Mechanisms of S1P-Induced Endothelial Barrier Enhancement

Natascha Guimarães Alves

University of South Florida, natagalves@gmail.com

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Mechanisms of S1P-induced Endothelial Barrier Enhancement

by

Natascha Guimarães Alves

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences with a concentration in Cardiovascular Biology
Department of Molecular Pharmacology and Physiology
College of Medicine
University of South Florida

Major Professor: Jerome W. Breslin, Ph.D.
Co-major Professor: Sarah Y. Yuan, M.D., Ph.D.
Thomas Taylor-Clark, Ph.D.
Srinivas Tipparaju, Ph.D.
Hana Totary-Jain, Ph.D.

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Dedication

This dissertation is dedicated to my parents, Eliane Moreno Guimarães and Harley Alves. Thank you for always giving your best to raise and educate me, and for many times sacrificing your own objectives to make me happy. Everything I am and everything I have achieved is because of your unconditional love and support. I am extremely proud to have you as my parents; you are my inspiration and my supporting rock.

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Abstract

Excessive microvascular permeability is a serious complication involved in traumatic injury and inflammatory diseases. Alcohol intoxication can exacerbate the physiological derangements produced by microvascular endothelial barrier dysfunction in such disease conditions. Sphingosine-1-phosphate (S1P) has known endothelial barrier-protective properties, and has been shown to ameliorate microvascular leakage in a model of combined alcohol intoxication and hemorrhagic shock and resuscitation (HSR). However, whether the barrier-protective properties of S1P extend to endothelial cells of the blood-brain barrier (BBB) is unclear. The mechanisms of S1P-induced barrier protection during alcohol intoxication or HSR are also unknown. In the current study, we tested the hypothesis that S1P could enhance endothelial barrier during alcohol intoxication or hemorrhagic shock by preserving the integrity of junction proteins and the endothelial glycocalyx, and protecting mitochondrial function.

Cultured primary human brain microvascular endothelial cell (HBMEC) monolayers were used to characterize endothelial-specific mechanisms of S1P protection of the BBB during alcohol treatment. Transendothelial electrical resistance (TER) and apparent permeability coefficients for albumin, dextran-4 kDa, and sodium fluorescein were used as indices of barrier function. Junctional localization was determined by immunofluorescence confocal microscopy. We also used an established in vivo rat model of conscious HSR and assessed microvascular leakage, endothelial glycocalyx integrity, and mitochondrial function by intravital microscopy. Cultured rat intestinal microvascular endothelial cell (RIMEC) monolayers were used to test the ability of S1P to protect against glycocalyx shedding and endothelial barrier dysfunction caused by direct disruption of mitochondrial integrity due to inhibition of mitochondrial complex III. The results show
that alcohol significantly impaired HBMEC TER and increased solute permeability, which was reversed with application of S1P after alcohol treatment. Alcohol caused the formation of gaps between cells. Treatment with S1P (after alcohol) increased junctional localization. Our *in vivo* results show that S1P protects against HSR-induced hyperpermeability, preserves the expression of adherens junctional proteins, and protects against glycocalyx degradation. S1P treatment during HSR also protects against mitochondrial membrane depolarization. Besides that, S1P protects RIMECs against mitochondrial dysfunction-induced endothelial barrier dysfunction and glycocalyx degradation by acting through mitochondrial complex III.

Our results indicate that S1P may be useful for restoring BBB function during alcohol intoxication. Moreover, S1P protects against HSR-induced mitochondrial dysfunction in endothelial cells, which in turn improves the structure of the endothelial glycocalyx after HSR and allows for better junctional integrity to prevention of excess microvascular permeability.
1.1 Overview of Endothelial Barrier Function

The microcirculation is essential to the function of the cardiovascular system. It is at the level of the microcirculation where the exchange of fluids, solutes and inflammatory cells between blood and tissue occurs [1]. The components of the microcirculation are arterioles, capillaries and venules. Together, these microvessels contribute to the control of blood pressure, distribution of blood throughout the body, and localized tissue perfusion [2]. Capillaries and post-capillary venules are the major site of blood-tissue exchange, and are important sites of inflammation [3]. A barrier composed of endothelial cells, endothelial glycocalyx, basement membrane and some accessory cells such as smooth muscle cells and pericytes, tightly controls the permeability of the microcirculation to fluids and solutes. Under physiological conditions, water, ions, and small molecules such as glucose are more likely to cross the endothelium by passing in between cells (paracellular transport), while larger molecules are more likely to cross the endothelium through active transport mechanisms, i.e. the transcellular pathway mediated by caveolae and vesiculo-vacuolar organelles [4,5]. Several pathophysiological conditions, such as trauma, ischemia-reperfusion injury, sepsis, diabetes, and cancer are characterized by increased microvascular permeability due to underlying changes in the structural conformation of endothelial cells. Such conformational changes are caused by inflammatory mediators such as histamine, platelet activating factor and cytokines. The resulting increase in microvascular permeability to fluids and plasma proteins causes edema formation [6]. If the exacerabated fluid loss in the onset of these diseases is not promptly controlled, an extended and aggravated inflammatory response can lead
to further tissue injury and eventual organ failure [2]. Therefore, finding strategies to minimize microvascular hyperpermeability during such inflammatory conditions may prevent or reduce edema-related complications and improve patient outcome. An effective approach to improve barrier function may also have application in multiple pathological conditions, given that many of them involve disruption of the endothelial barrier and microvascular leakage.

1.2 Determinants of Endothelial Barrier

1.2a Adherens Junctional Proteins and Endothelial Barrier

Under physiological conditions, hydrostatic and diffusional gradients drive the transport of water and solutes through the junctional clefts between endothelial cells. This paracellular pathway contains a fibrous matrix of proteins that can select and exclude solutes from ions to macromolecules based on their size and charge [6-8]. Inflammatory and pathophysiological conditions lead to opening of this paracellular pathway, increasing the permeability of the microvasculature to plasma proteins, and facilitating the transendothelial passage of inflammatory cells, pathogens and even metastatic tumor cells [9,10]. Integrity of the structural proteins that compose the junctional cleft is crucial for proper endothelial barrier function. These proteins control paracellular permeability by conferring cell-cell adhesion and barrier “tightness.” There are multiple families of junctional proteins present in the paracellular cleft of the microvascular endothelium: tight junctions (TJ), adherens junctions (AJ), gap junctions (connexons), and desmosomal complexes. TJ and AJ proteins have been of particular interest to the field, and are ubiquitously expressed along various vascular beds. However, there is variability in expression within specialized microvascular beds; for example TJ proteins are more prominently expressed in the microvasculature of the blood-brain barrier [11].

It is currently thought that AJ proteins play a major role in controlling the endothelial barrier in the microvasculature. Data from studies have shown that systemic microvascular permeability
is achieved by selectively disrupting the normal junctional organization of the protein vascular endothelial cadherin, or VE-cadherin, the major component of the AJs [12-14]. The VE-cadherin has intracellular and extracellular domains, spanning the plasma membrane. The extracellular domain of one VE-cadherin molecule in one cell forms calcium-dependent homotypic bonds with the extracellular domain of a VE-cadherin expressed in the membrane of an adjacent endothelial cell, limiting the passage of fluids and solutes from through the junctional clefts [15,16]. The mechanisms that regulate VE-cadherin expression at the junctions are of major importance for controlling microvascular permeability and leukocyte extravasation [17]. The intracellular domain of VE-cadherin connects to the actin cytoskeleton by linking to the catenin family of proteins [5], of which β-catenin is of interest in this dissertation. Dissociation of the VE-cadherin/β-catenin complex leads to increased microvascular permeability [18]. Other adhesion molecules such as E-cadherin, junctional adhesion molecules (JAM) and platelet-endothelial cell adhesion molecules (PECAM-1) are also present at the paracellular cleft [2]. However, their contribution to endothelial barrier function is not well understood. It is worth highlighting, however, that PECAM-1 is known to play a role in the development of inflammation by binding to integrins on leukocytes and facilitating their transmigration across the vascular endothelium [2].

The TJ proteins have similar structures to the adherens junctions, but their role in enhancing endothelial barrier integrity is thought to be more important for the blood-brain and blood-retinal barriers [19]. These proteins have transmembrane domains and their extracellular domains bind homotypically with the extracellular domains of tight junction molecules on neighboring endothelial cells. They include claudins, occludins and JAM-A. On the cytoplasmic side, they connect to the actin cytoskeleton via the zona occludin-1 proteins (ZO-1). Besides playing a role in the structure of the paracellular cleft, ZO-1 proteins have been shown to serve as signaling molecules to enhance tight junctions [20]. Together, TJs and AJs are essential for the functional integrity of the endothelial barrier and changes in their structure can modulate microvascular permeability.
1.2b The Endothelial Glycocalyx and Endothelial Barrier

The endothelial glycocalyx layer has also been demonstrated to be an important modulator of microvascular permeability [21,22]. The glycocalyx is a multilayer structure that covers the surface of endothelial cells and interacts with plasma proteins and lipids [23,24]. It is composed of proteoglycans, glycoproteins, and glycolipids that are associated with the endothelial cell membrane and bind extracellularly to oligosaccharide side chains such as Heparan Sulfate and Chondroitin Sulfate. Even though the glycocalyx layer is ubiquitously expressed in the circulatory system, its composition and thickness fluctuates throughout the vasculature [25]. In the rat mesenteric microcirculation, which is the microvascular bed we primarily studied, the glycocalyx is unevenly distributed, while it is more uniformly distributed in rat aorta [26]. The variations in glycocalyx thickness could be associated with different vessel permeability properties [27]. The glycocalyx has a net negative charge, protecting endothelial cells from negatively charged molecules, and repelling red and white blood cells (leukocytes) [28]. In addition, molecules larger than 70 kDa are excluded from the glycocalyx. Albumin, which is 67 kDa and has a negative net charge, is able to bind tightly to the glycocalyx due to its amphoteric nature, reducing the hydraulic conductivity across the vascular barrier [29]. The glycoproteins that compose the glycocalyx also act as adhesion molecules for plasma constituents to bind to endothelial cells. They express immunoglobulins including intercellular adhesion molecules 1 and 2 (ICAM-1 and -2) that act as ligands for integrins on the surface of leukocytes and platelets, mediating their adhesion to the endothelium. Therefore, the endothelial glycocalyx layer plays a role not only in the homeostasis of the vascular system, but also in protecting the vasculature from circulating inflammatory cells and pathogens.

The endothelial glycocalyx is one of the first components of the endothelial barrier to be involved in barrier dysfunction during inflammation. Damage to the glycocalyx can lead to microvascular permeability and edema [25]. For example, enzymatic degradation of the
glycocalyx in rat myocardial capillaries causes myocardial edema [30]. During acute inflammatory responses, disruption of the glycocalyx exposes endothelial cells to leukocyte adhesion, exacerbating the inflammation. Exposure to inflammatory factors such as tumor necrosis factor alpha (TNF-α) and bacterial lipopolysaccharide have also been shown to degrade the glycocalyx [29,31]. Ischemia-reperfusion injury also nearly completely degrades the glycocalyx layer by generating oxygen free radicals [32].

Studying how different challenges affect the glycocalyx and paracellular junctional proteins may have a positive impact in the development of new strategies to improve endothelial barrier function during pathophysiological conditions. To this end, some interventions to prevent or repair glycocalyx damage during injury have been proposed. For example, maintaining the physiological concentrations of plasma proteins, particularly albumin, has been demonstrated to be quite effective [33]. However, the mechanisms of glycocalyx protection and its interaction with paracellular junctional proteins to maintain endothelial barrier function have been understudied.

1.3 S1P and Endothelial Barrier

Sphingosine-1-phosphate (S1P) is a blood-borne bioactive lipid. S1P is synthesized by red blood cells and platelets [34], and in the circulation it is largely bound to high-density lipoproteins (HDL) and albumin for systemic distribution [35]. Its plasma concentration can vary depending on the context, and is typically found between 10 nM to 4 µM [36]. S1P contributes to a variety of physiological functions, which are attributable to its binding to G-protein-coupled receptors on the cell membrane and to intracellular receptors [37,38]. The receptor-mediated effects of S1P have been studied in a variety of cell types. Endothelial cells have been demonstrated to express S1P receptors 1, 2 and 3 (S1PR1, S1PR2, and S1PR3, respectively), which together regulate endothelial cell functions associated to vascular development, tone and permeability [39-41].
In contrast to work done in endothelial cells \textit{in vitro}, the protective effects of S1P on permeability and barrier function have not as widely been studied \textit{in vivo}. One study reported that intravenous injection of S1P reduces edema formation and inflammation in a murine lung model of LPS-induced acute lung injury (ALI) \cite{42}. The same study shows that FTY720, a S1P analogue that is used for the treatment of multiple sclerosis, also decreases LPS-induce microvascular leakage. A canine model of ALI showed that infusion of S1P reduces protein accumulation and edema formation after intrabronchial LPA administration \cite{43}. Our lab has also recently demonstrated the potential of S1P to attenuate endothelial barrier dysfunction in a rat model of acute alcohol intoxication and hemorrhagic shock and resuscitation \cite{44}. These exciting results from \textit{in vivo} models of hyperpermeability show the potential of S1P in the treatment of microvascular barrier dysfunction. However, further investigation is needed to elucidate the mechanisms of S1P-mediated barrier protection.

\textbf{1.4 Gap in Knowledge and Objective of the Dissertation}

As we have seen, traumatic injury and inflammatory diseases can lead to microvascular hyperpermeability, edema, organ failure, and death. Degradation of junctional proteins and the endothelial glycocalyx layer have been shown to be involved in such complications. Even though efforts have been made to understand the mechanisms underlying barrier dysfunction, there is a lack of effective therapeutic interventions and the morbidity is still high. S1P is a powerful barrier enhancer, however it is not known how the signaling mechanisms activated by S1P can be targeted to enhance barrier function during diseases that involve endothelium breakdown and vascular leakage.

Our lab has successfully demonstrated that S1P protects endothelial barrier function in a combined model of acute alcohol intoxication (AAI) and hemorrhagic shock and resuscitation (HSR). In that study, we investigated whether the administration of different concentrations of S1P (0.003, 0.03 and 0.1 mg/kg) could limit microvascular hyperpermeability in rat mesenteric
microvessels. Our results demonstrated that the 0.1 mg/kg concentration effectively rescued albumin extravasation following AAI + HSR [44]. However, the effects of S1P on the structure of endothelial barrier and the mechanisms of S1P-mediated protection were not assessed. Therefore, the **objective** of this study was to investigate how different challenges affect the components of the endothelial barrier and to elucidate the potential mechanisms of S1P-induced barrier protection during such challenges. We used two different insults that are known to disrupt barrier function to pursue our objective. The first method was the treatment of brain microvascular endothelial cells with alcohol *in vitro*, and the second method was an *in vivo* model of HSR. We used these two models of endothelial barrier dysfunction to test the protective effects of S1P on paracellular junctions, glycocalyx health, and cell viability.

We focused on this gap of knowledge and generated the central **hypothesis** that “S1P enhances endothelial barrier during alcohol intoxication or hemorrhagic shock by preserving the integrity of junction proteins and the endothelial glycocalyx, and protecting mitochondrial function”. We tested our hypothesis by executing three **specific aims**: Aim 1 was to determine whether S1P protects brain endothelial cells from alcohol-induced barrier disruption. Aim 2 investigated the effects of S1P on endothelial structural components following HSR. Lastly, Aim 3 was to determine whether S1P protects against mitochondrial dysfunction-induced endothelial barrier disruption. We designed our study to provide foundational knowledge for the development of therapeutic interventions for hyperpermeability-related disease conditions. Figure 1 summarizes the three specific aims in a conceptual figure.
Figure 1. Schematic Graph of two Adjacent Endothelial Cells and Specific Aims.

A. Aim (1) investigates whether S1P protects brain endothelial cells against alcohol (EtOH)-induced endothelial hyperpermeability and junctional disruption. B. Aim (2) investigates the effects of S1P in the endothelium structural components (junctions and glycocalyx) following HSR. Aim (3) investigates whether S1P protects mitochondrial function following HSR, and whether mitochondrial dysfunction can lead to junction and glycocalyx disruption.
Chapter Two:
S1P Protects Brain Endothelial Cells Against Alcohol-induced Hyperpermeability and Junctional Disorganization

2.1 Introduction

The blood-brain barrier (BBB) performs an important role in maintaining homeostasis in the central nervous system. This selectively permeable layer is composed of endothelial cells that protect the brain from blood-borne cytotoxins and regulate the entrance of molecules, while also ensuring the supply of nutrients for proper neural function. Brain microvascular endothelial cells are reported to have tighter intercellular junctions than peripheral endothelial cells [45]. Claudin-5 and Occludin are the major tight junction proteins, which are highly expressed in brain endothelium [46]. Endothelial cells in the BBB also express vascular-endothelial cadherin (VE-cadherin), bound to the cytoskeleton via β-catenin. Translocation of β-catenin to the nucleus has been shown to mediate transcriptional repression of claudin-5 in brain microvascular endothelial cells [47]. The functional integrity and low permeability of the BBB depends in part on high levels of expression of junctional proteins, and their high degree of localization at the junctional clefts [48]. Disruption of these proteins by disease or certain drugs may result in increased BBB permeability, exposing the brain to cytotoxins and inflammatory mediators, and consequently impairing neural function [47]. Hence, understanding how various factors can affect BBB junctional proteins is critical for improving methods for prevention of neurological diseases and for the development of novel treatments.

Alcohol intoxication not only impairs brain function, but also results in neuroinflammation and neurodegeneration in humans and animals [49]. The mechanisms involved in alcohol-induced
pathologies include cellular changes, inflammasome activation, apoptosis, and reactive oxygen species (ROS) production [50]. Haorah et al. (2005) showed that alcohol causes disruption of brain endothelial cell monolayers, accompanied by decreased expression of ZO-1, claudin-5 and occludin [51]. Despite these advances in understanding, no strategies to improve BBB function under such conditions have been developed.

S1P is an abundant sphingolipid present in plasma at nanomolar to micromolar concentrations [52], and has been shown to enhance endothelial barrier function by inducing cytoskeletal, junctional and adhesion changes [53-55]. In the first part of this dissertation, we investigated the potential of S1P to enhance the barrier function of brain microvascular endothelial cell monolayers in the presence of alcohol. We assessed to what extent alcohol impairs barrier function and we hypothesized that S1P could ameliorate barrier dysfunction and junctional protein disruption caused by alcohol treatment.

2.2 Materials and Methods

2.2a Endothelial Cell Culture

Human Brain Microvascular Endothelial Cells (HBMEC) were used as an in vitro BBB model. Cells were obtained from ScienCell Research Laboratories (Carlsbad, CA) and grown in 10 cm petri dishes in endothelial cell medium (ECM; ScienCell) supplemented with fetal bovine serum, endothelial growth supplement and penicillin/streptomycin (ScienCell), on 1.5% porcine gelatin matrix (Sigma-Aldrich, St. Louis, MO), in an incubator at 37 °C and 5% CO₂. HBMECs were used at passages 1 to 3.
2.2b Pharmacological Agents

Ethyl alcohol was obtained from Pharmco by Greenfield Global (Shelbyville, KY), sphingosine-1-phosphate (S1P) was from Tocris Biotechne (Minneapolis, MN), and 8-CPT-2'-O-mcAMP-2'-O-Me-cAMP (8-CPT) was purchased from Merck-Millipore (Billerica, MA).

2.2c in vitro Assessment of Endothelial Barrier Function

We assessed endothelial barrier function using two distinct methods. The first was to determine transendothelial electrical resistance (TER) of confluent cell monolayers using the Electric Cell-Substrate Impedance Sensing (ECIS) system (Applied Biophysics, Troy, NY) as previously described [56,57]. ECIS is a well-established indicator of endothelial barrier function based on the tightness of cell-cell junctions [20,58,59]. The advantages of this method are its sensitivity to detect small changes in barrier function due to nanometer-scale changes in the junctions between cells, and the ability to make moment-to-moment measurements, typically every 1-10 seconds apart [59]. Cells were subcultured onto gelatin-coated gold electrode arrays and allowed to grow at 37 °C for 3 - 5 days post-confluence before experiments to allow for maturation of paracellular junctions, with medium changed every other day. On the day of the experiment, the medium was changed to basal endothelial cell medium (bECM) 1 h before the start of the experiment, and the arrays were attached to the ECIS system. A 1 μA AC signal at 4 kHz was applied through a 1-MΩ resistor to a constant-current source, and the in-phase and out-of-phase voltage, respectively proportional to resistance and capacitance, were recorded with the ECIS software. The data are expressed as TER normalized to the 0-min time-point. Treatment was added at time = 0, followed by addition of S1P (1 μM) or vehicle. The ECIS system allows cells to be treated while simultaneously recording TER.

The second method we used to evaluate barrier function was to determine the apparent solute permeability ($P_{solute}$) of HBMEC monolayers to molecules of different sizes, as previously described [57,60,61]. Briefly, HBMEC were seeded on the upper chamber of gelatin-coated
Transwell membranes (0.4 µm; Corning, Corning, MA), and allowed to grow until 3 days post-
confluence so that they formed mature junctions. On the day of the experiment, the medium was
changed to ECM. After 1 hour of baseline, a stock concentration of the molecular tracers sodium
fluorescein (0.4 kDa) or sodium fluorescein isothiocyanate (FITC)-conjugated to albumin (66.4
kDa) or dextran (4 kDa) was added to the upper chamber. HBMECs were immediately treated
with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, or alcohol + S1P. The S1P was
added 5 minutes after alcohol treatment. After 60 minutes of total treatment, samples of media
were collected from both upper (luminal) and lower (abluminal) chambers for fluorometric
analysis. For the sodium fluorescein tracer, the samples were collected after 10 minutes of alcohol
treatment, to avoid equilibration of this tracer in both chambers, which happens within less than
an hour. The concentration of the tracer in each chamber was determined using a standard curve,
and the permeability coefficient was calculated using the equation $P_{\text{solute}} = \frac{C_A}{t} \times \frac{1}{A} \times \frac{V}{C_L}$,
where $C_A$ is the abluminal concentration, $t$ is the time (in seconds), $A$ is the area of the membrane
(in cm$^2$), $V$ is the volume of the abluminal chamber, and $C_L$ is the luminal concentration.

2.2d Experimental Protocols

To assess whether alcohol causes concentration-related changes in TER, the cells were
treated with 50, 75 or 100 mM ethyl alcohol. These concentrations are equivalent to two to three
times the legal limit for blood alcohol concentration in the United States. The alcohol was diluted
in 0.1% BSA dissolved in PBS (vehicle) prior to addition to the cell media. Controls were treated
with isovolumic vehicle. To assess the effects of the barrier enhancers, cells were treated with
either S1P (1 μM), 8-CPT (100 μM), or isovolumic vehicle (0.1% BSA in PBS). To test whether
S1P could reverse any effects of alcohol, cells were treated with alcohol, followed by 1 μM S1P
or isovolumic vehicle, which were administered 5 minutes after alcohol treatment. 100 μM 8-CPT
, a well-studied endothelial barrier enhancer, was used as a positive control for barrier
enhancement. It is worth noting that for S1P, the barrier-protective properties occur at
physiological concentrations (0.1-1.0 μM). At higher concentrations (10 μM), S1P significantly can impair barrier function by disrupting ZO-1 structure and the distribution of F-actin in endothelial cells [62]. Therefore, the concentrations of S1P and 8-CPT used in this study were based on the effective concentrations for enhancing barrier function as previously reported [63,64].

2.2e Immunofluorescence Labeling and Laser Confocal Microscopy

To assess the impact of alcohol on intercellular junctions and the potential of S1P to restore junctional integrity, cultured brain endothelial cells were immunolabeled for VE-cadherin (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), Claudin-5 (4C3C2, Invitrogen) and β-catenin (L54E2, Cell signaling) following treatment with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, or alcohol + S1P. S1P was added 5 minutes after the treatment with alcohol. The cells were fixed for immunofluorescence labeling 15 minutes after alcohol was added, to match the time-point of maximum drop in TER caused by alcohol, as observed in the ECIS assay. Briefly, HBMEC were seeded onto gelatin-coated glass coverslips (Fisher Scientific, Hampton, NH) and incubated in endothelial growth medium until confluent. The medium was changed to bECM and allowed to incubate for 1 hour before the treatment. After the various treatments, cells were fixed in 4% paraformaldehyde in PBS for 10 minutes, permeabilized in 0.1% Triton-X-100 in PBS for 10 minutes and blocked for nonspecific binding with 10% serum in PBS for 30 minutes. Cells were incubated with primary antibodies overnight at 4 °C. They were then washed 3X for 10 minutes with 0.1% Tween-20 in PBS, followed by incubation for 1 hour with Alexa 488- and Alexa-555-conjugated secondary antibodies (Life Technologies, Carlsbad, CA). The samples were washed 3X again and the coverslips were mounted onto microscope slides with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were collected with an Olympus FV1200 spectral inverted laser scanning confocal microscope (Olympus, Center Valley, PA), and the images were analyzed with NIH ImageJ software [65].
2.2f Data Analysis

For time-course data, the tracings show mean normalized TER over time for each experimental group. Other summarized data are presented as the mean ± SE. Immunofluorescence confocal images were analyzed using ImageJ software. Junction protein expression is given as the integrated optical intensity (IOI) of regions of interest (ROI) created on the borders of endothelial cells. Values of N are for the number of cell assays, representing independent experiments, unless otherwise stated. Differences between two groups were assessed using unpaired t-tests. When three or more groups were compared, analysis of variance (ANOVA), and when appropriate, post-hoc tests to compare treatment groups to the control (Dunnett’s test) or to compare all groups (Tukey’s test) were performed. For the permeability coefficient analysis, a two-way ANOVA was performed with the treatments (control, alcohol, and alcohol+S1P) and solutes (fluorescein, dextran-4kDa, and albumin) as factors, and Holm-Sidak multiple comparisons test used for post-hoc analysis. Significance was accepted when P < 0.05.

2.3 Results

2.3a Alcohol Elicits Barrier Dysfunction of Brain Endothelial Cell Monolayers.

The direct impact of different alcohol concentrations (50, 75, and 100 mM) on barrier function of human brain microvascular endothelial cell monolayers was assessed using ECIS. Consistent with a previous study on the effects of alcohol on BBB function [66], we found that alcohol causes a concentration-related decrease in HBMEC barrier function (Fig. 2). Alcohol applied at 75 mM and 100 mM alcohol significantly decreased TER compared to vehicle (0.1% BSA in PBS) control (Fig. 2B). The maximum drop in TER was within the first 15 minutes of treatment, and was followed by a sustained decrease in TER compared to baseline that lasted for over 2 hours (Fig. 2A).
**2.3b S1P Rescues Alcohol-induced Barrier Dysfunction on Brain Endothelial Cells.**

Next, we tested whether S1P’s barrier-enhancing properties extend to HBMEC monolayers. S1P increased TER to 38% above baseline on average (Fig. 3A), while the positive control 8-CPT caused an average 28% increase (Fig. 3C). Vehicle controls caused no change in TER. To examine whether S1P could rapidly restore barrier dysfunction caused by alcohol treatment, we first treated HBMEC monolayers with 75 mM alcohol to elicit a drop in TER, and added S1P 5 minutes later. S1P restored TER well above baseline levels (Fig. 3E). This response was quite rapid, and significantly reduced the amount of the time needed for TER to recover to at least baseline levels after the addition of alcohol (Fig. 3F). Treatment with 8-CPT also ameliorated alcohol-induced barrier dysfunction and decreased the time of recovery, compared to alcohol alone (Fig. 2G, H). These data indicate that S1P’s barrier enhancing properties apply to brain endothelium, and that S1P has the potential to protect barrier function in the presence of alcohol.

**2.3c S1P Rescues Alcohol-Induced Increases in Endothelial Permeability.**

While TER is an indicator of endothelial cell-cell “tightness”, it does not necessarily represent permeability to plasma proteins or other solutes. Therefore, we also determined how alcohol impacts permeability coefficients of HBMEC monolayers to molecules of three different sizes: sodium fluorescein (0.4 kDa), FITC-dextran (4 kDa) and FITC-albumin (66.4 kDa). Three groups were compared (control, alcohol, and alcohol + S1P), and two-way ANOVA revealed a significant difference due to these treatment groups, independent of which tracer was used (p<0.001). Post-hoc analysis of the main treatment groups revealed that alcohol treatment significantly increased permeability compared to control, and that addition of S1P in the presence of alcohol significantly decreased permeability compared to alcohol alone (Fig. 4). It is worth noting that when a similar post-hoc analysis was performed with subgroups for each individual tracer, no significant differences were found, likely due to the smaller sample sizes in each
subgroup. These data suggest that alcohol can increase the permeability of brain endothelial cells monolayers, and that S1P has the potential to decrease permeability in the presence of alcohol.

2.3d S1P Enhances Paracellular Junction Protein Localization in the Presence of Alcohol

To assess whether the alcohol-induced decrease in barrier integrity may be due to the disruption of junctional protein organization, we investigated the localization of Claudin-5, VE-cadherin, and β-catenin (Fig. 5). We also tested the ability of S1P treatment (5 min. after addition of alcohol) to rescue any changes caused by alcohol. In control HBMEC monolayers, claudin-5, VE-cadherin and β-catenin form a continuous border between cells, with junctions that appear very tight. We observed that treatment with 75 mM alcohol for 15 minutes caused an apparent disruption on the normal junctional integrity of all three proteins. In many cells treated with alcohol, instead of the typical continuous junctional protein belt between cells, there was an apparent shift of the proteins to the cytoplasm. We also found that treatment with alcohol caused formation of gaps between HBMECs. In contrast, when S1P was added to alcohol-treated cells, these effects appeared to be reversed. To quantify our observations, we evaluated content of these proteins at the paracellular junctions by measuring the intensities of the immunofluorescence labeling with regions of interests drawn at cell borders. Alcohol treatment did not cause a significant change in junctional content of Claudin-5, VE-cadherin, and β-catenin. However, addition of S1P after alcohol significantly elevated the intensities of all three when compared to the cells treated with alcohol alone (Fig. 5). These data suggest that the ability of alcohol to reduce barrier function and cause junctional gaps does not necessarily involve a significant loss of VE-cadherin, claudin-5, and β-catenin at intercellular junctions. However, the enhanced barrier function elicited by S1P appears to involve increasing or stabilizing the amounts of VE-cadherin, Claudin-5, and β-catenin within intercellular junctions.

2.4 Discussion
Exogenous S1P has been shown to enhance endothelial barrier function at physiological concentrations, while prolonged or high-dose supplementation with S1P can reverse these beneficial effects [62,67]. Previous studies have typically shown a rapid and brief response caused by low-dose S1P treatment in HUVEC, significantly increasing the TER within 2 min, followed by a peak at 5 min, and a decline by 10 min [62,68]. The current results extend the barrier-protective effects of S1P to HBMEC, and show some similarities with these previous results. For example, TER also starts to decrease 10 min after S1P treatment, although our data show that S1P-induced enhancement of barrier function is sustained above baseline levels for at least 60 minutes in HBMEC monolayers (Fig. 3A).

The key finding in this study is that S1P can improve the integrity of HBMEC monolayers that have been exposed to alcohol-induced stress. We first demonstrated that alcohol disrupts barrier function in this model of the BBB, as evidenced by the drop in TER on HBMEC monolayers (Fig. 2). We also showed that S1P treatment, at a physiological concentration, reversed the effects of alcohol by significantly decreasing the time needed for recovery to baseline TER in alcohol-treated brain endothelial cell monolayers (Fig. 3E, F). We found that these findings extend to solute permeability (Fig. 4). Furthermore, we observed that alcohol causes a displacement of junctional proteins from the cell borders to the cytoplasm, and causes gap formation between endothelial cells. S1P significantly reverses the effects of alcohol by recruiting these proteins back to the cell borders, significantly increasing their junctional content and reinforcing a tight endothelial monolayer (Fig. 5). A possible explanation for the increase in interendothelial junctional proteins above baseline levels after S1P treatment is that S1P recruits all the available Claudin-5, ß-catenin, and VE-cadherin to the cell periphery, including protein molecules that were not yet localized to the cell border before alcohol treatment. This experimental evidence supports the overall concept that exogenous S1P has potential to protect barrier function of brain microvessels during alcohol intoxication.
An important limitation of this study is that our model of the BBB utilized only HBMEC and not additional supporting cells that would be found in the brain. While this model enabled us to examine the direct impact of alcohol and S1P on brain endothelial cells, we would expect a tighter BBB *in vivo*. The observation that alcohol rapidly reduces TER of HBMEC and increases the permeability might not be relevant in the healthy brain, but is likely to be relevant in a “double-hit” scenario, such as when an injury occurs during alcohol intoxication, or if a chronic neuroinflammatory disease is already present, amplifying the overall detrimental challenge faced by the brain endothelial cells. In such situations, the BBB might become more permeable to larger molecules that typically do not enter the realm of the central nervous system in the presence of alcohol alone. We have observed this type of effect in the mesentery, in which alcohol intoxication exacerbates hemorrhagic shock and resuscitation-induced hypotension and mesenteric microvascular leakage *in vivo* [44]. Importantly, treatment with S1P can reverse these detrimental effects [44].

Based on these previous observations and the current findings, we conclude that there may be potential benefit of the use of S1P or activation of its receptors in conditions that involve alcohol-induced disruption of the BBB. We speculate that S1P-induced blood-brain barrier enhancement may also be useful for “two hit” scenarios, which represent a topic for future investigation.
Figure 2. Alcohol-induced endothelial barrier dysfunction in cultured human brain endothelial cell (HBMEC) monolayers. A. Traces from an individual experiment showing the time course of changes in transendothelial electrical resistance (TER) in HBMEC monolayers following application of vehicle (0.1% BSA in PBS) or 50, 75, or 100 mM alcohol. B. The mean maximum alcohol-induced changes in TER in HBMEC, observed 5 minutes post-alcohol treatment were compared. *p<0.05, alcohol vs. vehicle control (one-way ANOVA, Dunnett’s multiple comparisons test). N = 4 experiments, 4 monolayers for each concentration.
Figure 3. S1P restores TER in alcohol-treated human brain endothelial cell monolayers. 

A. Traces from an individual experiment showing the time course of changes in TER of HBMEC treated with 1 µM S1P. 

B. The bar graph represents the mean maximum % increase in TER in
HBMEC treated with 1 µM S1P, observed 10 minutes after addition of S1P. C. Time course of changes in TER of HBMEC treated with 100 µM of the Epac Activator 8-CPT, used as a positive control. D. The bar graph represents the maximum % increase in TER in HBMEC monolayers treated with 100 µM 8-CPT, observed 15 minutes post-treatment. E. Traces from an individual experiment show the time-course change in TER of HBMEC monolayers treated with alcohol (75 mM) followed by S1P (1 µM) or vehicle. Alcohol was added at the 0 min time point, and caused a drop in TER. S1P, added 5 minutes later, enhanced barrier function and restored TER in alcohol-treated cells above baseline levels. F. Cells treated with S1P took significantly less time to recover back to baseline levels, compared to vehicle controls. G. Traces from an individual experiment show the time course change in TER of HBEMC treated with 75 mM alcohol followed by 100 µM 8-CPT or vehicle (0.1% BSA in PBS). H. Cells treated with alcohol followed by vehicle took above 150 minutes to recover TER to baseline levels. Cells treated with alcohol followed by 8-CPT took significantly less time. *P < 0.05 S1P/8-CPT vs. vehicle treated group (unpaired t-test). N = 5 - 6 experiments each group.
Figure 4. Impact of alcohol treatment on HBMEC monolayer permeability. Monolayers were treated with vehicle + vehicle (0.1% BSA in PBS), 75 mM alcohol + vehicle, or 75 mM alcohol + 1 µM S1P. Transwell permeability assays were performed to calculate the apparent permeability coefficients for FITC-albumin (66.4 kDa), FITC-dextran (4 kDa) and sodium (Na)-fluorescein (0.4 kDa). The permeability coefficients ($P_{\text{solute}}$) represent an average over 1 hour for FITC-albumin and FITC-dextran, while only a 10-min time period was used for sodium fluorescein due to its relatively high permeability. Two-way ANOVA analysis revealed a significant difference among the three treatment groups (p<0.001). *Indicates p<0.05 between the groups indicated in post-hoc analysis (Holm-Sidak test multiple comparisons test, all solutes included). A similar post-hoc analysis with subgroups for each individual tracer was also performed and revealed no significant differences. N=3 independent experiments (with 4 replicates within each experiment) were performed for the sodium fluorescein and FITC-dextran-4 kDa groups, and N=4 experiments were performed for the FITC-albumin group.
Figure 5. Alcohol causes intercellular gap formation, while S1P enhances paracellular protein organization at intercellular junctions in the presence of alcohol.

**A.** Representative confocal immunofluorescence microscopy z-projections of claudin-5 (green) and VE-Cadherin (red) in confluent cultured HBMEC treated with vehicle + vehicle (0.1% BSA in PBS), alcohol + vehicle, and alcohol + S1P. Arrows indicate gap formation associated with alcohol treatment. Cells treated with 75 mM alcohol followed by 1 μM S1P do not present gap formation. While the quantification (bar graphs) of VE-cadherin and claudin-5 at the paracellular cleft do not
show a significant change upon alcohol treatment, S1P treatment after alcohol significantly increases their localization at junctions compared to alcohol alone.

**B.** Representative immunofluorescence microscopy images of β-catenin of confluent HBMEC monolayers treated with vehicle + vehicle, alcohol + vehicle, and alcohol + S1P. Arrows indicate gap formation associated with alcohol treatment. Treatment with S1P after alcohol was able to restore junctional integrity. Quantification of β-catenin at the paracellular junctions of HBMECs monolayers treated with vehicle + alcohol do not show a significant change compared to vehicle-only treated cells. However, S1P treatment significantly increases β-catenin content at the junctions (bar graph). Cell nuclei are labeled in blue. Scale bar = 45 μm. Images were analyzed using ImageJ software, **P < 0.01, *P 0.05 alcohol + S1P vs. alcohol + vehicle (one-way ANOVA, with Tukey’s multiple comparisons test). N = 7 - 8 areas for each treatment group.**
3.1 Introduction

Increased microvascular permeability to plasma proteins is a hallmark of traumatic injuries and sepsis, and is involved in the pathophysiology of diabetes, ischemia-reperfusion, and, cancer [2,69]. Failure to control the excessive extravasation of fluids and proteins results in edema, impairing normal tissue homeostasis. Hemorrhagic shock involves significant loss of intravascular volume that decreases tissue perfusion and leads to systemic microvascular hyperpermeability [6]. If the extravasation of fluids is not readily resolved, the complications can progressively worsen and culminate in multiple organ failure, which accounts for 50-60% of trauma related deaths [70]. Fluid resuscitation following hemorrhage remains a major challenge, as initial resuscitation with standard care fluids may not improve patient outcome and may exacerbate endothelial damage [71,72].

Paracellular junctional proteins play a major role in controlling the endothelial barrier function in the microvasculature. VE-cadherin is an important adherens junctional protein that is connected to the actin cytoskeleton via association with catenins, particularly β-catenin [73]. Dissociation of the VE-cadherin/β-catenin complex leads to increased microvascular permeability [18,74]. Early studies on the effects of inflammatory mediators on barrier integrity suggested that increases in endothelial permeability are associated with changes in actin cytoskeleton organization and formation of paracellular gaps [75-77]. More recent studies using in vitro models of burn injury showed increased endothelial monolayer permeability and barrier disruption upon
treatment with plasma from mice that had suffered burn injury. This was associated with actin stress fiber formation, loss of VE-cadherin/β-catenin complex from the cell periphery and gap formation in the paracellular cleft [78,79]. In vivo studies using models of hemorrhagic shock have shown increased permeability of the microvasculature, suggesting disruption of junctional proteins [44,80].

In addition to coordination between paracellular junctional proteins and the actin cytoskeleton, the endothelial glycocalyx layer (EGL) has also been elegantly demonstrated to be a modulator of microvascular permeability and immune cell-vessel wall interactions [81]. Shedding of glycocalyx components has been shown in both small and large animal models of hemorrhagic shock [82-85]. Moreover, severely injured trauma patients display glycocalyx degradation in association with increased microvascular permeability [86]. Studies aimed at diminishing microvascular permeability following hemorrhage suggest the involvement of plasma components in glycocalyx health. For example, one study compared the in vivo effects of resuscitation with normal saline, lactated Ringer's, 5 % albumin or fresh frozen plasma (FFP), focusing on maintenance of the endothelial glycocalyx and microvascular barrier integrity after hemorrhagic shock. The outcome was that the crystalloids failed to protect against shedding and thinning of the glycocalyx, increased permeability, and leukocyte adhesion in rat mesenteric microvessels, while FFP conferred protective effects [87]. Resuscitation with fresh whole blood can also restore normal endothelial surface levels of the glycocalyx components syndecan-1 and heparan sulfate and restore permeability changes in a rat model of HSR when compared to LR resuscitation [88]. An additional, innovative study used dried or lyophilized plasma, which can be easily maintained, transported and reconstituted, and demonstrated that it has similar properties to FFP in protecting endothelial function. It enhanced barrier function and preserved adherens junction in vitro, and reduced inflammation and edema in vivo after hemorrhagic shock [89]. These studies show that disruption of glycocalyx is involved in HSR-induced permeability, and suggest that plasma components can improve barrier dysfunction associated with traumatic conditions.
The endogenous bioactive lipid S1P is one plasma component of particular interest because of its endothelial barrier-protective properties and because it is synthesized by erythrocytes and platelets [34], which becomes diluted in the blood upon administration of crystalloid fluids. As shown by multiple studies, including those outlined in Chapter 2, treatment of cultured endothelial cell monolayers with S1P causes an increase in their barrier function, and recruitment of junctional proteins such as VE-cadherin and β-catenin to intercellular junctions [90,91]. S1P has also been demonstrated to inhibit the degradation, and in fact stimulate, the synthesis of components of the glycocalyx such as syndecan-1 and heparan sulfate in endothelial cells [92,93]. Moreover, S1P’s contribution to the maintenance of barrier integrity in isolated microvessels is associated to preservation of the glycocalyx [94]. In vivo, intravenous administration of S1P reduces inflammation and edema formation in the lungs of both rat and canine models of LPS-induced microvascular leakage [42,95]. However many questions remain about the ability of S1P to restore the structure of the endothelium in vivo during hemorrhagic shock or other traumatic injuries, including the mechanism(s) of action.

Our laboratory recently demonstrated that administration of S1P during resuscitation following combined alcohol intoxication and hemorrhage ameliorated microvascular leakage in rat mesenteric microvessels and alleviated hypotension after hemorrhagic shock [44]. However, in that particular study we did not investigate the mechanisms of S1P-induced protection of barrier function in the context of hemorrhagic shock and resuscitation (HSR). Therefore, in the current study we assessed to what extent S1P protects the structure of the microvascular endothelium during HSR. We hypothesized that S1P improves microvascular barrier function during HSR by preserving intercellular junction structure, preventing loss of the glycocalyx layer and reducing inflammation.

3.2 Materials and Methods
3.2a Pharmacological Agents and Antibodies

Sphingosine-1-phosphate (S1P) was obtained from Tocris Biotechne (Minneapolis, MN). The polyclonal rabbit anti-VE-cadherin (CD144), and all fluorescence-labeled secondary antibodies were obtained from Invitrogen (Carlsbad, CA). Mouse anti-β-catenin monoclonal antibody (L54E2) was purchased from Cell Signaling Technology (Boston, MA). Mouse anti-β-actin (C4) horseradish peroxidase-conjugated was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit anti-Caveolin-1 polyclonal antibody (aa 1-17) was from Abcam (Cambridge, MA). Fluorescein isothiocyanate (FITC)-conjugated albumin, FITC-dextran and FITC-lectin were purchased from Sigma-Aldrich (St. Louis, MO). Lactated Ringers and 0.9% sodium chloride solutions were obtained from Henry Schein (Ocala, FL). All other reagents were obtained from Sigma-Aldrich unless otherwise specified.

3.2b Animals

This study was approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC permit number IS00005044) and performed in accordance with the U.S. Animal Welfare Act, U.S. Public Health Service Policy (PHS) on the Humane Care and Use of Laboratory Animals and the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. 12-15 week old male Sprague-Dawley rats (330 – 355 g) were purchased from Envigo (Indianapolis, IL) and housed in a controlled environment (22°C, 12h light/12h dark cycle) in the vivarium, and provided a standard diet (Purina Rat Chow, Ralston Purina) and water ad libitum for a one-week acclimation period prior to surgery. After the acclimation, surgery to implant vascular catheters was performed on anesthetized rats, followed by a one-week recovery period, and then the experimental protocols, all detailed below. All animals were humanely euthanized with Somnasol Euthanasia-III Solution (87 mg/kg i.v., Henry Schein, Dublin, OH) following experimental protocols.
3.2c Surgical Preparation

The surgical procedures for this model have been recently described in detail by our lab and others [44,96,97]. Briefly, the rats were anesthetized with isoflurane, an incision on the ventral side of the neck was made, and sterile catheters flushed with 0.9% sterile sodium chloride USP (Baxter, Deerfield, IL) were implanted in the left common carotid artery and the right external jugular vein. Catheters were secured with a purse-string suture, thermally sealed, subcutaneously routed to the dorsal nape of the neck and exteriorized through an incision. The catheters were secured to the closed incision with suture, coiled and wrapped with masking tape to prevent the rat from accessing it. Carprofen (5 mg/kg subcutaneously; Putney, Portland, ME) was administered every 12 h for the first 48 h following surgery to alleviate any pain during recovery. Before experiments, animals were allowed to recover for 5 to 7 days in the same conditions as the acclimation period.

3.2d Fixed-pressure Hemorrhagic Shock and Resuscitation Protocol

After the post-surgery recovery period, rats were subjected to a fixed-pressure hemorrhagic shock and resuscitation (HSR) protocol while conscious and unrestrained, as previously described [44,97]. A fixed-pressure hemorrhage model was chosen over a fixed-volume model because constant-pressure models are more reliable for experimental standardization and reproducibility [98]. With this method, reliable evaluations of the physiological changes in the tissue of interest that occur during a specific central pressure can be made. Moreover, the model with conscious, unrestrained rats is superior to other models because handling, restraint and anesthetics all significantly impact blood pressure [99,100]. The experimental groups were Sham control rats, HSR, and HSR + S1P. The Sham control group received the same catheter implantation surgical procedure as the other groups but did not undergo the HSR protocol. Briefly, the rats were placed in small cages and the catheters were
routed through the top of each cage. The carotid catheter was connected to a pressure transducer (ADInstruments PowerLab 4/35 with Quad Bridge amplifier system and LabChart software, ADInstruments, Colorado Springs, CO) for continuous blood pressure recording and monitoring throughout the whole protocol. The rats were allowed to acclimate by roaming freely in the cage for 60 minutes before the start of hemorrhage. At the beginning of hemorrhage, arterial blood was withdrawn from the carotid catheter (blood pressure recording is momentarily interrupted at this point) to achieve a blood pressure of 50-60 mmHg, which is maintained for 60 minutes. If necessary, additional blood was withdrawn during this period to maintain the blood pressure. At the end of the 1-h hemorrhage period, warm resuscitation solution (Lactated Ringer’s) was delivered intravenously through the jugular catheter as an initial bolus with a volume of 40% of the total blood volume removed (TBR) followed by an infusion of 2 X TBR over 60 minutes. For the HSR + S1P group, S1P was dissolved in the resuscitation fluid to achieve a dose of 0.1 mg/kg for the overall 1-h resuscitation period. Our previous findings from a dose-response study demonstrated that this dose is most effective to protect against microvascular permeability induced by combined acute alcohol intoxication and HSR [44]. The HSR group received warm lactated ringers without S1P for resuscitation.

3.2e in vivo Assessment of Microvascular Permeability

Following the HSR protocol, microvascular leakage of the mesenteric microcirculation was assessed by intravital microscopy (IVM) as previously described [44,57,97] Briefly, rats were anesthetized with isoflurane (4% induction, 2% maintenance), the ventral abdominal fur was shaved and the skin was sterilized with 4% chlorohexidine gluconate solution (CareFusion, Leawood, KS), 100% ethyl alcohol, and 7.5% Povidone-iodine (Purdue Products L.P., Stamford, CT). Next, a midline laparotomy was performed, and a segment of the small intestine and associated mesentery was exteriorized, spread out over an optical stage and superfused with warm Ringer’s solution. The animal’s body temperature was maintained using a heating plate
placed under the rat’s body and monitored by a rectal thermometer. For assessing microvascular leakage, FITC-conjugated albumin dissolved in Lactated Ringer’s solution was administered intravenously through the jugular catheter as a 1 ml bolus (100 mg/kg) followed by continuous infusion (0.15 mg/kg/min). Observation of mesenteric microvessels was done with a fluorescent microscope (Nikon Eclipse E600) using a 10X objective (Nikon Instruments Inc., Natick, MA) at 488 nm excitation. Fluorescent images were captured via Photometrics HQ2 digital camera (Photometrics, Tucson, AZ), controlled by MicroManager software.

3.2 Data Analysis: Microvascular Permeability

After acquiring images with MicroManager, they were opened with Fiji/ImageJ software. Analysis of FITC-albumin extravasation was done by measuring the integrated optical intensity (IOI) of extravascular areas close to post-capillary venules [6]. Briefly, at least three regions of interest (ROI) were drawn in areas just outside the venules, and the intensity of the grey values of the ROIs were measured and averaged to obtain IOI. The number of replicates for each group was at least 3 rats. Data were analyzed using one-way ANOVA to detect overall differences, and post-hoc assessment with Tukey’s test to detect differences between specific pairs of groups. Significance accepted when p < 0.05.

3.2f Immunofluorescence Labeling and Confocal Microscopy of Mesenteric Microvessels

For some experiments, immunofluorescence confocal microscopy of the junctional protein VE-cadherin on small intestine mesenteric windows was performed following the HSR protocol. At the end of the resuscitation period, the rats were anesthetized with isoflurane, a small laparotomy was performed, and the small intestine and mesentery were harvested and rinsed in ice-cold albumin-physiological salt solution (APSS; 120 mM NaCl, 4.7 mM KCl, 2 mM CaCl$_2$·2H$_2$O, 1.2 mM MgSO$_4$·7H$_2$O, 1.2 mM NaH$_2$PO$_4$, 2 mM Na pyruvate, 5 mM glucose, 0.02 mM EDTA, 3 mM MOPS, and 1% BSA). Rats were then immediately euthanized. A section of the mesentery was pinned in a dissection dish containing ice-cold APSS and, with the aid of a
dissection microscope, mesenteric windows were carefully dissected from the surrounding tissue. Each window was fixed with 4% paraformaldehyde for 15 minutes at room temperature, washed twice with 100 mM glycine buffer to quench the fixation, and then washed once with Ca\(^{2+}\)/Mg\(^{2+}\)-free Dulbecco’s PBS (DPBS). Windows were then permeabilized with 0.1% TritonX-100 in 1X PBS for 30 minutes and washed three times with DPBS for 5 minutes to remove the TritonX-100 detergent. After permeabilization, windows were blocked in 5% donkey serum in DPBS for 45 to 60 minutes, and then incubated overnight at 4 °C with anti-VE-cadherin (1:400) or anti-β-catenin (1:250) primary antibodies diluted in antibody buffer (151 mM NaCl, 17 mM trisodium citrate, 2% donkey serum, 1% BSA, 0.05% Triton X-100, and 0.02% NaN\(_3\)). After overnight incubation with primary antibody, the mesenteric windows were rinsed (four times for 10 min) with an antibody wash solution (151 mM NaCl, 17 mM trisodium citrate, and 0.05% Triton X-100) and then incubated with secondary antibodies (1:500-diluted AlexaFluor-488 donkey anti-rabbit for VE-cadherin or AlexaFluor-594 donkey anti-mouse for β-catenin) diluted in antibody buffer for 60 min at room temperature, followed by four rinses of 10 min in antibody wash solution. After the last wash, each window was splayed on a glass slide with a SecureSeal imaging spacer and 20 µl of 50% glycerol and covered with a glass coverslip. Confocal image stacks (0.5 - 1 µm z sections) of the microvessels in each window were taken with an Olympus FV1200 spectral inverted laser scanning confocal microscope with 40X objective at the Lisa Muma Weitz Advanced Microscopy and Cell Imaging Core at the University of South Florida. The images were analyzed using Imaris image analysis software (Bitplane, Concord, MA). The confocal image stacks were processed into maximum intensity z-projections for presentation as figures using ImageJ software.

For some experiments, immunofluorescence (IF) labeling of VE-cadherin was performed immediately after IVM visualization of the mesenteric windows with FITC-albumin, for correlation of endothelial barrier failure with VE-cadherin redistribution, as previously described [101] with modifications. Briefly, after IVM animals were euthanized and the mesenteric windows were isolated and washed once with Ca\(^{2+}\)/Mg\(^{2+}\)-free Dulbecco’s PBS (DPBS), followed by immediate
incubation with fixative. IF labeling of VE-cadherin was performed normally as described above. Because the IVM with FITC-albumin was followed by a light wash with DPBS and immediate fixation, areas of FITC-albumin leakage could be identified by observation of extravascular FITC-albumin labeling [101]. Confocal image stacks (1-µm z-sections) were collected at excitation wavelengths of 488 nm (for FITC-albumin) and 594 nm (for secondary antibody for VE-cadherin). Images for each fluorophore were collected separately, to eliminate bleed-through due to the intensity of FITC-albumin labeling. For presentation, the images from separate channels were processed with Fiji/ImageJ software into maximum intensity z-projections and merged.

3.2f i Data Analysis: Mesenteric Immunofluorescence Images

The Z-projection 3-D images were saved as Olympus oib format files and opened for analysis using Imaris image analysis software (Bitplane, Concord, MA). Thresholds and parameters were determined and ROIs were created around the length of the microvessels. The sum of the intensities of the grey values was divided by the area of the ROI to obtain the mean intensity value of the vessels. The confocal oib files were exported to Fiji/ImageJ, processed into maximum intensity Z-projections and saved as TIF format for presentation. At least three areas from different venules in each rat were used for analysis. Significance was determined by one-way ANOVA followed by Tukey’s multiple comparisons test and accepted when p < 0.05.

3.2g in vivo Measurements of Glycocalyx Thickness and Integrity

Integrity of the endothelial surface glycocalyx layer (EGL) was assessed in Sham control, HSR and HSR + S1P rats by direct visualization using Bandeiraea simplicifolia (BSI)-lectin conjugated to FITC (FITC-lectin; Sigma-Aldrich, St. Louis, MO), which has high specificity for sugar moieties and can bind to the polysaccharide side chains that compose the EGL [102,103]. Following the experimental protocol, rats were prepared for IVM as described above. Once an area with good blood flow was detected, we administered FITC-lectin (6.25 mg/kg diluted in 1 ml
saline) via the jugular catheter and allowed for systemic distribution before images were taken using the 10X objective at 488 nm excitation. At least three post-capillary venules were used for quantification of the EGL in each rat. All vessels were comparable in blood flow and diameter.

Additionally, we used the dye-exclusion method \([104-106]\) to estimate the thickness of the glycocalyx. After preparing each rat for IVM, a bolus of FITC-labeled dextran (150 kDa) at 50 mg/kg diluted in 1 ml Ringer’s solution was administered via jugular catheter, and fluorescence was allowed to reach a steady level. Post-capillary venules with steady blood flow were selected, and both brightfield and fluorescent (488 nm) images were captured with a 10X objective. At least three different areas were imaged per rat.

3.2 Data Analysis: FITC-lectin

After acquiring the images with MicroManager, they were saved in TIF format and opened with Fiji/ImageJ software for analysis. A region of interest (ROI) was created by manually tracing a measurement path along the wall of each vessel. The grey intensity values of each pixel along the path were measured and averaged to obtain the mean integrated optical intensity (IOI) for that vessel wall. This IOI was averaged with the average IOI obtained from a path traced on the contralateral wall in focus \([107]\). Significance was determined with one-way ANOVA followed by Tukey’s multiple comparisons test, and accepted when \(P < 0.05\).

3.2 Data Analysis: Dye Exclusion

Large-size molecules such as 150-kDa dextrans are excluded from the vessel wall by the glycocalyx, so the difference in vessel diameter at a given location between brightfield and fluorescence images represents the thickness of the glycocalyx on the two vessel walls \([105,106]\). Briefly, after acquiring brightfield and fluorescent images with MicroManager, they were saved as TIF and later opened with ImageJ for analysis. On the brightfield image, a straight line was traced from one vessel inner wall to the opposite inner wall and the length of the line was measured in microns. This process was repeated on the fluorescent image, measuring the diameter of the
luminal FITC-Dextran-150 at the same location as the corresponding brightfield image. The difference in vascular widths between the brightfield and fluorescent images was divided by two to obtain the thickness of the glycocalyx for that particular area. This process was repeated at least three times along the length of each vessel and the values were averaged to obtain the mean glycocalyx thickness. Significance was determined with one-way ANOVA followed by Tukey’s multiple comparisons test, and accepted when P < 0.05.

3.2h Leukocyte-Endothelium Rolling and Adhesion

Leukocyte rolling and adhesion were analyzed in Sham, HSR and HSR + S1P rats following the experimental protocol. Rats were prepared for IVM and mesenteric venules (30 - 60 µm diameter) with comparable blood flow were selected for visualization of leukocyte dynamics. The exteriorized mesenteric was continuously superfused with 37 °C Ringer’s solution. The microcirculation was observed using a Nikon Eclipse E600 microscope and 10X objective, and rapid time-lapse brightfield image sets were acquired over a 30-s period. The microscope was focused above and below the diametral plane in order to capture all leukocytes present in that segment.

3.2h.i Data Analysis: Leukocyte Rolling and Adhesion

Rolling and adhesion were measured by assessing the frame-by-frame playback of the digital image set for individual leukocytes. The number of leukocytes that were rolling in a velocity noticeably slower than that of cells in the central blood stream was counted for each selected vessel segment, and the length of that segment was also recorded. A leukocyte was defined adherent if it remained stationary for at least 5 s. Significance was determined by one-way ANOVA, with Tukey’s multiple comparisons test, and accepted when p < 0.05. Leukocyte rolling and adhesion are expressed as the number of leukocytes per 100 µm of vessel length as previously described [58,108,109].
3.2i Western Blotting of Mesenteric Microvessels

Following the experimental protocol for each group, some rats were used for protein expression analysis of the mesenteric microvasculature. Briefly, at the end of the experiment, the rats were humanely euthanized and the small intestine and mesentery were excised and immediately placed in a beaker containing ice-cold APSS. The tissue was splayed on a dissecting dish and the mesenteric microvasculature was carefully dissected away from the surrounding tissue, as previously described [80]. For total protein extraction, the isolated microvasculature was homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors (Thermo Fisher, Rockford, IL). Tissue homogenates were centrifuged for 15 min at 14,000 rpm at 4 °C, and the supernatant was collected for protein concentration estimation using Pierce's bicinchoninic acid assay (BCA) kit (Thermo Fisher). Each sample consisted of microvessels pooled from 7 - 10 rats per experimental group. To extract the cytosolic protein fraction, the microvessels were dissected as described above, and a plasma membrane protein extraction kit (Abcam, Cambridge, MA) was used according to the manufacturer’s protocol to extract the membrane and cytosolic protein fractions. Our samples yielded concentrations sufficient for immunodetection of proteins in the total and cytosolic fractions, but not in the membrane fraction by Western blot. The protein samples were heated at 100 °C for 5 minutes and subjected to SDS-PAGE, then transferred to polyvinylidene (PVDF) membranes. The membranes were blocked for 1 hour at room temperature with 5% bovine serum albumin (BSA) in Tris-buffered saline solution with 0.1% Tween-20 (TBS-T) and were incubated with mouse anti-β-catenin (1:300), rabbit anti-Caveolin-1 (1.5 μg/ml) or HRP-conjugated anti-β-actin (1:1000) diluted in blocking solution overnight at 4 °C. The membranes were washed three times with TBS-T. For the membranes incubated with the anti-β-catenin and anti-caveolin-1 antibodies, an additional incubation with HRP-conjugated-anti-mouse IgG secondary antibodies (1:1000) was performed at room temperature for 1 h. Following three washes with TBST-T, the protein bands
were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher, Rockford, IL) and the Chemidoc XRS+ Molecular Imager with Image Lab software (Bio-Rad, Hercules, CA).

3.2.1 Data Analysis: Western Blot

Band intensities were quantified using ImageJ software. Regions of interest were drawn around each β-catenin and β-actin bands and the mean grey intensity value was measured. Cytosolic β-catenin bands were normalized to their respective cytosolic actin levels for comparison between groups. Significance was determined using one-way ANOVA for comparison between groups, and an unpaired t-test was used to compare the HSR and HSR+S1P groups. Significance was accepted at P < 0.05.

3.3 Results

3.3a S1P Rescued Mean Arterial Blood Pressure and FITC-Albumin Extravasation after HSR.

To evaluate how S1P impacts HSR-induced hypotension and microvascular leakage following HSR, we monitored the mean arterial blood pressure (MAP) throughout the protocol and assessed FITC-albumin extravasation by IVM. Administration of S1P in the resuscitation fluid (lactated Ringer’s solution) significantly raised MAP at the end of the resuscitation period, compared to rats that received lactated Ringer's only (Fig. 6). When examining microvascular leakage, in time-matched Sham rats, the FITC-albumin remained in the luminal compartment of the microvessels, with minimal fluorescence intensity in the extravascular space. HSR rats showed high levels of fluorescence in the areas surrounding the mesenteric microvasculature. Rats that received S1P in their resuscitation fluid had relatively low fluorescence outside the vasculature (Fig. 7A). Quantification of these data showed that the HSR group had a significantly higher mean integrated optical intensity (IOI) in the extravascular areas near the microvessels.
compared to sham controls, indicating elevated microvascular leakage of FITC-albumin. HSR rats that received S1P (HSR + S1P) had significantly lower IOI compared to HSR rats (Fig. 7B). These data suggest that the S1P improves mean arterial pressure after hemorrhage, and attenuates the microvascular hyperpermeability induced by HSR, thus preventing loss of central fluid volume.

3.3b Resuscitation with S1P following Hemorrhage Preserved Paracellular Junction Protein Integrity.

Because the expression and localization of junctional proteins such as VE-cadherin at the paracellular junctions is pivotal for endothelial barrier integrity, we performed immunofluorescence confocal microscopy of mesenteric post-capillary venules to determine to what extent S1P may reduce HSR-induced microvascular leakage by improving junctional location of VE-cadherin. Fig. 8A shows that VE-cadherin is localized between endothelial cells in Sham rats, outlining a clear boundary for each cell. HSR disrupted the localization of VE-cadherin, and also significantly decreased the intensity of labeling. HSR + S1P rats showed high localization of VE-cadherin at the junctions, and significantly higher VE-cadherin expression compared to HSR rats (Fig. 8A, B). Disorganization of the adherens junctional protein β-catenin was also observed with HSR, and its localization in the paracellular junction was significantly increased in HSR + S1P rats compared to HSR rats (Fig. 8C, D).

β-Catenin is known to stabilize VE-cadherin and aid in paracellular junction maturation and function. Loss of cadherin-mediated adhesion in the cell-cell junction can promote the release of β-catenin into the cytoplasm, where it acts as a signaling molecule [110]. Moreover, the high expression of cadherin molecules at the cell membrane can act as a “trap” for free, cytoplasmic β-catenin [111]. While immunofluorescence microscopy enabled us to view disorganization of β-catenin in microvessels, it did not allow us to determine the precise membrane or cytosolic localization of β-catenin. Therefore, in order to better understand the dynamic changes in β-
catenin localization during HSR, we performed western blot on the mesenteric microvessels following experiments. Our results, in which loading of samples was performed after overall protein levels in the lysates were equalized, showed no change in the total amount of β-catenin protein in mesenteric microvessels of sham, HSR or HSR + S1P rats. However, we observed a noticeable increase in the cytosolic levels of β-catenin following HSR compared to Sham rats. HSR + S1P rats had significantly lower cytosolic levels of β-catenin compared to HSR rats (Fig. 9 A, B). To verify cytosolic protein fraction isolation, we also performed Western blots for caveolin-1, a protein known to be localized to lipid rafts of the plasma membrane, where it performs several roles in membrane dynamics [112]. The band intensities for caveolin-1 were lower in the cytosolic fraction than those for the total protein fraction of all three groups, indicating successful fractionation. Interestingly, higher cytosolic levels of caveolin-1 in the HSR group were observed and might indicate its dissociation from membrane caveolae as a response to injury. Given that total β-catenin expression did not change, this data suggests that HSR causes a shift in β-catenin localization from the junctions to the cytosol, destabilizing VE-cadherin and contributing for the increase in permeability. Treatment with S1P seems to recruit the available β-catenin in the cytosol back to the paracellular junctions during HSR, and this might be contributing to rescue albumin permeability.

3.3c VE-Cadherin Breakdown Correlated with Endothelial Barrier Failure.

In order to determine whether adherens junction breakdown correlated with areas of microvascular barrier dysfunction, we performed confocal microscopy to examine VE-cadherin morphology specifically at areas where endothelial barrier had failed as evidenced by FITC-albumin accumulation in vessel walls and extravasation. We performed these experiments in rats that underwent Sham HSR, HSR or HSR with S1P treatment. Examination of the mesenteric venules of Sham and S1P-treated rats showed interendothelial VE-cadherin labeling pattern more
often than HSR rats, whereas microvessels of HSR-treated rats consistently revealed a strong degree of disruption in the intercellular borders, as evidenced by a diffuse VE-cadherin labeling (Fig. 10). Moreover, we observed that VE-cadherin discontinuity corresponded to albumin labeling in and just outside the vessel walls, independently of the treatment given (Fig. 10).

3.3d Resuscitation with S1P Protected the Endothelial Glycocalyx Layer (EGL).

Hemorrhagic shock and resuscitation has been shown to cause degradation of the EGL [85,113]. In the current study, we performed direct visualization of the EGL using fluorescence-labeled lectin, and we found that HSR caused a significant drop in the accumulation of FITC-lectin bound to the walls of post-capillary venules compared to Sham rats (Fig. 11 A, B). Rats that received S1P in the resuscitation fluid (HSR + S1P) had significantly higher FITC-lectin fluorescence compared to HSR rats (Fig. 11 A, B). We also measured the thickness of the glycocalyx in the mesenteric post-capillary venules by using the dye-exclusion method. We found that rats subjected to hemorrhage and resuscitation without S1P had significant reduction in glycocalyx thickness compared to Sham rats. Rats that received S1P resuscitation displayed significantly thicker glycocalyx compared to HSR rats (Fig. 11 C, D). These data suggest that S1P’s barrier-protective effects during HSR are partly due to its ability to maintain glycocalyx integrity.

3.3e Leukocyte-endothelium Interaction Was Reduced With S1P Administration.

Degradation of the glycocalyx plays a role in inducing a pro-inflammatory response and promoting leukocyte adhesion to endothelial cells [114,115]. Therefore, as an indicator of the level of inflammation, and indirect indicator of glycocalyx integrity, we evaluated leukocyte interaction with the endothelium of mesenteric post-capillary venules in Sham, HSR, and HSR + S1P rats. All venules chosen were comparable to flow rate and diameter. Leukocyte rolling and adhesion data are shown in Fig. 12. Treatment with S1P resulted in significantly lower number of slow-
rolling leukocytes in mesenteric venules after HSR compared to HSR rats that did not receive S1P (Fig. 12A). HSR rats exhibited significantly greater leukocyte adhesion (leukocytes that were stationary for 5 seconds or more) to the endothelium compared to Sham rats. This effect was reversed by resuscitation with S1P (Fig. 12B). These data support the role of S1P in protecting against glycocalyx degradation during HSR, attenuating the inflammatory response that leads to disruption of endothelial barrier and hyperpermeability.

3.4 Discussion

The complications that arise from fluid resuscitation following hemorrhagic shock remain a serious problem, including resuscitation-related changes in endothelial cell function and loss of endothelial integrity, contributing to microvascular leakage and edema formation [44]. In this part of the study, we demonstrate that S1P protects against microvascular leakage and improves blood pressure when administered during resuscitation following hemorrhagic shock. We also show that resuscitation with S1P prevents HSR-induced disruption of endothelial junctions and the glycocalyx layer. Likewise, rolling and adhesion of leukocytes, which can be facilitated by glycocalyx shedding, are also attenuated with S1P treatment.

Our findings that S1P can ameliorate HSR-induced hypotension and microvascular leakage are in agreement with previous work utilizing administration of S1P or its analog FTY720 in resuscitation fluids [44,116]. S1P or its mimetics have been shown to reduce microvascular leakage in a variety of other animal models, including platelet activating factor- and bradykinin-induced elevations of hydraulic conductivity in rat mesenteric microvessels [117], histamine-induced extravasation in the rat cremaster muscle [118], endotoxin-induced acute lung alveolar permeability [41], and bleomycin-induced lung edema [119]. The mechanisms involved in S1P-mediated reduction of microvascular leakage are not completely understood, but involve activation of one or more S1P receptors, followed by rapid activation of Rho family small GTPases, changes in the cytoskeleton including increased protrusion of local lamellipodia, plus
changes in junctional and focal adhesions [64,68,120,121]. In addition, S1P also has been shown to promote glycocalyx integrity in cultured endothelial cells [93,94]. As nearly all of the mechanistic studies have been performed in cultured cells, our current findings provide novel in vivo evidence that S1P is effective at preventing HSR-induced disruption of VE-cadherin and β-catenin localization at endothelial junction in combination with preserving glycocalyx integrity. Moreover, we provide the first evidence of that microvascular hyperpermeability induced by HSR correlates with areas where the organization of the adherens junction VE-cadherin is disrupted.

As described above, structural changes that are likely involved in the mechanism of S1P-mediated barrier enhancement have been elucidated. However, at this stage their linkage in a cause-effect paradigm remained to be determined. In the next section of this dissertation, we investigate a novel mechanism linking S1P-induced enhancement of paracellular junction protein localization, endothelial glycocalyx integrity, and endothelial barrier function during HSR through protection of mitochondrial function.
Figure 6. S1P Treatment Rescues Hypotension Induced by Hemorrhagic Shock and resuscitation (HSR).

Time-course tracing of mean arterial blood pressure (MAP) of HSR (n = 3) and HSR + S1P (n = 4) rats during the fixed-pressure HSR. A 30-minute baseline measurement (BL) was taken before the start of hemorrhage. Values are shown as means ± SEM, **P < 0.01 between groups at the last three time-points of resuscitation. The groups were compared with repeated measures ANOVA and Fisher’s LSD test.
Figure 7. S1P Treatment Rescues hemorrhagic-shock and Resuscitation-induced Microvascular Leakage.

A. Representative fluorescent images of the mesenteric microvasculature of Sham (N = 3), HSR (N = 4), and HSR + S1P (N = 3) groups. Arrows indicate “hot-spots” of albumin leakage just outside the vessel wall. The circles show areas of albumin leakage into the extravascular space.

B. Quantification of FITC-albumin extravasation was done by measuring the integrated optical intensity (IOI) in the extraluminal space adjacent to postcapillary venules. **P < 0.01, *P < 0.05 (one-way ANOVA, with Tukey’s multiple comparisons test).
Figure 8. Resuscitation with S1P preserves adherens junction organization and expression following hemorrhage.

A, C. Representative maximum intensity confocal z-projections of immunofluorescence-labeled VE-cadherin (green) and β-catenin (red) in mesenteric windows from Sham (N = 4), HSR (N = 5), and HSR + S1P (N = 4) rats. The representative images show disorganization of the junctional protein pattern in post-capillary venules following HSR, in contrast to organized localization between endothelial cells in the sham group and HSR + S1P group. B, D. Quantification of VE-cadherin and β-catenin expression on the microvessels was done using the Imaris image analysis software, and is shown as integrated optical intensity (IOI) of the vessel area. At least three windows from each rat were used for quantification. *P < 0.05 (one-way ANOVA followed by Tukey’s test).
Figure 9. HSR Increases Cytosolic Expression of β-catenin, and Resuscitation with S1P Reverses this Effect.

A. Representative Western Blot showing an increase in the cytosolic concentration of β-catenin following HSR. Specific antibodies were used to determine β-catenin expression in dissected mesenteric microvessels. In the HSR + S1P group, an apparent decrease in cytosolic expression of β-catenin, with no apparent change in total protein expression was observed. Caveolin-1, a protein that is expressed on the plasma membrane of most cell types, is shown as a control for cytosolic fraction separation. These data suggest a shift in β-catenin and caveolin-1 localization during HSR. Pooled samples of microvessels isolated from 7 - 10 rats were needed for sufficient protein for analysis. B. Quantification of the β-catenin band intensity from multiple Western blots. The data represent N = 2 pooled samples for sham, N = 3 pooled samples for HSR, and N = 4 pooled samples for HSR + S1P. *P < 0.05, HSR vs. HSR + S1P (unpaired t-test). The sham group was not used in the statistical analysis and is only shown for visual comparison.
Figure 10. Correlation of VE-cadherin disruption with endothelial barrier dysfunction.

Representative confocal images showing colocalization of VE-cadherin immunofluorescence labeling and sites of FITC-albumin permeability in mesenteric microvessels of Sham, HSR, and HSR + S1P rats. Disruption in VE-cadherin organization following HSR colocalizes with FITC-albumin labeling around the microvessel.
Figure 11. S1P protects the mesenteric microcirculation against glycocalyx degradation induced by HSR.

A. Representative images showing FITC-lectin binding to the endothelial glycocalyx on the vessel walls of post-capillary venules. FITC-lectin was administered intravenously following the sham, HSR, or HSR + S1P protocols. Sham rats present high concentration of lectin bound to the venular wall, while HSR rats have much smaller amounts of FITC-lectin fluorescence, as indicated by the arrows. Animals that received resuscitation with S1P (HSR + S1P) retain FITC-lectin on the endothelium. B. Analytical quantification of FITC-lectin bound to the walls of post-capillary venules. Each data point represents the average integrated optical intensity (IOI) of at least 3 separate vessels in one rat. **P < 0.01, *P < 0.05 for the indicated groups, one-way ANOVA.
followed by Tukey’s multiple comparisons test. N=3 rats in each group. C. Measurement of rat mesenteric microvascular glycocalyx thickness using FITC-dextran (150 kDa) exclusion assay was performed with brightfield and fluorescent images of mesenteric venules. D. Quantification of glycocalyx thickness from the imaging data is shown. Each data point represents the average of glycocalyx measurements on 4 - 8 vessel areas per rat. **P < 0.01, one-way ANOVA, with Tukey’s multiple comparisons test. N = 3 rats per group.
Figure 12. Resuscitation with S1P decreases leukocyte-endothelium interactions following hemorrhage.

A. S1P administration during resuscitation after hemorrhage significantly reduces the number of slow-rolling leukocytes in mesenteric postcapillary venules, compared to HSR alone. B. The number of leukocytes firmly adhered to the endothelium following HSR is significantly higher than sham control rats. Resuscitation with S1P significantly decreases firm leukocyte adhesion. *P < 0.05 (one-way ANOVA, with Tukey’s multiple comparisons test) for the indicated groups. Sham group, N = 3; HSR, N = 6; HSR + S1P, N = 4.
Chapter Four:
S1P Protects Endothelial Glycocalyx/Junctional Structure and Barrier Function by Limiting Mitochondrial Depolarization

4.1 Introduction

The mitochondria are the key metabolic regulators in most types of cells. In addition to their classic role in the aerobic production of ATP, they regulate a variety of processes from cell proliferation, to apoptosis and nuclear signaling [122-124]. However, the mitochondrial content of endothelial cells is relatively modest. In rat endothelial cells, they occupy only 2 - 6 % of the cell volume, in contrast to 28 % in hepatocytes and 32 % in cardiac myocytes [125]. Indeed, endothelial cells obtain a great portion of their energy from anaerobic glycolysis [126], from where they generate more than 80% of their ATP [127]. Although the mitochondria are not the main source of energy production in endothelial cells, they do have important signaling roles in the vascular endothelium [125]. For example, the mitochondria are considered important regulators of the intracellular calcium buffering system in endothelial cells. Although the ER is the major site for calcium storage, up to 25 % of cellular calcium is located in the mitochondria [125]. The mitochondria and the ER have been shown to operate together to regulate calcium trafficking in endothelial cells [128]. Calcium signaling regulates the actin cytoskeleton in endothelial cells, influencing cell motility, shape, and barrier function [129]. Mitochondrial reactive oxygen species (mROS) are also associated to endothelial cell dysfunction, apoptosis, and senescence. Altered mitochondrial dynamics has been associated with endothelial dysfunction in patients with vascular diseases such as diabetes mellitus, atherosclerosis and hypertension [130,131]. Although mitochondrial dysfunction has been associated to endothelial cells in diseases that involve
vascular damage, the direct mechanisms of mitochondria-induced disruption of endothelial barrier are still unknown.

Previous studies have demonstrated that activation of the mitochondrial intrinsic apoptotic signaling pathway could play a role in HSR-induced hyperpermeability in vivo. They reported that hemorrhagic shock resulted in an increase in the expression of the pro-apoptotic protein BAK, disruption in mitochondria transmembrane potential with release of cytochrome-c into the cytoplasm, and caspase-3 activation. In vivo transfection of BAK had similar effects [80]. Caspase-3 is responsible for proteolytic cleavage of β-catenin, which maintains endothelial cell morphological homeostasis by associating with VE-cadherin and protecting it from degradation [132,133]. Besides that, the mitochondrial complex III was shown to be involved in endothelial hyperpermeability induced by BAK protein in vitro [134]. Interventions to rescue or improve mitochondrial functions during the pathogenesis of various vascular diseases have been proposed as potential strategies to improve vascular conditions in both animal models and human patients [125].

S1P is an important regulator of cell viability and survival by modulating apoptotic signaling. Activation of S1PR1 has been shown to decrease cell death by preventing mitochondrial dysfunction [135,136]. The role of S1P in supporting mitochondrial function specifically has been elegantly showed in a recent study. They investigated how T-cells survive for years while constantly traveling, and their results suggest that S1PR1 signaling is required for T-cells to maintain mitochondrial content and function, providing them with energy to continue their constant migration [135]. In a study about the neuroprotective effects of S1P in an in vitro model of ischemia, they found that treatment with S1P reduced oxygen-glucose deprivation (OGD)-induced mitochondrial inner membrane depolarization and also reduced the increase in mitochondrial Ca\(^{2+}\) during OGD [136], suggesting a mechanism of S1P-mediated enhancement of cell viability.
In this part of the dissertation, we investigated the role of the mitochondria in mediating endothelial barrier integrity and function, and tested a possible mechanism of S1P-mediated endothelial barrier protection through the mitochondria. We hypothesized that disruption of mitochondrial membrane potential induces endothelial barrier dysfunction through breakage of junction proteins and glycocalyx, and S1P is able to reverse these effects by protecting mitochondrial function.

4.2 Materials and Methods

4.2a Pharmacological Agents and Antibodies

JC-1, a fluorescent mitochondrial membrane potential sensor, was obtained from Invitrogen (Carlsbad, CA). The mitochondrial complex III inhibitor Antimycin-A and the ionophore Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP), both inducers of mitochondrial depolarization by disrupting different aspects of the electron transport chain, were purchased from Abcam (Cambridge, MA). The mouse monoclonal cytochrome-c antibody (37BA11) was also purchased from Abcam. Mouse monoclonal anti-heparan sulfate antibody (F58-10E4) was obtained from Amsbio (Cambridge, MA). Polyclonal rabbit anti-ZO-1 (Mid) was purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-β-catenin (L54E2) was obtained from Cell Signaling Technologies (Boston, MA). The rabbit anti-BAK (N-terminus) and anti-fractin (C-terminus) polyclonal antibodies were obtained from Millipore (Burlington, MA). All fluorescence-labeled secondary antibodies were obtained from Invitrogen.

4.2b Cell Culture

Rat Primary Intestinal Mesenteric Vascular Endothelial Cells (RIMEC) and complete rat endothelial cell medium (ECM) were purchased from Cell Biologics (Chicago, IL). The cells were grown in ECM on 0.2 % porcine gelatin matrix (Sigma-Aldrich, St. Louis, MO) in a 37°C and 5 %
CO₂ incubator. The ECM was designed and optimized for the culture of rat endothelial cells. It contains 2% fetal bovine serum (FBS), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), L-glutamine, and antibiotic-antimycotic solution. On the day of experiments, the ECM was replaced with basal ECM (bECM), a serum-free basal medium that lacks FBS and the other growth factors found in ECM to minimize confounding effects of these additives on experimental outcomes.

4.2c Animals

The *in vivo* experiments described in this chapter were done with Sprague Dawley rats, using the same surgical preparation and HSR protocol described in Chapter 3, sections 3.2b-e. All experiments were performed after IACUC approval and in accordance with PHS guidelines and the NIH *Guide for Care and Use of Laboratory Animals*.

4.2d *in vivo* Assessment of Mitochondrial Transmembrane Potential

Following the HSR protocol, some rats were prepared for *in vivo* visualization of mitochondrial transmembrane potential of mesenteric microvessels using IVM. Once animals had a section of their small intestine and mesentery exposed on an optical stage, the mesentery was superfused with warm JC-1 solution (10 μg/ml in sterile 0.9% saline). Before image capture, the JC-1 was rinsed off the mesentery. JC-1 exhibits potential-dependent accumulation within the mitochondria and its response is specific to depolarization [137]. JC-1 is able to enter the matrix of healthy mitochondria, where it selectively accumulates and forms aggregates that fluoresce in red. When the mitochondrial membrane depolarizes, JC-1 cannot accumulate, remaining in the cytoplasm as a monomeric green fluorescent form [80]. Consequently, a decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. Images were captured with a 20X objective, by exciting both the monomer and the J-aggregate at 488 nm, and detecting green and red emission at 530 and 590 nm wavelengths.
4.2d.i Data Analysis: in vivo Mitochondrial Membrane Potential

The TIF image files obtained during IVM were opened with ImageJ for analysis of mitochondrial function. The multi-channel images were split into single emission channels and the brightness and contrast were adjusted for viewing but without altering the original pixel data. Using the green channel image, which was easier to visualize the vessel, a ROI was created along the length of a vessel wall, and the grey intensity value of each pixel along the ROI was measured and averaged to obtain the mean IOI. The same ROI was used in the red channel image to obtain the mean IOI. The same process was repeated on the contralateral wall of the same vessel. Changes in mitochondrial membrane potential were determined by calculating the red to green fluorescence intensity ratio on each vessel wall and averaging the values of at least three areas for each rat. Significance of differences between means was determined by one-way ANOVA followed by Tukey’s multiple comparisons test, and accepted when P < 0.05.

4.2e Assessment of Apoptotic Signaling Activation in Mesenteric Microvessels

In order to determine activation of apoptotic signaling on mesenteric microvessels following experimental protocol, we performed immunodetection by western blot (WB) of pro-apoptotic protein BAK, cytosolic Cytochrome-c, and Fractin, a novel marker for caspase-3 activity [138]. The procedure of protein extraction, immunodetection and data analysis by WB in mesenteric microvessels has been described in Chapter 3, section 3.2i. The BAK and fractin antibodies were used at 1:500 and 1:1000 dilutions, respectively. The mouse monoclonal cytochrome-c antibody was used at concentration 1 μg/ml.

4.2f Transendothelial Electrical Resistance

RIMEC were grown to confluence in complete endothelial cell medium (ECM) containing 2% fetal bovine serum (FBS) at 37 °C and 5% CO₂. Cells were seeded onto gold electrode arrays pre-coated with 0.2% gelatin for assessment of transendothelial electrical resistance (TER) using
the ECIS system, as described in Chapter 2, section 2.2b. Vehicle (bECM), Antimycin-A (10 µM) or CCCP (10 µM) were added at time = 0, immediately followed (~1 min later) by addition of S1P (1 µM) or vehicle, and the TER was recorded for 10 hours to determine changes in barrier function over time.

4.2f.i Data Analysis: Transendothelial Electrical Resistance

After each experiment, the data text file produced by the ECIS software was opened as comma-separated values in Microsoft Excel for analysis. The “0-min” time-point is defined as the time when the first treatment was applied to the monolayer. All data points were normalized to the 0-min time point, and the TER values for each treatment at each time-point recorded were averaged. The data is presented as the mean resistance ± S.E. for each hour following treatment vs. time in hours. N = 5 independent experiments were performed, with 4 - 8 monolayers per group in each experiment. Statistical analysis of normalized TER was done with repeated-measures ANOVA followed by Tukey’s multiple comparison test, and significance was accepted if p < 0.05.

4.2g in vitro Assessment of Endothelial Cell Death

RIMECs were seeded on 6-well plates pre-coated with 0.2% gelatin for analysis of cytosolic levels of cytochrome-c by immunodetection. Cells were allowed to grow to confluence in complete ECM, at 37 °C and 5% CO₂. Before the experiment the medium was changed to bECM (vehicle) and cells were allowed to equilibrate for 1 h. Vehicle (bECM), Antimycin-A (10 µM) or CCCP (10 µM) were added, immediately followed (~1 min later) by addition of S1P (1 µM) or vehicle. The conditions of treatment were 37 °C and 5% CO₂, and the total time was 5 h. Cytosolic protein extraction was done using the Plasma Membrane Protein Extraction Kit (Abcam, Cambridge, MA) according to the manufacturer’s protocol. Samples were subjected to protein concentration estimation using Pierce’s bicinchoninic acid assay (BCA) assay kit (Thermo Fisher),
followed by immunodetection and analysis using the WES Separation Module system (Protein Simple, San Jose, CA) according to the manufacturer’s instructions.

Propidium Iodide Viability Assay

In order to assess cell viability following AA or CCCP treatment, we used propidium iodide (PI) staining, according to the manufacturer’s protocol (Life Technologies, Carlsbad, CA). Briefly, RIMECs were seeded onto gelatin-coated 35-mm glass bottom dishes (MatTek Corporation, Ashland, MA) and allowed to grow to confluence. On the day of the experiment the medium was changed for bECM and allowed to equilibrate for 1 hour. Cells were treated with vehicle, AA or CCCP and the total time of treatment was 5 hours. After the various treatments, cells were rinsed and equilibrated with a PI solution for 15 minutes at 37 °C, according to the manufacturer’s instructions. After 15 minutes, cell viability was assessed using an ASI Imaging Rapid Automated Modular Microscope system (ASI Imaging, Eugene, OR) with a 40X objective and Micromanager/ImageJ software for image collection. Each treatment was repeated 5 times. In each petri dish, a field of view was selected at random and the number of PI-positive cells were counted and divided by the number of total cells.

4.2h in vitro Immunofluorescence (IF) of Endothelial Glycocalyx and Junction Proteins

RIMEC were grown at 37 °C and 5 % CO2, as described above, and seeded onto glass coverslips pre-coated with 0.2 % gelatin and allowed to mature for 3 days post confluence. Medium was changed every other day. On the day of the experiment, the medium was changed to 37 °C bECM 30 minutes before treatment. Cells were treated with either vehicle (bECM) or AA (10 µM), followed by treatment with S1P (1 µM) for 1 hour. For labeling of the endothelial glycocalyx, cells were washed once with DPBS and fixed with 2% paraformaldehyde (PFA) and 0.1 % glutaraldehyde solution in DPBS for 30 min. After fixation, PFA was rinsed with DPBS and cells were blocked with 2 % donkey serum in DPBS for 1 hour. Cells were incubated with mouse
monoclonal anti-heparan-sulfate antibody overnight at 4 °C. Three washes of 10 min each were
done with DPBS, followed by incubation with secondary donkey anti-mouse antibody conjugated
to Alexa Fluor 488 for 1 hour at room temperature. After three washes of 10 min with DPBS, the
coverslips were mounted onto slides with Prolong Gold antifade reagent with DAPI (Invitrogen,
Carlsbad, CA). For the junction proteins ZO-1 and β-catenin, the same IF labeling protocol was
followed as described in Chapter 2, section 2.2e. Confocal images were taken with an Olympus
FV1200 spectral inverted laser scanning confocal microscope with a 60X objective at the Lisa
Muma Weitz Advanced Microscopy and Cell Imaging Core at the University of South Florida. The
image stacks were processed and analyzed using ImageJ.

4.2i Data Analysis: Glycocalyx Immunofluorescence

The confocal images were saved as Olympus oib files and opened later with ImageJ for
analysis. Regions of interest (ROI) were drawn in the four quadrants of each field of view, and the
intensities of the grey values of that ROI were measured and averaged to obtain the integrated
optical intensity (IOI) for that field of view. The IOIs of three areas for each experiment were
averaged and presented as an estimate of Heparan Sulfate expression (± SEM). Significance
of differences between means was determined by one-way ANOVA with Tukey’s multiple
comparisons test, and accepted when P < 0.05.

4.2i in vitro Assessment of Mitochondrial Membrane Potential

Rat Primary Intestinal Mesenteric Vascular Endothelial Cells (RIMEC) were grown to
confluence in complete endothelial cell medium (ECM) containing 2% fetal bovine serum (FBS)
at 37 °C and 5% CO₂. For live-cell imaging of mitochondrial function, the cells were seeded onto
glass-bottom dishes pre-coated with 0.2 % gelatin and allowed to mature for 3 to 5 days post
confluence. On the day of the experiment the medium was changed to warm basal endothelial
cell medium (bECM) containing JC-1 (2 µg/ml) and allowed to stabilize at 37 °C for 15 min. The
dye-containing medium was washed off with basal medium and the cells were randomized into
groups that were treated with bECM (controls), bECM containing antimycin-a (10 µM final
congestion), or treated with antimycin-a followed by S1P treatment (1 µM final concentration).
At 10 min post-treatment, images were captured with a Olympus FV1200 spectral inverted laser
scanning confocal microscope using a 40X objective, by exciting JC-1 monomer at 488 nm and
selectively exciting the J-aggregate at 554 nm, and detecting green and red emissions at 530 and
590 nm.

4.2.i Data Analysis: in vitro Mitochondrial Potential

The confocal images were saved as oib Olympus format files and transferred to ImageJ
for analysis. The multi-channel images were split into single emission channels. In each field of
view analyzed, a region of interest (ROI) was created around each individual cell on both red and
green emission channels. The mean grey value intensity of the ROI was measured and expressed
as integrated optical intensity (IOI). The IOI of the red channel was divided by the IOI of the green
channel for each cell. The red/green IOI values (mean ± SEM) of at least 4 cells per field-of-view
is presented as a measurement of mitochondrial depolarization levels. Significance of the
differences between means was determined by one-way ANOVA followed by Tukey’s multiple
comparisons test, and was accepted when P < 0.05.

4.3 Results

4.3a Resuscitation With S1P Protects Mitochondrial Function.

Previous studies have reported that changes in mitochondrial function in response to HSR
could be associated with microvascular hyperpermeability [80]. In order to assess the effects of
S1P in mitochondrial function of mesenteric microvessels in the context of HSR, we used intravital
observation of microvessels loaded with the mitochondrial membrane potential sensor JC-1. In
cells with healthy mitochondria, some JC-1 exists as a monomeric form in the cytosol (green fluorescence) and but a significant portion also accumulates in the mitochondria and forms aggregates, fluorescing red. Upon depolarization, JC-1 cannot accumulate in the mitochondria and produces only green fluorescence [139]. Mitochondrial depolarization is seen as a decrease in the red to green fluorescence ratio. We found that HSR caused mitochondrial depolarization in mesenteric postcapillary venules, as seen as a significant decrease in red/green ratio when compared to sham rats (Fig. 13). Treatment with S1P during resuscitation protected mitochondrial membrane potential and reversed the effect of HSR, significantly increasing red/green fluorescence ratio (Fig. 13).

**4.3b HSR Does Not Induce Apoptotic Signaling Activation on Mesenteric Microvessels.**

Previous studies have associated “intrinsic” apoptotic signaling activation to microvascular permeability using a different model of HSR, in which rats were anesthetized [80]. In order to investigate whether the increase in microvascular permeability observed in our studies could be due to endothelial cell death, we subjected the mesenteric microvasculature to immunoblot analysis of pro-apoptotic proteins BAK, cytochrome-c and fractin. Fractin is the name given to the N-terminal fragment of β-actin when cleaved by activated caspases. It has been established as a marker for caspase-3 activity, and it is used to distinguish apoptotic from necrotic cells [138]. Our results showed no significant difference in the levels of BAK in mesenteric microvasculature following HSR. The expression levels of cytosolic cytochrome-c and fractin were also not significantly changed (Fig. 14). These data suggest that, even though HSR caused disruption of mitochondrial membrane potential, apoptotic signaling and caspase activation were not involved in microvascular permeability in our model of HSR. This led us to hypothesize that: 1) apoptotic signaling activation is not necessarily involved in endothelial permeability induced by mitochondrial dysfunction; and 2) S1P-mediated attenuation of microvascular permeability is associated with its ability to promote normal mitochondrial function on endothelial cells.
4.3c Mitochondrial Complex III is Involved in S1P-mediated Endothelial Barrier Enhancement.

To directly test whether mitochondrial depolarization impairs the endothelial barrier, we treated cultured rat intestinal mesenteric endothelial cells (RIMEC) with two different inhibitors of oxidative phosphorylation: the ionophore Carbonyl Cyanide m-chlorophenyl Hydrazone (CCCP), and the inhibitor of mitochondrial respiratory chain complex III antimycin-A (AA) [140], that has been previously been associated with endothelial monolayer permeability [134]. Fig. 15A shows a time course of changes in TER, an index of endothelial barrier function, for RIMEC treated with vehicle alone (control), AA + Vehicle or AA + S1P. AA treatment immediately caused the TER to begin to drop and the reduction in TER was sustained throughout the time course. For the cells treated with S1P given just after the addition of AA, only a small drop in TER occurred, which was insignificant compared to control (Fig. 15A), indicating that S1P is able to protect RIMEC from AA-induced barrier dysfunction. Fig. 15B shows the time course changes in TER of RIMEC treated with vehicle control alone, CCCP + Vehicle or CCCP + S1P. Treatment with CCCP caused the TER to gradually decrease throughout the 11-hour time course. Treatment with S1P following the addition of CCCP was not able to significantly rescue the drop in TER (Fig. 15B). These data indicate that disruption of mitochondrial membrane leads to endothelial barrier dysfunction, and that complex III is involved in the maintenance of barrier function. Moreover, S1P is able to rescue barrier dysfunction induced by complex III inhibition.

To investigate whether endothelial cell death could be involved in AA- and CCCP-induced barrier dysfunction, we analyzed cytosolic levels of cytochrome-c, and measured cell viability using PI stain. Treatment with AA or CCCP was not correlated with significant changes in cytochrome-c levels in the cytosol (Fig. 16 A, B). PI stain analysis did not show an increase in cell death upon AA or CCCP treatment (Fig. 16 C, D). These data support our in vivo data (Fig. 14)
and suggest that endothelial barrier dysfunction induced by mitochondrial membrane disruption does not necessarily involve cell death.

**4.3d S1P Protects Against Paracellular Junctional Disruption and Glycocalyx Degradation Induced by Mitochondrial Complex III Inhibition.**

Mitochondrial complex III has been reported to be involved in endothelial permeability induced by BAK transfection. In order to determine whether the complex III is involved in the maintenance of paracellular junctional structure, we treated RIMEC with AA and assessed the morphological changes in tight and adherens junction proteins ZO-1 and β-catenin. AA caused disruption of ZO-1 and β-catenin organization and formation of paracellular gaps. Treatment with S1P following addition of AA maintained the localization of β-catenin, and preserved the continuous expression of ZO-1 between endothelial cells (Fig. 17).

In order to assess whether the complex III inhibition causes degradation of the endothelial glycocalyx layer (EGL), we treated cells with AA and evaluated the surface expression of the glycocalyx component heparan sulfate (Fig. 18). We found that treatment with AA degraded heparan sulfate surface expression on cultured RIMEC. Treatment with S1P after AA preserved the surface expression of heparan sulfate (Fig. 18A, B). These results suggest that normal mitochondrial complex III activity, and by extension normal mitochondrial membrane integrity, are required for normal maintenance of junction proteins and the EGL, and show that S1P has the potential to attenuate endothelial structure disorganization caused by mitochondrial dysfunction during inhibition of mitochondrial complex III.

**4.3e S1P Inhibits Mitochondrial Depolarization Caused by Blockade of Mitochondrial Complex III Activity.**
In order to support our *in vivo* findings that S1P protects mitochondrial function by blocking mitochondrial depolarization, we performed imaging of live RIMEC loaded with JC-1 and treated with antimycin-a in the absence or presence of S1P (Fig. 19). We found that inhibition of complex III with AA caused depolarization of the mitochondria in RIMEC monolayers, and that treatment with S1P after AA was able to rescue this effect (Fig. 19). This result shows that S1P-induced protection of mitochondrial function is likely due its ability to protect against inhibition of mitochondrial respiratory chain complex III. These results also suggest that S1P-induced protection of endothelial barrier function is due to its ability to protect against mitochondrial depolarization at the level of complex III.

4.4 Discussion

In this series of experiments, we tested our hypothesis that disruption of mitochondrial function could lead to endothelial barrier dysfunction through disruption of junction protein organization and glycocalyx integrity, and that S1P could rescue this effect. Consistent with previous reports [80], we show that HSR causes a disruption in mitochondrial transmembrane potential and depolarization in mesenteric venules, suggesting the mitochondria as potential modulators of endothelial barrier function. In addition, we provide the novel finding that S1P administration in the resuscitation fluid attenuates the HSR-induced disruption of mitochondrial membrane integrity. Even though we show that mitochondrial depolarization occurs during HSR, analysis of proapoptotic proteins BAK, cytosolic cytochrome-c and the caspase-mediated fragmentation of β-actin (fractin), in which no differences were observed between the different treatment groups, suggest no activation of apoptotic signaling in HSR when performed in conscious, unrestrained rats.

To determine whether protection of mitochondrial membrane potential may mediate S1P-induced attenuation of microvascular leakage caused by HSR, we utilized a cultured RIMEC model in which we could study the direct impact of mitochondrial disruption on endothelial barrier
function. The results demonstrate that mitochondrial depolarization occurs after inhibition of mitochondrial complex III with AA [141] or uncoupling of the proton gradient with CCCP, both of which also cause endothelial barrier dysfunction. These results support previous reports that complex III is indeed involved in endothelial cell hyperpermeability [134]. However, those studies showed involvement of complex III in BAK-induced endothelial hyperpermeability, with AA alone having no impact in the basal permeability. Our results, which arose utilizing a more sensitive assay for barrier function, ECIS, differ from those studies by showing that complex III is in fact involved in endothelial barrier function. In addition, our results show that normal activity of complex III is required for the maintenance of junctional proteins and the glycocalyx. Taken together with previous reports that direct disruption of the glycocalyx increases endothelial permeability [92,93], our findings suggest that complex III activity, and by extension normal mitochondrial function, helps maintain the microvascular barrier by promoting a healthy glycocalyx layer on endothelial cells. Moreover, our in vitro studies show that mitochondrial membrane disruption can lead to endothelial permeability without activation of apoptotic signaling, or cell death, ruling out these pathways. We also present the novel finding that treatment with S1P partially restores the AA-induced loss of mitochondrial membrane potential, in combination with prevention of 1) AA-induced shedding of the glycocalyx component heparan sulfate, and 2) AA-induced barrier dysfunction.

Collectively, the current results, in context with the previous literature, suggest that S1P-induced enhancement of endothelial barrier function is due at least in part to stabilization of mitochondrial membrane potential, which in turn promotes junctional and glycocalyx integrity. These results also show that S1P can protect the mitochondrial membrane from disruptions of the mitochondrial transport chain at the level of Complex III.

An interesting part of our results is that S1P only partially rescues mitochondrial depolarization induced by AA (Fig. 19 B). However, our data show that S1P completely reverses the effect of AA on endothelial barrier function (Fig. 15 A), which is accompanied by significant
preservation of junctional proteins and the glycocalyx. This either suggests that S1P’s ability to block mitochondrial depolarization is sufficient to maintain barrier function, or that S1P signaling compensates for other pathways downstream of complex III. The production of reactive oxygen species (ROS) during endothelial injury and disruptions in the calcium signaling mechanism might provide a possible explanation for S1P-induced protection during mitochondrial dysfunction. During inflammatory state conditions, there is an increase in levels of cytosolic calcium, due to release from the ER or extracellular calcium influx. This results in increased mitochondrial calcium levels, enhanced activity of the electron transport chain, and subsequent superoxide production [142]. The mitochondrial complex III has been regarded as a main source of superoxide [143], which upon release into mitochondrial matrix and inter-membrane space, is converted to hydrogen peroxide, elevating mROS levels. Mitochondrial ROS can diffuse to the cytoplasm, where it interacts with and modifies calcium signaling, usually resulting in further increase in intracellular free calcium levels [142]. The stability and control of vascular barrier function is dependent on calcium-based homotypic linkage between neighboring adherens junction proteins, such as VE-cadherin [95]. S1P is implicated in mediating the interaction between adheres junction proteins and the actin cytoskeleton through enhancing the interaction between the actin-binding proteins cortactin and endothelial myosin light chain kinase (MLCK), an event that is dependent on calcium. Treatment of endothelial cells with S1P rapidly localizes both of these proteins to the cell periphery, where they participate in rearranging the cytoskeleton, leading to enhanced barrier function [55]. The elevation in intracellular levels of calcium that occurs during mitochondrial dysfunction, particularly dysfunction of complex III, might intensify the ability of S1P to mediate the interaction between cortactin and MLCK, possibly explaining the overshoot in TER above baseline levels observed when AA-treated cells are co-treated with S1P (Fig. 15A). This mechanism of S1P-mediated endothelial barrier protection during mitochondrial dysfunction is a possibility for future investigation.
Figure 13. S1P protects against HSR-induced mitochondrial dysfunction.

A. Representative *in vivo* IVM of mesenteric windows from the sham, HSR and HSR + S1P groups. In sham rats, JC-1 aggregated in the mitochondria of post-capillary venules and fluoresced red (590 nm images), indicating healthy mitochondria. HSR rats show impaired mitochondrial membrane integrity, causing elevated diffusion of JC-1 to the cytoplasm (green fluorescence, 530 nm images) and decreased red aggregate. S1P-treated rats show intact mitochondria as JC-1 red aggregate. B. Analysis of red to green fluorescence ratio in the walls of post-capillary venules show that HSR causes significant mitochondrial depolarization, which is rescued by resuscitation with S1P. Each dot represents the average ratio value for one rat. *P < 0.05, ***P < 0.01. The groups were compared with one-way ANOVA and Tukey’s multiple comparisons test. Sham, N = 5; HSR N = 6; HSR + S1P, N = 5.
Figure 14. HSR Does Not Induce Apoptotic Signaling Activation in Mesenteric Microvessels

Graphs show the quantifications of the band intensity from Western blots of proteins BAK, cytosolic cytochrome-c and fractin following Sham control treatment, HSR, or HSR + S1P. One-way ANOVA with Tukey’s multiple comparisons test showed no significant difference in the expression levels of these proteins between all treatment groups. The data represent N = 3 or 4 pooled samples of microvessels isolated from 7 - 10 for each group.
Figure 15. S1P rescues endothelial barrier dysfunction induced by inhibition of mitochondrial complex III.

A. Dynamic changes in TER of RIMEC monolayers treated with vehicle control (bECM) followed by vehicle (VEH + VEH), antimycin-a followed by vehicle (AA + VEH) and AA followed by S1P (AA + S1P). RIMEC treated with antimycin-A (AA) display a time-dependent decrease in TER, which is rescued by treatment with S1P (N = 5 independent experiments for, with 4 - 8 monolayers per group in each experiment). B. Dynamic changes in TER of RIMEC treated with vehicle followed by vehicle, CCCP followed by vehicle (CCCP + VEH) and CCCP followed by S1P (CCCP + S1P). Treatment with CCCP causes a time-dependent decrease in TER, which was not rescued by S1P treatment (N = 4 independent experiments, 4 - 8 monolayers per group in each experiment). Tracings indicate the mean resistance normalized to t = 0 (indicated by arrow) ± S.E. for each hour following treatment. Statistical analysis of normalized TER was done with repeated-measures ANOVA, with Tukey’s multiple comparisons test, *P < 0.05.
Figure 16. Mitochondrial membrane disruption does not involve endothelial cell death.

A. Representative WES showing cytosolic cytochrome-c in RIMECs treated with vehicle followed by vehicle (V/V), antimycin-a followed by vehicle or S1P (A/V, A/S), or CCCP followed by vehicle or S1P (C/V, C/S).  

B. Quantification of cytochrome-c cytosolic levels from 3 independent experiments (one-way ANOVA).

C. Representative Propidium iodide (PI) stain images of RIMECs.
treated with vehicle control, antimycin-a (AA) or CCCP. The number of PI-positive cells does not increase upon AA or CCCP treatment. D. Quantification of PI stained cells show no significant change in cell viability (one-way ANOVA). N = 5 independent experiments.
Figure 17. S1P Rescues Paracellular Junctional Disorganization Induced by Mitochondrial Complex III Inhibition

Z-projection confocal immunofluorescence microscopy images of AJ and TJ proteins β-catenin and ZO-1 are shown. Vehicle treated control cells display continuous expression of junction proteins between endothelial cells (VEH + VEH). Inhibition of complex III with antimycin-a (AA) (AA + VEH) leads to gap formation (indicated by the arrows) and discontinuity in paracellular localization of junctional proteins. Treatment with S1P (added ~1 min after AA) maintains junction protein organization (AA + S1P). Images are representative of three separate experiments.
Figure 18. S1P rescues endothelial glycocalyx degradation induced by inhibition of mitochondrial complex III.

A. Representative z-projections of confocal immunofluorescence images of the glycocalyx component heparan sulfate. High expression of heparan sulfate can be seen on RIMECs treated with vehicle only (VEH + VEH). Lower heparan sulfate fluorescence is seen in cells treated with AA + Vehicle. Cells treated with S1P show high heparan sulfate fluorescence (AA + S1P).

B. Quantification of heparan sulfate fluorescence on RIMEC show a significant decrease induced by
AA that is rescued by S1P treatment. Heparan sulfate expression is expressed as the mean integrated optical intensity (IOI) from three independent experiments. *P < 0.05, **P < 0.01 (one-way ANOVA, Tukey’s multiple comparisons test).
Figure 19. S1P protects endothelial cells from mitochondrial depolarization induced by inhibition of complex III.

A. Representative confocal fluorescence images of JC-1-loaded RIMECs treated with antimycin-a (AA) followed by S1P or vehicle. Control cells (VEH + VEH) show JC-1 aggregates (red fluorescence, indicated by arrows) in the mitochondria, indicating healthy mitochondria. Cells treated with AA show an increase in cytoplasmic JC-1 monomer (green fluorescence), indicating mitochondrial depolarization. Cells treated with S1P after antimycin-a (AA + S1P) show some intact mitochondria.

B. Quantification of mitochondrial depolarization is shown in terms of Red/Green fluorescence ratio. AA treatment causes a significant decrease in red to green fluorescence compared to controls. AA treatment followed by S1P significantly increases red to
green fluorescence ratio on RIMECs, indicating protection of complex III by S1P. All images were acquired 10 minutes after addition of S1P or vehicle. **P < 0.01, ***P < 0.001 one-way ANOVA, followed by Tukey’s multiple comparisons test. Each dot represents the average of each independent experiment. N=4 experiments for each group, with at least four cells analyzed in each independent experiment.
Chapter Five:
Overall Conclusions and Future Directions

5.1 Overall Questions and Findings of this Dissertation

Endothelial barrier dysfunction is a common feature of several vascular-related diseases, including hypertension, diabetes, traumatic injury, and cerebral diseases like stroke, subarachnoid hemorrhage and Alzheimer’s [144]. Understanding how different stimuli affect endothelial cell function is essential for the development of novel therapeutics for treatment of such diseases. With this in mind, in this study we have investigated the potential of S1P to rescue endothelial barrier function after challenge by different insults that produce hyperpermeability. Based on previous literature showing the detrimental effects of alcohol on the blood-brain barrier, and the barrier-enhancing properties of S1P, we hypothesized that S1P could ameliorate barrier dysfunction and junctional protein disruption caused by alcohol treatment in an in vitro model of blood-brain barrier. We found that alcohol elicits brain endothelial cell monolayer barrier dysfunction, confirming previous findings. We then showed that brain endothelial cell monolayer barrier disruption is accompanied by increased permeability to solutes of different sizes. This is accompanied by breakage of junction proteins thought to be determinants of endothelial barrier function in the central and peripheral circulation, thus having a role in blood-brain barrier homeostasis, such as Claudin-5, VE-cadherin, and β-catenin. We show that S1P is able to reverse the detrimental effects of alcohol in brain endothelial cell function and junctional integrity, confirming our hypothesis.

We further investigated the barrier-protective effects of S1P during challenge with hemorrhagic shock and resuscitation in vivo. This model has clinical significance, as traumatic
injuries cause systemic microvascular hyperpermeability. We hypothesized that S1P could improve the microvascular barrier function during HSR by preserving paracellular junction integrity, preventing loss of glycocalyx on endothelial cells, and reducing inflammation. Our hypothesis was proven correct when administration of S1P in resuscitation fluids preserved the localization of adherens junctions between endothelial cells, attenuated thinning of the glycocalyx layer, and reduced the interaction between leukocytes and the endothelium. Due to increasing evidence that the mitochondria play a role as signaling molecules in the endothelium rather than an exclusively metabolic part, we investigated whether mitochondrial dysfunction could disrupt endothelial barrier, and whether S1P is able to reverse these effects by protecting mitochondrial function. We found that S1P was able to rescue mitochondrial dysfunction produced by HSR, suggesting that the mitochondria are important regulators of barrier function. Interestingly, we found that blockage of the mitochondrial complex III leads to endothelial barrier dysfunction, supported by breakage of junction proteins β-catenin and ZO-1, and shedding of glycocalyx component heparan sulfate. We also found that uncoupling of electron transport chain (ETC) also leads to endothelial barrier dysfunction. However, we unexpectedly found that S1P is only able to prevent barrier dysfunction when it is caused by complex III inhibition, and not by direct disruption of mitochondrial membrane potential with an ionophore, suggesting that S1P acts through a mechanism that involves or is downstream of complex III, but is not immediately affected by uncoupling of the proton gradient across the inner mitochondrial membrane. When testing the mechanisms of S1P-induced barrier protection, we confirmed our hypothesis and showed that S1P at least partly protects mitochondrial function by preventing depolarization as a result of complex III inhibition.

Collectively, these data support our overall hypothesis that S1P can protect endothelial barrier during alcohol intoxication and hemorrhagic shock by preserving the integrity of junction proteins and the glycocalyx, and protecting mitochondrial function.
5.2 Future Directions

Our results suggest that alcohol intoxication could exacerbate the effects of a secondary inflammatory insult in the endothelial barrier. One immediate future direction as a result of this work is to study the effects of alcohol on the BBB \textit{in vivo}, in the presence of secondary stimuli such as traumatic brain injury, as well as the potential therapeutic effect of S1P. In a previous investigation we observed that S1P confers a beneficial effect to the mesenteric microcirculation when HSR is combined with acute alcohol intoxication [44]. Under such conditions, alcohol appears to act by causing additional insult to the endothelial barrier. Investigation of this model in the BBB would likely expand the scope of possible therapeutic applications for S1P. The signaling mechanisms underlying S1P’s protection under a “two-hit” scenario can also be subject of future investigation. Our data suggest that some common mechanisms probably underlie the ability of S1P to enhance the endothelial barrier whether hyperpermeability is caused by HSR or alcohol. For example, paracellular junction integrity would likely be further disrupted with alcohol treatment preceding HSR. On the other hand, the effects of alcohol on the endothelial glycocalyx have been largely understudied, making it harder to predict the outcomes. However, the development of techniques for glycocalyx visualization suggest that alcohol promotes a considerable collapse of this structure [145], providing a foundation for future investigation.

Another important future direction of this study is to investigate the molecular mechanism that explains how mitochondrial function contributes to the integrity of intercellular junctions and endothelial glycocalyx. We have provided insight by showing involvement of the mitochondrial complex III in barrier function and S1P-mediated barrier enhancement. However, the specific molecular mechanisms that link the complex III to junctional and glycocalyx integrity are not known. The possible involvement of complex III-generated mitochondrial ROS and disturbances in calcium homeostasis may be subject of future studies. Furthermore, future studies on whether the mitochondria is involved in alcohol-induced barrier dysfunction may provide additional
information on how to enhance the function of endothelial cells during inflammatory challenges that involve alcohol intoxication.

In summary, our findings present the first evidence that S1P can rescue the functional and morphological integrity of endothelial cells disrupted by alcohol. In addition, we provide the first demonstration that S1P can protect against HSR-induced endothelial mitochondrial membrane disruption in combination with endothelial junctional and glycocalyx damage, increased leukocyte rolling and adhesion, and microvascular leakage. Our findings provide new insights on the therapeutic potential of S1P, and the importance of endothelial mitochondrial function as a potential pharmacologic target for future interventions. We expect that future work will produce additional advances that may lead to improved therapeutics to prevent excessive microvascular leakage in inflammatory disease conditions.
References


**APPENDIX A**
IACUC APPROVAL FOR ANIMAL RESEARCH

RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO: Jerome Breslin,

FROM: Farah Mouavi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 5/22/2018

PROJECT TITLE: 51P fluid therapy to reduce hemorrhagic shock & intoxication-induced injury

FUNDING SOURCE: National Institutes of Health
USF department, institute, center, etc.

IACUC PROTOCOL #: R ISO00050/44
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 5/22/2018:

Rat: Sprague-Dawley (7-13 wk/280-360 gM-F) 656

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
MEMORANDUM

TO: Jerome Breslin,

FROM: Farah Moulvi, MSPH, IACUC Coordinator
       Institutional Animal Care & Use Committee
       Research Integrity & Compliance

DATE: 5/15/2015

PROJECT TITLE: Using S1P to improve fluid therapy for alcohol intoxication and hemorrhagic shock

FUNDING SOURCE: National Institutes of Health; USF department, institute, center, etc.

IACUC PROTOCOL #: R IS00001315

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 5/15/2015:

Rat: Sprague-Dawley (7-13 wk/280-360 952 g/M-F)

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the

5/15/2015
APPENDIX B

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