barrier function. Endothelial cells are the major origin of S1P, knockout of the exporter spns2, which is responsible for exporting S1P to the blood stream, severely affected blood S1P levels [213]. It is therefore interesting to compare and contrast the effect of spns2 knockdown on both baseline endothelial barrier function and in inflammatory condition. Our results also suggest that none of the S1P1-S1P3 receptors alone are responsible for the endothelial barrier enhancement effect of S1P. In the future, we will test if multiple receptors are responsible for the effect by combining

multiple S1P receptor inhibitors. We will also apply S1P receptor agonists and antagonists on the S1P1 knockout mice to test the involvement of different receptors in regulating endothelial barrier function.

In summary, although there are some technical difficulties and limitations with this dissertation, overall our results suggest that 1) endothelial barrier function changes strongly correlate with lamellipodia protrusion changes seen after S1P; 2) We propose lamellipodia protrusions help to enhance endothelial barrier function by increasing paracellular diffusion distance and junctional stability; 3) S1P-mediated endothelial barrier enhancement occurs in a Rac1 independent, RhoA dependent mechanism; 4) S1P1 is critical for maintaining baseline endothelial barrier function, and further investigation is still needed to elucidate which receptors mediate the endothelial barrier enhancement effect of S1P.

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