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Endothelial Protrusions in Junctional Integrity and Barrier Function

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Abstract

Endothelial cells of the microcirculation form a semi-permeable diffusion barrier between the blood and tissues. This permeability of the endothelium, particularly in the capillaries and postcapillary venules, is a normal physiological function needed for blood-tissue exchange in the microcirculation. During inflammation, microvascular permeability increases dramatically and can lead to tissue edema, which in turn can lead to dysfunction of tissues and organs. The molecular mechanisms that control the barrier function of endothelial cells have been under investigation for several decades, and remain an important topic due to the potential for discovery of novel therapeutic strategies to reduce edema. This review highlights current knowledge of the cellular and molecular mechanisms that lead to endothelial hyperpermeability during inflammatory conditions associated with injury and disease. This includes a discussion of recent findings demonstrating temporal protrusions by endothelial cells that may contribute to intercellular junction integrity between endothelial cells and affect the diffusion distance for solutes via the paracellular pathway.

1. INTRODUCTION

Microvascular permeability is a normal physiological function of the circulatory system required for delivery of fluids and nutrients to the tissues of the body. The microvascular wall is composed of endothelial cells, along with the endothelial glycocalyx, basement membrane, and some accessory cells, which collectively form a tight and selective barrier. Disruption of endothelial barrier function is a hallmark of vascular inflammation in several pathological and injury conditions such as trauma, ischemia-reperfusion injury, sepsis, diabetes, and cancer. During such pathological conditions, changes in the structural conformation of the endothelial cells can be caused by inflammatory mediators such as histamine, bradykinin, and platelet activating factor, cytokines such as vascular endothelial growth factor (VEGF), metabolic factors like ATP, and activated neutrophils, collectively leading to higher permeability of the microvascular wall (Durán *et al.*, 2008). Elevated microvascular permeability results in increased leakage of fluids and plasma proteins to the extravascular space. In turn, their accumulation in the interstitium leads to tissue swelling, or edema. If edema formation becomes uncontrolled, the complications from the exacerbated

inflammatory response can lead to additional tissue injury and organ failure (Yuan & Rigor, 2010).

Acute respiratory distress syndrome is an example of such a pathological, edematous state, with mortality rates ranging from 26 to 58% (Ranieri *et al.*, 2012). The endothelial barrier dysfunction may result from infectious or sterile inflammatory stimulation, such as pneumonia or mechanical ventilators, respectively. It is generally thought that at the cellular and molecular level, the microvascular hyperpermeability is due to compromised endothelial cell-cell adhesion properties, as we will explore in this chapter.

In the following sections, we will review what is known about the control of microvascular permeability, focusing on cell-cell contact and junctional protein integrity. After a summary of the current available protocols to study endothelial barrier function, we will explore the responses of the endothelial barrier to injury and diseases. Finally, we will show evidence supporting that endothelial protrusions and local lamellipodia contribute to endothelial barrier function.

2. DETERMINANTS OF ENDOTHELIAL BARRIER FUNCTION

Under physiological conditions, passage of fluids and solutes across the endothelial barrier can occur via a combination of transcellular or paracellular pathways. In both cases, an active role for the endothelial cells is implied. Transcytotic pathways require energy for vesicle formation and passage. Paracellular junctions must be formed and continually maintained in an environment where there are constant stresses that could potentially cause intercellular adhesions to fail.

The transcellular pathway is thought to mediate the normal, physiological transport of macromolecules across the endothelium. Morphological data from several reports supports a process involving formation of endocytotic vesicles that can trap albumin and other plasma proteins at the luminal membrane, followed by transcytosis across the cell, and exocytosis at the basal membrane (Jennings & Florey, 1967; Bruns & Palade, 1968; Johansson, 1979; Clough & Michel, 1981; Simionescu & Simionescu, 1984; Yuan & Rigor, 2010). This process is likely mediated by caveolae, which are lipid raft microdomains that form invaginations in the plasma membrane, and by vesiculo-vacuolar organelles, which can fuse with trafficking vesicles and form transcellular pores, spanning the cell interior (Feng *et al.*, 1996; Mehta & Malik, 2006).

The paracellular pathway is thought to mediate transport of water and solutes ranging from ions to macromolecules. Paracellular passage occurs through the junctional clefts between endothelial cells, which contain a fibrous matrix of proteins that act as a molecular sieve that can exclude solutes based on size or charge (Chambers & Zweifach, 1947; Curry & Michel, 1980). Increased microvascular permeability to plasma proteins under acute inflammatory or pathophysiological conditions is thought to be due in large part to opening of the paracellular pathway (Majno & Palade, 1961). This route also serves as a migration path for pathogens, leukocytes and even metastatic tumor cells (Shen *et al.*, 2009). Interendothelial junctional proteins are responsible for cell-cell adhesion and barrier “tightness”, conferring

the control of paracellular permeability. Two families of junctional proteins present in the microvascular endothelium corresponding to the classical adherens junctions (AJ) and tight junctions (TJ) are present. The AJ proteins are thought to be the major determinants of endothelial barrier, due to findings that selective disruption of the normal junctional organization of the AJ protein vascular-endothelial cadherin (VE-cadherin) results in systemic microvascular permeability (Corada *et al.*, 1999; Corada *et al.*, 2001). The extracellular domain of a VE-cadherin molecule expressed on one cell forms a homotypic bond with the extracellular domain of another VE-cadherin molecule expressed on the membrane of an adjacent endothelial cell, binding them together. VE-cadherin molecules connect intracellularly to the actin cytoskeleton using catenins as linkers, including β -catenin (Yuan & Rigor, 2010). TJ proteins include claudins, occludins and junction adhesion molecule (JAM), and these connect to the actin cytoskeleton via the zona occludin-1 (ZO-1). The TJ proteins are also present in the peripheral microvasculature but are thought to be more prominently expressed in the microvascular endothelium of the blood-brain and the blood-retinal barriers (Hawkins & Davis, 2005).

The basal side of the endothelium is connected to the extracellular matrix by protein complexes called focal adhesions that are composed of integrins. The focal adhesions also connect intracellularly to the cytoskeleton via the linker proteins including focal adhesion kinase, talin, paxillin, and vinculin (Wu, 2005). The focal adhesions are an important component of endothelial barrier as they help form a tight connection between endothelial cells and their substratum. Focal adhesions also help coordinate migration and crawling movements of endothelial cells in their basal plane while maintaining barrier integrity.

The elevation in microvascular permeability during inflammation is due to the specific binding of inflammatory mediators to their cognate receptors on endothelial cells, which activates a coordinated cellular response. Several of the initial molecular signaling mechanisms often termed “second messengers” include the cascades involving elevations in intracellular free calcium (He *et al.*, 1990; Bates & Curry, 1997; Pocock *et al.*, 2000; Sandoval *et al.*, 2001a; Sandoval *et al.*, 2001b; Glass *et al.*, 2005) and activation of protein kinase C isoforms (Johnson *et al.*, 1990; Lynch *et al.*, 1990; Murray *et al.*, 1991; Kobayashi *et al.*, 1994; Huang & Yuan, 1997; Yuan *et al.*, 2000; Aramoto *et al.*, 2004; Pocock *et al.*, 2004; Tinsley *et al.*, 2004a; Tinsley *et al.*, 2005; Gaudreault *et al.*, 2008; Rigor *et al.*, 2012). Downstream signals such as eNOS-mediated NO release, cGMP production by guanylate cyclase, and PKG activation have also been implicated (Yuan *et al.*, 1992; Yuan *et al.*, 1993b; Wu *et al.*, 1996; Varma *et al.*, 2002; Breslin *et al.*, 2003; Sanchez *et al.*, 2006; Durán *et al.*, 2008; Sánchez *et al.*, 2009; Duran *et al.*, 2010; Sánchez *et al.*, 2011). Other notable signaling pathways that contribute to microvascular hyperpermeability include activation of Src (Eliceiri *et al.*, 1999; Kevil *et al.*, 2001; Paul *et al.*, 2001; Tinsley *et al.*, 2002) and the MAP kinase cascades including ERK-1/2 and p38 MAP kinase (Kevil *et al.*, 1998; Kevil *et al.*, 2000; Verin *et al.*, 2000; Becker *et al.*, 2001; Lal *et al.*, 2001; Garcia *et al.*, 2002; Nwariaku *et al.*, 2002; Varma *et al.*, 2002; Breslin *et al.*, 2003; Aramoto *et al.*, 2004; Wu *et al.*, 2005; Yu *et al.*, 2005a; Adderley *et al.*, 2015a; Adderley *et al.*, 2015b). Additional signaling molecules that more directly impact the activity and conformation of the actin cytoskeleton have also been shown to have importance. These include myosin light chain (MLC) kinase (MLCK) and RhoA/ROCK signaling, which control myosin activity (Moy *et*

al., 1993; Sheldon *et al.*, 1993; Garcia *et al.*, 1995; Verin *et al.*, 1995; Yuan *et al.*, 1997; Garcia *et al.*, 1999; Tinsley *et al.*, 2000; Yuan *et al.*, 2002; Huang *et al.*, 2003; Birukova *et al.*, 2004; Breslin & Yuan, 2004; Tinsley *et al.*, 2004b; Breslin *et al.*, 2006; Reynoso *et al.*, 2007; Kumar *et al.*, 2009; Dudek *et al.*, 2010; Beard *et al.*, 2014). The balance of activities among members of the Rho family of small GTPases such as RhoA, Rac1, and Cdc42 have also been shown to be of importance for both disruption and stabilization of the endothelial barrier (Waschke *et al.*, 2004a; Waschke *et al.*, 2004b; Waschke *et al.*, 2006; Baumer *et al.*, 2008a; Schlegel *et al.*, 2009; Schlegel & Waschke, 2009; Spindler *et al.*, 2010; Breslin *et al.*, 2015; Breslin *et al.*, 2016; Zhang *et al.*, 2016). In addition, there is increasing evidence that in addition to phosphorylation, other posttranslational modifications such as S-nitrosation of junctional proteins (Marin *et al.*, 2012; Sánchez *et al.*, 2013; Guequén *et al.*, 2016), or specific palmitoylation of PKC β by palmitoyl acyltransferase DHHC21 (Beard *et al.*, 2016) can lead to elevated microvascular leakage.

Ultimately the molecular signals converge to determine the integrity of junctions between endothelial cells. This is dependent upon the strength of the adhesions between adjacent cells, and the tension developed by the individual cytoskeletal networks of each cell. An increase in endothelial cell tension has been recorded after the administration of inflammatory mediators that also produce MLC phosphorylation, actin stress fiber formation, and endothelial barrier function (Goeckeler & Wysolmerski, 1995; Moy *et al.*, 1996; Moy *et al.*, 2002; Breslin *et al.*, 2006), which would be expected to put strain on intercellular junctions and open junctional clefts. In addition, a certain degree of basal tension within cells may cause cellular retraction if conformational changes in junctional proteins cause a decrease in adhesiveness, resulting in junctional cleft opening (Ingber, 2003; Kumar *et al.*, 2006; Luo *et al.*, 2008). In relation to this our laboratory has explored the temporal and fluid behavior of adhesions between endothelial cells as the push and pull against each other in a monolayer. One surprising finding, was temporary endothelial protrusions we have termed local lamellipodia as contributors to maintenance of endothelial barrier function (Breslin *et al.*, 2015). By studying these dynamic structures, we have been able to delineate the dynamic interaction between the cellular membrane and the actin cytoskeleton and how these interactions lead to elevated permeability in response to inflammatory conditions. In the next sections we will provide an overview of the models used to study endothelial barrier function, followed by current knowledge of the cellular and molecular mechanisms leading to endothelial barrier dysfunction in different models of injury or disease, and finally how our most recent findings pertaining to endothelial junctional dynamics may help with development of new therapeutic strategies for reducing microvascular leakage.

3. METHODS TO STUDY ENDOTHELIAL BARRIER FUNCTION

A variety of methods to study endothelial barrier function have been formulated over the past century and refined in the past few decades. With to the continual expansion of studies endothelial permeability in new contexts, encompassing multiple disease models and organs, choice of the most appropriate and relevant way to determine permeability or other indices of barrier function will help avoid potential pitfalls or inaccuracies. In this section we will

review several common methods to study endothelial barrier function, illustrating the advantages and disadvantages of each method.

3.1 Measurement of Protein Leakage: Evans Blue Assay

Evans blue dye (EBD) is a commonly used marker for measuring plasma volume and also for the study of microvascular leakage in animal models. EBD binds strongly to albumin, is not metabolically active, and its plasma concentration remains relatively constant within the first few hours following intravenous injection (Yen *et al.*, 2013; Wang & Lai, 2014). It has long been used as a marker for albumin extravasation. In the brain, its leakage into the parenchyma is considered an indication of blood-brain barrier (BBB) disruption (Wang & Lai, 2014). EBD assays have proven reliable for determining gross microvascular leakage in many models and organs including the brain and lung (Yen *et al.*, 2013; Beard *et al.*, 2016). The classical methodology of this assay is that EBD is injected intravenously then tissue is collected, proteins are lysed and homogenized before checking the absorbance at 620 nm. Alternatively dye distribution in section tissues can be observed and measured under transmitted light or epifluorescence microscopy (Borlongan *et al.*, 2004; Garbuzova-Davis *et al.*, 2013). Additionally, in the Miles assay which tests extravasation in the skin, gross dye levels can be observed visually on the skin surface (Miles & Miles, 1952). Despite the popularity of EBD, drawbacks with its use include: 1) potential effects of unbound dye on the analysis; 2) EBD can also bind other proteins other than albumin; 3) the affinity of EBD differs also according to the tissue; and 4) concerns of toxicity in certain animals (Saunders *et al.*, 2015). Lastly, the biggest general drawback to EBD assays is that the endpoint is typically only a crude measurement of extravasation, and finer endpoints such as plasma filtration or solute permeability cannot be obtained (Durán *et al.*, 2008).

3.2 Determination of the capillary filtration coefficient (K_f) in whole organs

This method, which is popular for estimating lung edema, is a gravimetric approach based on the Starling-Landis equation, in which the rate of change in interstitial fluid volume is related to the change in microvascular pressure (Dongaonkar *et al.*, 2011). The increase in interstitial fluid volume can be determined from measurement of the tissue wet-to-dry ratio or the increase in organ weight to a steady state (Guyton & Lindsey, 1959; Drake *et al.*, 1980). Some investigators favor continuous measurement of the weight gain over the steady state gravimetric technique in order to exclude the initial rapid filling of the vasculature from the calculations. The rate of fluid accumulation in the interstitial space is determined by dividing the change in interstitial fluid volume by the time needed to reach steady state or between time points, depending on which protocol is used. The K_f is estimated as the ratio of the rate of weight gain to the change in the microvascular pressure. These methods provide a fairly simple way to evaluate fluid imbalances. However, they do rely on the assumptions that interstitial fluid pressure is constant, and neglect interstitial compliance and the potential contribution of lymphatic resistance (Dongaonkar *et al.*, 2011).

3.3 Intravital Microscopy

Intravital microscopy is widely used to view the microcirculation *in vivo*. Parameters such as changes in diameter, red blood cell velocity, and leukocyte rolling and adhesion along post-capillary venules can be recorded. The use of fluorescent tracers and epifluorescent

illumination has enabled reliable measurement of microvascular leakage in a variety of tissues. An example from our recent work studying microvascular leakage in the rat mesentery is shown in Fig. 1. This procedure was performed in anesthetized rats that received intravenous administration of FITC-albumin, which is clearly visible inside the microvessels. Extravasation of this tracer was assessed by evaluating escape of the tracer into the surrounding tissues, which can be seen in Fig. 1B, where there are “hot spots” of FITC-albumin leakage. Methods to quantify the leakage include counting the number of “hot spots” or determining the integrated optical intensity (IOI) of extravascular regions near postcapillary venules. The IOI can be reliably used to estimate changes in permeability if there is no increase in local blood flow, which would increase capillary filtration. This can be evaluated by measuring local arteriolar diameter (Durán *et al.*, 2008). The advantages of this technique are the ability to view microvascular leakage in an *in vivo* setting, and the ability to view changes in leakage over time or before and after a given stimulus. The main limitation with intravital microscopy, at least with conventional widefield and single photon excitation is that it can only be applied to semitransparent membranes that serve as natural windows, such as the rodent mesentery, cremaster muscle, and hamster cheek pouch (Yuan & Rigor, 2010). However, intravital microscopy using multiphoton excitation has proven useful for determining microvascular leakage in deeper tissues or through the skin (Egawa *et al.*, 2013). As additional advances in deep tissue imaging become available, intravital microscopy will continue to be a very useful tool for studying microvascular leakage.

3.4 Single Microvessel and Isolated Microvessel Permeability Methods

While the aforementioned intravital microscopy methods are good for evaluating changes in microvascular leakage, there are protocols that allow for more precise determination of permeability in individual microvessels. One of these is the modified Landis technique to determine hydraulic conductivity (L_p), essentially the microvascular wall's permeability to water, which is reviewed in detail elsewhere (Michel, 1984). An additional method is to determine permeability coefficients of individual microvessels to fluorescently labeled solutes. A single microvessel must be cannulated to allow for infusion of the fluorescent tracer or a wash solution. The cannulation may be done on two upstream branches using two micropipettes, or with a single “theta” pipette that is loaded with both solutions (Huxley *et al.*, 1987; Scallan & Huxley, 2010). This configuration allows for tracer wash-in and washout (Fig. 2). The initial step increase in fluorescence intensity (I_{f0}) in a measuring window that includes the microvessel and surrounding area, when taking into account vessel geometry based upon the radius r , corresponds to the concentration difference of the solute across the microvessel wall. Following this step increase, the slope of the gradual increase in intensity $[(dI_f/dt)_0]$ reflects the flux of tracer across the microvessel wall into the surrounding space. These parameters can be used to determine permeability to the solute using Fick's first law of diffusion (Fig. 2B).

Because some tissues are not amenable to intravital microscopy methods, such as cardiac venules, the single-perfused microvessel method was modified to an isolated, perfused model measuring in which the microvessel was mounted onto glass micropipettes (Yuan *et al.*, 1992; Yuan *et al.*, 1993a). Venules ranging from 20 to 70 μm diameter and ideally 1 mm long are manually dissected from the epicardium (or other tissues of interest), and mounted

onto glass micropipettes containing an albumin physiological salt solution. Originally, a set of concentric pipettes was used to allow perfusion with either the tracer solution or wash solution (Yuan *et al.*, 1992; Yuan *et al.*, 1993a), although more recently a theta pipette was introduced (Scallan *et al.*, 2013). The protocol for wash-in and washout of tracer is the same as for single-perfused microvessels, and P_s is calculated in the same way (Fig. 2). The advantage of this particular model is that the chemical composition of the bath, the transmural pressure, and the flow velocity can all be tightly controlled. A disadvantage of this method is that it requires direct manipulation of the microvessel, which can potentially affect its permeability (Durán *et al.*, 2008).

3.5 Cultured Endothelial Cell Monolayer Diffusion Assays

Cultured endothelial cell monolayers are a popular model for determining endothelial permeability due to the fact that the contribution of these cells alone can be studied, and with advances in cell culture, the relative ease of the assays compared to the *in vivo* and isolated microvessel models. Many of the agents that have been used to produce hyperpermeability *in vivo* also enhance permeability of cultured endothelial cell monolayers (Durán *et al.*, 2008; Duran *et al.*, 2010). Protocols based upon Fick's first law of diffusion utilize an endothelial cell monolayer grown on a porous membrane that is placed between two compartments. Ideally, the cells are grown for several days to allow for junctions to mature. Two basic configurations are used. There is a vertical configuration with an upper and lower chamber (Transwell assay), and a horizontal configuration (Ussing chamber and Snapwell membranes). The chamber that faces the apical side of the endothelial cells is termed the luminal chamber, and the other chamber as the abluminal chamber. A tracer such as FITC-dextran or FITC-albumin is added in the luminal chamber and its gradual accumulation in the abluminal chamber is measured over a certain amount of time to determine the solute flux (Yuan & Rigor, 2010). The main advantage of this system is that it can be used to study the intracellular signaling pathways in endothelial cells that lead to changes in permeability. On a related note, strategies to transfect endothelial cells with DNA, siRNA, peptides, and proteins significantly expand the menu of manipulations that can be used to test hypotheses. This potentially powerful tool also has potential drawbacks. Experimental conditions such as sudden changes in temperature or medium pH when tissue culture incubator doors are opened and closed may affect the outcome. Pore size in the membrane and type of matrix used for culturing must be consistent to minimize variability. Another major limitation is these assays generally are not conducted under flow conditions present in microvessels. Moreover, it has been well documented that endothelial cells in cultures may behave differently from those *in vivo* due to some phenotypic differences between cells in culture and *in situ* (Uhlir *et al.*, 2014). For example, cells in culture have less caveolae and are 10 to 100 times more permeable to albumin than *in vivo* (Uhlir *et al.*, 2014). Factors that are known to increase permeability in different types of endothelial cells *in vitro* play little role in the development of pulmonary edema (Uhlir *et al.*, 2014). The phenotypic differences of cultured endothelial cells can be seen in the endothelial glycocalyx, which has been shown to be less thick in cultured cells compared to *ex-vivo* and *in-vivo* endothelium, as documented by Chappell *et al.* (Chappell *et al.*, 2009). Therefore, the limitations of cultured endothelial cells should be kept in mind when investigating the mechanisms of pathophysiological edema.

3.6 Transendothelial Electrical Resistance and Impedance Measurements

An indirect method to study the barrier function has been to measure electrical resistance across the endothelium. This was performed in frog capillaries/postcapillary venules of the mesentery, muscle, and brain, which yielded electrical resistances of 0.95, 23, and 1870 $\Omega\cdot\text{cm}^2$, respectively (Crone & Christensen, 1981; Crone & Olesen, 1982; Olesen & Crone, 1983). The findings suggested variability in endothelial barrier function between different tissues and reinforced the concept of a tight blood-brain barrier (BBB).

These *in vivo* measurements of electrical resistance supported the idea of performing these types of measurements on endothelial cell monolayers as an index of permeability.

Ussing chambers can be used to study epithelial or endothelial membrane properties by inserting electrodes into the luminal and abluminal chambers (Clarke, 2009). Similar assays are also available for vertical chamber systems. These systems allow for repeated measures of resistance over time, before and after stimuli. A drawback is that the transendothelial electrical resistance measurements may be lower than those observed in intact microvessels (Srinivasan *et al.*, 2015). (Chappell *et al.*, 2009)

An additional, popular method for determining endothelial barrier function is Electric Cell-substrate Impedance Sensing (ECIS) across endothelial cell monolayers as an index of barrier function. The cells are grown to confluence on gold microelectrodes, with a large counter electrode configuration. While the impedance measurements can serve as an index of endothelial barrier function, the cell monolayers have both resistive and capacitive properties which can be used to tease out the relative contributions of the resistance due to intercellular junctional tightness between cells (R_b) versus cell-matrix tightness (α) by applying different frequencies of alternating current (Giaever & Keese, 1991, 1993). Recently a competitor system, xCelligence, that uses the same technology as ECIS has also been introduced. Both systems have the advantage that they can provide high throughput, and rapid measures of transendothelial electrical resistance. The ability to utilize flow of media over the cells is also an advantage (Breslin & Kurtz, 2009), although most investigations do not take advantage of this feature. However, the impedance devices utilize cells grown on an impermeable surface, which is different from physiological conditions (Bischoff *et al.*, 2016).

3.6 Microfluidic Membrane Chip Assays

The new microfluidic membrane chips are a modified form of the Transwell solute flux assays that take into account flow induced shear stress effects. Shear stress can modify endothelial barrier function (Yuan *et al.*, 1992; Sill *et al.*, 1995; Seebach *et al.*, 2000; Breslin & Kurtz, 2009). For this reason, Young and colleagues developed a method to study permeability under flow conditions using a micro channel system separated by a porous membrane. Permeability was assessed by measuring leakage of FITC albumin under laser fluorescent microscopy (Young *et al.*, 2010). Microfluidic membrane chip methodology has been applied by other studies targeting blood and lymphatic permeability (Srigunapalan *et al.*, 2011; Sato *et al.*, 2015; Thomas *et al.*, 2017).

4. MICROVASCULAR HYPERPERMEABILITY IN INJURY OR DISEASE CONDITIONS

4.1 Hemorrhagic shock and Ischemia-reperfusion Injury

Hemorrhagic shock is a life-threatening condition related to traumatic injury, with estimated death rate of 55.9 in every 100,000 persons (Sawant *et al.*, 2014). A major problem with the resuscitation of hemorrhagic shock patients is that the intravenous fluids administered to restore central fluid volume and blood pressure leak out of the microcirculation. The consequence of the resulting edema formation is hypoperfusion of the end organs and multiple organ failure. Traumatic injuries that lead to multiple organ failure account for 51–60% of trauma-related deaths (Dewar *et al.*, 2009).

Our lab has investigated the impact of hemorrhagic shock and resuscitation (HSR), acute alcohol intoxication, and their combination on the microvascular barrier function of the gut mesentery (Doggett & Breslin, 2014; Doggett *et al.*, 2017), which has been demonstrated to be particularly susceptible to ischemic injury following hemorrhagic shock (Hollenberg, 2011). Using a conscious rat model of HSR, we found that alcohol intoxication exacerbated HSR-induced hypotension and microvascular hyperpermeability to albumin. The mechanism for hyperpermeability may be attributable in part to disorganization of VE-cadherin at the intercellular junctions between endothelial cells following injury or alcohol intoxication (Doggett & Breslin, 2014). Intravenous administration of the bioactive lipid sphingosine-1-phosphate (S1P) was tested as a pharmacological intervention during resuscitation to test whether it could be employed to attenuate the effects of combined alcohol intoxication and HSR (Doggett *et al.*, 2017). S1P is an endogenous bioactive lipid produced in cell membranes, and previous reports showed that S1P enhances barrier function in cultured endothelial cells and isolated rat microvessels, and reduced microvascular hyperpermeability *in vivo* in murine lung (Singleton *et al.*, 2005; Adamson *et al.*, 2010; Sammani *et al.*, 2010). We performed a dose-response study of S1P's efficacy and found that S1P administered at 0.1 mg/kg during resuscitation significantly reduced the combined HSR and alcohol intoxication-induced microvascular hyperpermeability to albumin. This effect was reflected in the mean arterial pressure, which was significantly elevated after resuscitation in rats that received S1P compared to controls (Doggett *et al.*, 2017).

Apoptotic signaling has also been suggested to contribute to HSR-induced endothelial cell barrier dysfunction through a series of studies by Childs and colleagues. Following HSR, an increase in BH3 pro-apoptotic protein BAK in the mesenteric microvasculature initiates the mitochondria-mediated intrinsic apoptotic signaling, with an increase in reactive oxygen species and the release of apoptogenic protein cytochrome-c. This leads to activation of the effector caspase-3, and subsequent cleavage and disruption of adherens proteins, resulting in an increase in paracellular endothelial cell permeability. In addition, transfection of BAK peptide in a rat model resulted in increased microvascular permeability through the activation of caspase-3 (Childs *et al.*, 2007). In a subsequent study, they investigated which mitochondrial complex involved in the electron transport chain was responsible for ROS formation and hyperpermeability. Using cultured rat lung microvascular endothelial cells, they found that inhibition of the mitochondrial electron transport chain complex III resulted

in a decrease in BAK-induced ROS formation and cytochrome-c release, as well as attenuation of microvascular permeability. Inhibition of other complexes and cytoplasmic enzymes did not show protection against BAK-induced ROS formation and endothelial hyperpermeability, confirming the role of mitochondrial-mediated intrinsic apoptotic signaling activation in endothelial barrier disruption by increasing ROS and releasing cytochrome-c. (Childs *et al.*, 2008).

The ischemia/reperfusion (I/R) injury created during hemorrhagic shock induces pro-inflammatory signals in the endothelial cells of postcapillary venules. Data from studies using hypoxia and reoxygenation on endothelial cell monolayers as a model of I/R injury suggest that the exacerbated traffic of leukocytes in these microvessels results from an increased expression of different endothelial cell adhesion molecules on the surface of both leukocytes and endothelium (Ichikawa *et al.*, 1997). Ischemia and reperfusion injury in an isolated rat lung model has been shown to increase lung permeability and weight, with upregulation of the adhesion molecule CD31 (Chiang *et al.*, 2011). ROS production induced by depolarization of endothelial cell membranes is also involved in I/R inflammation and leukocyte extravasation. The decrease in blood flow is a mechanical stimuli sensed by a complex consisting of the adhesion molecule PECAM, VEGF receptors, and VE-cadherin that is located in the caveolae of endothelial cells (Chatterjee *et al.*, 2014). Activation of this complex results in depolarization of the endothelial cell membrane and subsequent generation of ROS, which disrupts the integrity of endothelial cell-cell junction and compromises endothelial barrier, leading to hyperpermeability (Gilmont *et al.*, 1998).

The endothelial glycocalyx is another important component of the vascular endothelium that is degraded during hemorrhagic shock and I/R injury. The glycocalyx is composed of proteoglycans, glycosaminoglycan molecules and glycoproteins, and is located in the luminal side of blood vessels, spanning the membrane of endothelial cells (Reitsma *et al.*, 2007). Early studies established the endothelial glycocalyx as an important determinant of microvascular permeability (Henry & Duling, 1999; Vink & Duling, 2000). For example, enzymatic removal of the glycocalyx in rat myocardial capillaries causes myocardial edema (van den Berg *et al.*, 2003). Some studies have shown degradation of the endothelial glycocalyx following ischemia-reperfusion injury as well as after a prolonged, fixed-pressure model of hemorrhagic shock and resuscitation (Mulivor & Lipowsky, 2002; Kozar *et al.*, 2011). However, most studies evaluated glycocalyx based on post-mortem electron microscopy images. More recently, Torres Filho *et al.* showed a reduction in the glycocalyx thickness of microvessels *in vivo* during hemorrhagic hypotension. Using a rat model of fixed-volume hemorrhage, they showed a significant hemorrhage-induced decrease of 59% in glycocalyx thickness in skeletal muscle venules compared to control rats, and that venules with the largest decreases in glycocalyx presented the greatest reduction in local blood flow (Torres Filho *et al.*, 2013). In a more recent study, in which they tested the efficacy of seven different resuscitation fluids after fixed-volume hemorrhage, they found a negative correlation between microvascular permeability and glycocalyx thickness, and that plasma levels of glycocalyx components syndecan-1 and Heparan sulfate were positively correlated to microvascular permeability (Torres Filho *et al.*, 2016), suggesting an important role for the glycocalyx in endothelial barrier function during hemorrhage and resuscitation.

The studies done by Dr. Torres Filho and others have shown that resuscitation with fresh frozen plasma (FFP) and fresh whole blood following hemorrhagic shock is correlated with endothelial restoration of the glycocalyx and its component syndecan-1 (Sdc-1) (Torres Filho *et al.*, 2016). A different group showed the specific contribution of Sdc-1 to FFP-mediated protection in lung endothelium. Treatment with FFP enhanced the expression of Sdc-1 on pulmonary endothelial cell monolayers. When Sdc-1 expression was silenced, permeability was higher and stress fiber formation was evident with FFP treatment. In the same study, in a hemorrhagic shock (HS) model, lung microvascular permeability was significantly increased compared to sham controls. When comparing resuscitation with lactated ringers (LR) versus FFP, the FFP showed a reduction in the microvascular leakage (Peng *et al.*, 2013). Sdc1^{-/-} mice also presented enhanced lung permeability after HS, however FFP treatment showed no benefit over LR resuscitation. Loss of Sdc1 *in vivo* also led to no difference between LR and FFP in reducing pulmonary injury scores and markers of inflammation after hemorrhage. Furthermore, Sdc1^{-/-} mice presented a significant increase in pulmonary syndecan-4 expression after HS and FFP resuscitation compared to animals resuscitated with LR, showing that other members of the syndecan family may be compensating for the loss in Sdc1 to maintain the integrity of the glycocalyx. These results support the role of glycocalyx components in modulating FFP-mediated protection of pulmonary endothelial barrier after hemorrhagic shock (Peng *et al.*, 2013).

4.2 Burn-Induced Endothelial Barrier Dysfunction

Systemic microvascular hyperpermeability is an important manifestation of severe burn injury. In the early stages after burn, systemic leakage of plasma proteins and subsequent edema have been directly linked to the development of multiple organ dysfunction and hypovolemic shock (Kremer *et al.*, 2008; Zhao *et al.*, 2015). In later stages in those who have survived a severe burn injury, wound swelling and inflammation are also directly linked to local microvascular hyperpermeability (Demling, 2005). Many factors may contribute to interstitial accumulation of plasma fluid during burn including increased filtration pressure and fluid absorption, impaired lymphatic clearance and general cell membrane damage (Lund *et al.*, 1992; Gibran & Heimbach, 1993; Demling, 2005). However, the cellular mechanisms that lead to a dysfunctional endothelial barrier are the major contributors to the initiation and development of burn edema. A variety of inflammatory mediators of endothelial permeability have been identified in the serum of burn patients, such as histamine, cytokines, and prostaglandins. Leukocyte adhesion, the release of free radicals, proteases and other factors also contribute the complexity of the burn-induced inflammatory response (Gibran & Heimbach, 1993; Arturson, 1996, 2000; Dudek & Garcia, 2001).

Yuan and colleagues have investigated the cellular and molecular processes triggered by inflammatory mediators that lead to endothelial barrier dysfunction during burn. In a rat model of burn injury, with 25% of total body surface area receiving a scalding burn, plasma was collected and used to treat rat lung microvascular endothelial cells to identify potential molecular mechanisms activated by the burn-plasma components. They observed that exposure to burn-plasma caused marked serine phosphorylation of β -catenin and VE-cadherin, and a shift of these AJ proteins from the cell membrane to the cytosol, plus formation of gaps between cells. PKC activity was also found to be required for these

changes (Tinsley *et al.*, 2004b; Tinsley *et al.*, 2005). Treatment of endothelial cells with burn plasma also decreased barrier function, as measured by albumin extravasation and transendothelial electrical resistance (TER), which were also attenuated in a concentration-related manner by PKC inhibitors (Tinsley *et al.*, 2005). Previous studies showed that phosphorylation of MLC by MLCK is required for burn-induced stress fiber formation and pulmonary endothelial hyperpermeability (Tinsley *et al.*, 2004b). They further investigated the role of MLC-dependent endothelial barrier injury during burns, focusing on the long isoform MLCK-210, which was been identified as the predominant isoform expressed in vascular endothelial cells. Using a mouse model of third-degree scald burn, they found that in wild-type mice, the burn injury causes a 2-fold increase in albumin flux and a 4-fold increase in hydraulic conductivity in the mesenteric microcirculation, which was associated with high mortality within 24 hours. However, in MLCK-210-deficient mice hyperpermeability to albumin was attenuated survival was significantly improved and (Reynoso *et al.*, 2007). The role of MLCK-210 in susceptibility to lung injury has been demonstrated by other studies *in vivo* through an MLCK-210 knockout mouse model that retains the production of the other MLCK isoforms. The MLCK-210 knockout mice were less susceptible to acute lung injury induced by endotoxin lipopolysaccharide and showed enhanced survival during mechanical ventilation (Wainwright *et al.*, 2003). Taken together, these studies suggest that modifications in both adherens junctions and actomyosin complex by PKC and MLCK, respectively, may contribute to the progression of pulmonary edema following burn injury, and that these effector molecules may be effective therapeutic targets for fighting the complications of endothelial barrier dysfunction.

Toll-like receptors (TLR) also have been shown to play an important role in sensing tissue damage and mediating the response of the vascular endothelium to sterile inflammatory stimulation. TLR-4 knockout mice display much less microvascular leakage in response to burn injury compared to wild-type mice. The increase in leukocyte adhesion is also attenuated in the mesenteric venules of TLR-4 knockout mice following burn, compared to wide-type controls. Complementary results using rat lung endothelial cell monolayers showed a rapid reduction in TER induced by burn plasma treatment, which was blunted by reducing the expression of TLR-4 receptor with specific siRNA knockdown (Breslin *et al.*, 2008). In a separate study, TLR-4 was shown to be required for the development of acute lung injury. Hemorrhage and endotoxemia caused the production of TNF- α , neutrophil accumulation, and extravasation of protein in the lungs of wide-type mice, while mice with a mutated, nonfunctional TLR-4 receptor showed a significant reduction in these parameters (Barsness *et al.*, 2004).

Mitogen-activated protein kinases (MAPKs) are also an important family of proteins that, during burn injuries, are activated and mediate the signal transduction involved in endothelial permeability. One group showed that p38 MAPK regulates the interaction of endothelial cell actin and myosin, leading to stress fiber formation, cellular contraction and enhanced vessel permeability (Wang *et al.*, 2010). Based on this previous work, they further investigated the role of other MAPKs, such as c-JUN N-terminal kinase (JNK) and extracellular signal-related kinase (ERK) that like p38 are activated by MAPK kinases but had an unknown role in thermal injury. They showed that exposure of endothelial cells to burn serum results in rapid membrane damage and relocation of the tight-junction protein

ZO-1 from the membrane to the cytosol. This phenomenon is completely abolished through pharmacological inhibition of p38 MAPK, whereas inhibition of JNK and ERK had no effect. The expression of adhesion molecule ICAM-1 was shown to be specifically regulated by JNK, as only inhibition of this MAPK was able to blunt the overexpression of ICAM-1 following burn serum exposure. These results were confirmed *in vivo*, as the aortic endothelium of thermally injured mice expressed enhanced ICAM-1 amounts. Adenoviral transfection of dominant negative JNK mutants attenuated this expression, whereas dominant negative p38 MAPK mutants did not influence ICAM-1 expression. However, the adhesion molecule P-selectin was found to be specifically regulated by p38 MAPK during thermal injury both *in vitro* and *in vivo* (Wu *et al.*, 2011). Putting these findings together, we conclude that MAPKs have a fine-tuned role on the regulation of endothelial permeability. Understanding the different signal transduction pathways that disrupt the endothelial barrier might help identify possible targets to prevent the pathophysiological responses induced by burn injury.

4.3 Endothelial Barrier Disruption in Stroke and Brain Injury

Stroke is defined by the world health organization as a clinical syndrome consisting of rapidly developing clinical signs of focal (or global in case of coma) disturbance of cerebral function lasting more than 24 hours or leading to death with no apparent cause other than a vascular origin. Stroke is the second most common cause of death worldwide and a leading cause of serious long-term disabilities (Katnik *et al.*, 2014). The vascular origin of the complications induced by stroke refers to the disruption of the BBB, a semi-permeable endothelial and glial barrier that regulates passage of blood and molecules into the brain (Ballabh *et al.*, 2004). Its integrity is significantly important for protection of the brain against passage of toxins or cells (van Sorge & Doran, 2012). The BBB is composed of three main cellular elements: endothelium of the blood vessel, astrocytic end-feet and pericytes (Persidsky *et al.*, 2006). Basement membrane, vascular smooth muscle cells and some immune cells also help maintain the integrity of the BBB (Daneman & Prat, 2015). The brain endothelium shares similarities with other types of endothelial cells in the expression of common endothelial junctional proteins such as VE-cadherin (Katt *et al.*, 2016). However, the endothelial junctions of the brain exhibit a higher expression of tight junctional proteins such as claudins, occludins and junctional adhesion molecules (Luissint *et al.*, 2012). This makes the cellular barrier relatively tighter than other types of endothelium (Joo, 1996).

Most strokes are ischemic, in which blood supply to the brain is interrupted. Exposure of neuronal tissue to ischemia leads to a shift from aerobic to anaerobic metabolism that results in acidosis and ATP depletion (Kogure & Schwartzman, 1980), leading to loss of membrane channel protein functions including Na/K ATPase (Huang *et al.*, 2015). The net result is calcium influx due to membrane depolarization and acid sensing ion channel activation (Cuevas *et al.*, 2011). Accumulation of calcium leads to mitochondrial dysfunction and disruption of many intracellular signaling pathways and eventually apoptosis as well as glutamate release (Nishizawa, 2001; Broughton *et al.*, 2009). The dead neurons release certain mediators that activate brain microglia to release inflammatory mediators such as IL1- β (Yenari *et al.*, 2010). The inflammatory cytokines as well as high glutamate levels have major effects on endothelium and are known to disrupt the BBB (Campos *et al.*, 2012;

Galea & Brough, 2013). Disruption of the BBB leads to brain edema which is a hallmark of stroke pathology that aggravates neuronal tissue damage (Dostovic *et al.*, 2016).

Similar to stroke, other types of brain injury are known to disrupt the BBB by various mechanisms. For example, traumatic brain injury (TBI) is well documented to negatively regulate BBB integrity by a 2-step process. The primary damage is characterized by the direct or indirect mechanical injury of the parenchyma and vascular wall. The secondary damage occurs after the BBB disruption and is marked by edema and inflammation (Price *et al.*, 2016). After the injury, astrocytes and microglia release many inflammatory mediators such as MMPs, interleukins and TGF beta, which are well known to disrupt the BBB (Lu *et al.*, 2005; Lakhan *et al.*, 2013; Guilfoyle *et al.*, 2015). Patients with stroke or TBI are commonly presented with some degree of brain edema and blood brain barrier disruption that can be assessed and quantified using dye extravasation methods and imaging techniques such as magnetic resonance imaging (MRI) (Tomkins *et al.*, 2011; Kassner & Merali, 2015). Several studies have used animal models of brain injuries to assess microvascular leakage of the brain. Most of these studies use Evans Blue extravasation assays to assess endothelial hyperpermeability during brain injury (Wang & Lai, 2014).

Tissue ischemia and hypoxia is one of the known factors modulating microvascular permeability in brain injury (Wang & Lai, 2014; Vespa, 2016). Engelhardt and co-workers have demonstrated that hypoxia causes disruption of rat brain endothelial cells tight junction by activating Hypoxia-Inducible Factor-1 (HIF-1) (Engelhardt *et al.*, 2014). Other studies have shown that HIF-1 α is involved in activation of matrix metalloproteinases (MMPs) that also downregulate endothelial tight junctions, enhancing permeability in cerebrovascular diseases (Yang & Rosenberg, 2011).

It has been well documented that brain injury is usually accompanied by some degree of inflammation in the brain. Activated microglia in stroke and TBI are known to secrete numerous inflammatory mediators such as IL-1 β and TGF- β , which has been linked to hypoxic conditions (Patel *et al.*, 2013; Loane & Kumar, 2016). Engelhardt and co-workers have shown that the inflammation caused by hypoxia-activated astrocytes causes brain endothelial dysfunction that depends on IL-1 β (Engelhardt *et al.*, 2014). Others have shown that IL-1 β released from activated microglia disrupts blood brain barrier by downregulating Sonic hedgehog protein in astrocytes that in turn transcriptionally decreases tight junction protein expression in endothelial cells thus promoting BBB disruption (Wang *et al.*, 2014). Beard et al have further investigated the mechanisms of IL-1 β -induced BBB disruption and have shown that the underlying mechanism involves β -catenin and FoxO1-dependent downregulation of the tight junction protein claudin-5 via non muscle myosin light chain kinase (Beard *et al.*, 2014).

Endothelial cytoskeleton and actin fiber dynamics are of significant importance in regulating endothelial permeability (Adderley *et al.*, 2015a; Zhang *et al.*, 2016) and they have been studied in the context of brain injury. One study showed that actin filaments are important to maintain the BBB, as treatment with cytochalasin-B, which disrupts actin integrity, caused BBB hyperpermeability (Nag, 1995). Shi and coworkers have shown that cytoskeletal dynamics were associated with early BBB disruption as they demonstrated that oxygen and

glucose deprivation rapidly induced actin stress fibers in brain endothelial cells, leading to hyperpermeability (Shi *et al.*, 2016).

Another important factor regulating microvascular permeability during brain injury is the endothelial glycocalyx, which is present on cerebral endothelium and is of significant importance in maintaining BBB integrity (Yoon *et al.*, 2017; Zhu *et al.*, 2017). Stroke and some other brain injury conditions are manifested by focal or global brain ischemia or hypoxia, which has been linked to endothelial barrier disruption by affecting endothelial glycocalyx (Ward & Donnelly, 1993; Rehm *et al.*, 2007). Glycocalyx degradation was reported associated with higher BBB permeability and aggravated brain edema in a rat model of cardiac arrest and cardiopulmonary resuscitation (Zhu *et al.*, 2017). However, more studies using animal models of stroke and brain injury are needed to characterize the role of the endothelial glycocalyx in protecting the BBB.

Glutamate and matrix metalloproteinases (MPPs) are also important in regulating BBB permeability during brain injury. Glutamate is an excitatory neurotransmitter that is abundant in the brain following stroke (Davalos *et al.*, 1997). The role of glutamate in BBB disruption is well documented (Hawkins, 2009; Xhima *et al.*, 2016). For example, Mayhan *et. al.* have shown that glutamate disrupts the BBB via a nitric oxide-dependent mechanism. They investigated BBB function in the pial microcirculation of rats using intravital microscopy, and found that topical application of glutamate and the nitric oxide donor S-nitroso-acetyl-penicillamine (SNAP) produced an increase in the BBB permeability to FITC-dextran-10K, which was attenuated by treatment with NG-monomethyl-L-arginine (Mayhan & Didion, 1996). MPPs are a family of enzymes that cleave protein substrates. Their role in BBB disruption in ischemic stroke has been well documented. They act by breaking down the extracellular matrix and endothelial tight junctions to enhance BBB permeability (Lakhan *et al.*, 2013).

As we have seen, multiple factors activated during stroke and brain injury are associated with BBB disruption. Those factors include but are not limited to inflammatory cytokines, HIF-1- α , glutamate and MMPs. Structurally, brain injury causes changes in endothelial cytoskeleton, glycocalyx shedding and downregulation of endothelial tight junctions. Most of the studies that have addressed this issue focused on brain endothelium to investigate junctional protein expression under brain injury condition. However, there is still a need to investigate at the microvascular level the changes of junctional proteins in isolated microvessels from brain injury models to comprehensively evaluate the status of endothelial junctional protein expression and localization under these disease conditions.

4.4 Diabetes-induced Vascular Disease

Diabetes mellitus is a group of metabolic disorders characterized by impaired insulin production and utilization, leading to high levels of blood sugar over prolonged periods of time. The major cause of morbidity and mortality in diabetic patients is cardiovascular disease, which initiates with dysfunction of the microvascular barrier, leading to end organ diseases such as nephropathy, neuropathy, cardiomyopathy and foot disease (Yuan *et al.*, 2007). The endothelial dysfunction in diabetes causes plasma leakage and deposition of proteins and lipid in the vessel wall, altering the basement membrane composition and

forming microangiopathic lesions characterized by a thicker and weaker microvessel, which contribute for the development of hypertension. Early studies in the structural changes in the coronary microcirculation caused by diabetes showed that hyperglycemia induces the formation of paracellular gaps, consistent with increased permeability (Ward & al-Haboubi, 1997). Other studies of high glucose treatment and streptozotocin (STZ)-induced diabetes show stress fiber formation and other cytoskeleton and endothelial cell structural changes (Salameh *et al.*, 1997; Yu *et al.*, 2005b). To support these findings, reduced expression of membrane-bound VE-cadherin has been reported in cultured endothelial cells treated with advanced glycation end-products (Otero *et al.*, 2001) as well as in the diabetic retina (Davidson *et al.*, 2000). The expression of tight junction protein ZO-1 was also reduced in the cerebral microvessels of STZ-induced diabetic rats (Chehade *et al.*, 2002).

The biochemical and molecular mechanisms involved in microvascular dysfunction during diabetes are directly related to disturbed glucose metabolism and inflammation. Three main pathways have been identified during hyperglycemia-induced microvascular hyperpermeability. The first pathway involves oxidative stress-induced alteration of endothelial barrier function. Excessive intracellular glucose and free fatty acids cause stimulation of mitochondrial respiration, modification of the redox states of various intracellular molecules, and changes in nitric oxide bioavailability in the microvascular endothelium (Yuan *et al.*, 2007). The second pathway involves generation of advanced glycation end-products (AGEs), which have been identified in the blood and vasculature of diabetic patients (Goldin *et al.*, 2006). These molecules, which are produced by auto-oxidation and nonenzymatic glycation of proteins or lipids (Yuan *et al.*, 2007), have been associated with breakdown and decreased expression of VE-cadherin in the endothelium (as mentioned above) in earlier experiments. More recent studies show that AGEs cause phosphorylation of VE-cadherin, dissociation of adherens junction and endothelial hyperpermeability through binding to their receptor (RAGEs). The cellular responses to AGEs are mediated and exacerbated by mammalian diaphanous-related formin (mDia1), which regulates cell adhesion by binding to the cytoplasmic domain of RAGEs and acting as a potent actin and microtubule polymerization factor (Zhou *et al.*, 2018). AGEs are also shown to cause endothelial dysfunction and hyperpermeability through activation of NF- κ B, p38 MAPK and Rho/ROCK (Wang *et al.*, 2017; Zhang *et al.*, 2017). Finally, the third mechanism involves synthesis of diacylglycerols (DAGs) due to increased levels of glucose metabolites. DAGs can bind and activate PKC, which is then translocated from the cytosol to the cell membrane, where it acts as a hyperpermeability factor (Yuan *et al.*, 2007). The apparent hyperpermeability to albumin in isolated porcine coronary venules was prevented by blockage of PKC during treatment with D-glucose. More importantly, selective inhibition of PKC- β attenuated the elevated permeability to albumin in porcine coronary venules at the early onset of STZ-induced diabetes (Yuan *et al.*, 2000). When investigating whether diabetes causes a change to PKC in transcriptional and translational levels, they found that PKC- β 2 was significantly upregulated in the heart and aorta of STZ-induced diabetic pigs at both transcriptional and translational levels (Guo *et al.*, 2003). These studies suggest that PKC- β 2 is a potential target for diabetic injury in the cardiovascular system.

Clinical studies have shown a relationship between obesity and insulin resistance in the development of acute respiratory distress syndrome (ARDS) (Gong *et al.*, 2010), a

pathological condition that involves increased pulmonary vascular permeability and pulmonary edema (Clemmer *et al.*, 2016). Besides that, alterations in the structure and function of the complex pulmonary microcirculatory network have been observed, as well as a positive relationship between lung permeability and pulmonary microangiopathy in diabetic patients (Kuziemska *et al.*, 2015). A few molecular mechanisms of pulmonary microvascular permeability during diabetes have been elucidated. One hypothesis presented was whether pulmonary endothelial permeability was increased during chronic hyperglycemia due to superoxide activation of the transient receptor potential channel TRPM2, which is known to increase calcium entry and pulmonary permeability coefficient (K_f) (Townsend *et al.*, 2006; Wu *et al.*, 2009). The results showed that STZ-induced diabetes in Zucker rats increased vascular superoxide levels and K_f , and decreased lung vascular TRPM2 expression compared to control Zucker rats. The increase in superoxide and K_f was significantly reversed with apocynin treatment, a NOX inhibitor. TRPM2 channel inhibition also reversed the increase in K_f . Furthermore, they induced oxidative stress in the lungs of control rats, which increased K_f . This response was attenuated with TRPM2 inhibition, suggesting a TRPM2-mediated pulmonary hyperpermeability associated with increased superoxide levels during diabetes (Lu *et al.*, 2014). These studies show that hyperglycemia-induced oxidative stress is a significant mediator of pulmonary permeability. However, there is a great need for further investigation on the effects of diabetes on ARDS. A few studies have identified diabetes mellitus as a risk modifier of acute lung injury (ALI) in patients with predisposition (Moss *et al.*, 2000; Gong *et al.*, 2005). A bigger study aimed to validate and refine a lung injury prediction score in at-risk patients found that diabetes mellitus decreased the risk to develop ALI by 1 point (Gajic *et al.*, 2011). While these studies do not prove that diabetes has a protective effect against lung injury, they do show some disconnect between the animal models and human studies, and of the complexity of the interaction between multiple pathologies.

5. EVIDENCE OF A ROLE FOR LOCAL LAMELLIPODIA IN THE CONTROL OF ENDOTHELIAL BARRIER FUNCTION

As shown above, mechanisms such as actin-myosin-mediated contraction are thought to increase centripetal tension of endothelial cells, putting stress on the junctions and widening junctional clefts to increase permeability (Moy *et al.*, 1996; Moy *et al.*, 2002; Breslin *et al.*, 2006). Many inflammatory stimuli that cause endothelial barrier dysfunction also elicit actin stress fibers in endothelial cells, which have been proposed as a possible source of this increased centripetal tension (Bogatcheva & Verin, 2008; Shen *et al.*, 2010). MLCK-mediated phosphorylation of MLC on Thr-18/Ser-19 promotes actin-myosin-mediated contraction in endothelial cells. Pharmacologic inhibition of MLCK has been shown to decrease the baseline permeability to albumin in isolated coronary venules (Yuan *et al.*, 1997). Inhibition of MLCK has also been shown to reduce neutrophil-induced microvascular hyperpermeability, and mice that lack the MLCK-210 isoform display reduced microvascular leakage in response to burn injury (Reynoso *et al.*, 2007). The MLC phosphatase (MLCP) reduces phosphorylation of MLC on Thr-18/Ser-19. MLCP-mediated dephosphorylation of MLC is inhibited by the RhoA/ROCK pathway via phosphorylation of its myosin targeting subunit MYPT-1, resulting in accumulation of phosphorylated MLC

(Bogatcheva & Verin, 2008; Shen *et al.*, 2010). Inhibition of ROCK has been shown to attenuate actin stress fiber formation in conjunction with inhibition of endothelial barrier function normally elicited by activated neutrophils, thrombin, histamine, and VEGF (Carbajal *et al.*, 2000; van Nieuw Amerongen *et al.*, 2000; Wojciak-Stothard *et al.*, 2001; Breslin & Yuan, 2004; Breslin *et al.*, 2006; Sun *et al.*, 2006).

While actin stress fibers were proposed as mediators of endothelial cell contraction, agents that protect the endothelial barrier such as S1P and cAMP analogs were found to promote cortical actin fibers, which were proposed to stabilize endothelial junctions (Waschke *et al.*, 2004b; Waschke *et al.*, 2004c; Adamson *et al.*, 2008; Bogatcheva & Verin, 2008; Spindler *et al.*, 2010). The small GTPase Rac1 is thought to promote cortical actin fibers, as cAMP analogs that promote Tiam1-mediated activation of Rac1 cause cortical actin network stabilization (Birukova *et al.*, 2007; Adamson *et al.*, 2008; Baumer *et al.*, 2008b; Birukova *et al.*, 2009; Birukova *et al.*, 2010), while selective inhibition of Tiam1-mediated activation of Rac1 increases endothelial permeability (Baumer *et al.*, 2009; Breslin & Kurtz, 2009).

In an effort to gain more detail in the understanding of how these actin structures contribute to the control of endothelial barrier function, we developed a protocol to express a green fluorescent protein- β -actin fusion protein (GFP-actin) in cultured endothelial cells in order to study dynamic changes in the actin cytoskeleton in response to stimuli that are known to increase or decrease endothelial permeability (Doggett & Breslin, 2011). We chose this model because GFP-actin was previously found to polymerize with native actin molecules and incorporate into normal actin structures, enabling study of cytoskeletal dynamics (Ballestrem *et al.*, 1998; Choidas *et al.*, 1998). We verified successful expression of GFP-actin in human umbilical vein endothelial cells (HUVEC), and that it was incorporated to the same structures as native actin molecules (Fig. 3). In addition, we confirmed that the typical changes in TER elicited to thrombin or S1P were similar between HUVEC expressing GFP-actin, and those that did not (Fig. 3).

In our initial experiments, we observed the expected filamentous actin structures. However, an unexpected finding was that in a monolayer of confluent endothelial cells, a large number of local lamellipodia were observed at junctions. The local lamellipodia essentially were active extensions of one cell's membrane containing GFP-actin that protruded over an adjacent cell, then retracted back toward the starting point. In addition, we observed actin stress fibers drifting from the cell edges toward the center of the cell, where they disassembled (Doggett & Breslin, 2011). These findings prompted us to form analysis procedures to study these structures so that we could determine their relative contributions to the control of endothelial barrier function. In addition to counting all the local lamellipodia events (protrusion frequency), we adopted kymograph analysis to characterize the protrusion and withdrawal distances, times, and velocities (Fig. 4). Kymograph analysis can also be used for studying the number of actin stress fibers crossing an imaginary vertical plane through the center of the cell, plus their lateral movements, as shown in Fig. 5.

Initial studies were aimed at determining how agents that modulate endothelial barrier function affect local lamellipodia and actin stress fibers. Data from a study performed with thrombin are shown in Fig. 6. HUVEC expressing GFP-actin were observed with time-lapse

fluorescent microscopy (Breslin *et al.*, 2015). During the baseline period, frequent local lamellipodia formation and withdrawal was observed, shown by the small arrows in the top left image in Fig. 6A. An additional transient structure we observed in the more central areas of cells was formation of cluster of GFP-actin that then expanded into a ring and dispersed away from its starting point, shown by the large arrow in the top left image of Fig 6A. These dynamic structures were previously been described and named “actin clouds” (Ballestrem *et al.*, 1998). When near the edge of a cell, actin clouds typically gave rise to a local lamellipodia. When 1 U/ml thrombin was added to the cells, within a few minutes there was a brief increase in GFP-actin intensity was apparent near the edges of cells (medium arrows, bottom left image of Fig. 6A) and borders of vesicles (very small arrows, same image), followed by less lamellipodia and actin cloud activity. Quantification of this data showed that the lamellipodia protrusion frequency significantly declined from baseline in the first 20 minutes after thrombin (Fig. 6B), corresponding to the time frame when thrombin typically also causes endothelial barrier dysfunction (Fig. 3F). At later time points, specifically 30, 40, and 50 min, the quantity of actin stress fibers was significantly increased (Fig. 6C). Two types of actin stress fibers were apparent, and their increase in numbers could be accounted for by two apparent independent mechanisms. First, de novo assembly/bundling of new actin stress fibers and the extension of smaller, preexisting fibers in the central area of the cell was observed, and these best fit the description of the ventral stress fiber subset (Hotulainen & Lappalainen, 2006; Pellegrin & Mellor, 2007). Second, thrombin caused an increase in the movement of cortical actin fibers toward the cell center, in a similar fashion as the actin stress fiber subset known a transverse arcs. Of these two subtypes, transverse arcs appeared to be more stable than the ventral stress fibers, which began disassembling 30–40 min after the addition of thrombin. Notably, the thrombin-induced increase in actin stress fiber number was significantly increased compared to baseline at the 30, 30, and 50 min time points (Fig. 6C), corresponding to the time period when the endothelial barrier dysfunction is at its greatest and begins to recover (Fig. 3F). Based on these findings, it appeared that the decline in lamellipodia may be a cause of endothelial barrier dysfunction, while actin stress fiber formation may be associated with later events or possibly recovery (Breslin *et al.*, 2015).

The hypothesis that there is a cause-effect relationship between local lamellipodia and the degree of endothelial barrier function was rigorously tested through a series of experiments examining how different agents that impact endothelial barrier function affect lamellipodia activity. Histamine, which causes brief endothelial barrier dysfunction, also caused a significant yet brief decrease in local lamellipodia protrusion frequency (Adderley *et al.*, 2015a). S1P, which enhances endothelial barrier function, elicited an increase in lamellipodia protrusion frequency (Breslin *et al.*, 2015). Overexpression of the atypical Rho GTPase Rnd3 decreased local lamellipodia protrusion frequency in association with its ability to attenuate increases in endothelial permeability (Breslin *et al.*, 2016). In addition to this correlative data, the impact of (–)blebbistatin on endothelial barrier function was tested because it specifically inhibits local lamellipodia formation without affecting actin stress fibers. Application of (–)blebbistatin caused a decrease in HUVEC barrier function, and also significantly increased the permeability of isolated rat mesenteric venules compared to its inactive control, (+)blebbistatin (Breslin *et al.*, 2015). Rac1, which has an important role in cellular protrusions (Machacek *et al.*, 2009), was also tested. Pharmacologic inhibition of

Rac1 decreased lamellipodia protrusion frequency and increased endothelial permeability, while overexpression of Rac1 had opposite effects (Breslin *et al.*, 2015). Additional studies have reported that local lamellipodia strengthen cell-cell contacts (Abu Taha *et al.*, 2014) and can selectively close pores produced by leukocytes migrating across the endothelium (Martinelli *et al.*, 2013). Interestingly, it was previously reported that S1P can enhance endothelial barrier function independently of VE-cadherin (Xu *et al.*, 2007), which might be due to the extension of local lamellipodia beyond VE-cadherin junctional complexes (Breslin *et al.*, 2015). Collectively, these findings suggest that local lamellipodia contribute to overall endothelial barrier function, likely due to a combination of their aforementioned contribution to promoting the integrity of intercellular junctions (Abu Taha *et al.*, 2014) and by increasing the net diffusion distance for solutes to cross via the paracellular pathway (Breslin *et al.*, 2015).

The molecular mechanisms that control lamellipodia formation remain to be fully elucidated. Underneath the protruding cell membrane is a highly organized meshwork of actin filaments meshwork with that appears to polymerize and push the cell edge forward during protrusion and develop shallower angles during withdrawal (Koestler *et al.*, 2008). This process likely involves the Arp2/3 complex in endothelial lamellipodia (Martinelli *et al.*, 2013; Abu Taha *et al.*, 2014). The protrusions also may involve localized, temporally alternating increases in the activities of RhoA and Rac1 (Danuser, 2005). Rac1 activity appears to correlate with lamellipodia protrusion frequency (Breslin *et al.*, 2015). In addition, application of S1P, which transiently increases local lamellipodia formation (Breslin *et al.*, 2015), also increases RhoA-mediated MLC phosphorylation and recruitment of vinculin near intercellular junctions between endothelial cells (Zhang *et al.*, 2016). Other actin-myosin-independent mechanisms such as microtubule growth may also contribute to the overall mechanism (Ingber, 2003; Birukova *et al.*, 2006).

6. CONCLUSIONS

As we have seen, dysregulation of the endothelial barrier occurs in a variety of disease and injury conditions. Independent of the pathological challenge, they all involve inflammation of the microvasculature, which is directly linked to disruption of the cell-cell adhesive properties and loss of membrane-bound junctional protein integrity. The disrupted control of permeability during such inflammatory challenges seems to be the root of complications that lead to organ dysfunction. The cellular and molecular mechanisms described in this review share a common ultimate target: integrity of the endothelial junctions. Although targeting specific hyperpermeability-mediators may confer some degree of effectiveness in certain pathological conditions, under more complicated situations where multiple organs are involved, this strategy only delivers partial success, and mortality rates due to multiple organ dysfunction are still high. Therefore, identifying an effector molecule that is common to all these pathological challenges might be a more effective strategy in treating the complications involved in endothelial barrier dysfunction, thus improving patient outcome. Our recent findings that endothelial local lamellipodia contribute to endothelial barrier function suggest that therapeutics should maintain or enhance these dynamic structures to successfully reduce microvascular hyperpermeability.

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Fig. 1.

Example of the rat mesenteric microcirculation viewed by fluorescence intravital microscopy. FITC-albumin served as the tracer, and is visible within the microvessels. Panels *A* and *B* show the microcirculation from a control rat (sham) and a rat that underwent a hemorrhagic shock and resuscitation procedure. The arrows in panel *B* show “hot spots” of leakage of FITC-albumin from the microvessels into the surrounding tissues. Adapted from (Doggett *et al.*, 2017) with permission.

Fig. 2.

Determination of permeability of a single-perfused microvessel. **A.** Schematic view of the cannulated microvessel, with an inflow pipette that allows perfusion with either a tracer solution or the wash solution, and a defined window for measuring fluorescence intensity in the microvessel and extravascular surrounding area. Note that this configuration can also work for isolated venules, although an outflow pipette is also needed. **B.** The fluorescence readings over time can be used to calculate permeability coefficients for the tracer molecules. When tracer is washed in, the step increase in fluorescence intensity (I_f) divided by the volume of the microvessel (V) represents the concentration of tracer in the vessel, and at this stage, the concentration difference across the microvessel wall. Following the step increase is a more gradual fluorescence intensity over time, and the slope of this increase, designated $(dI_f/dt)_0$, represents solute flux out of the microvessel. These parameters can be used to calculate the solute permeability coefficient (P_s) Fick's first law of diffusion. Taking into account the vessel geometry, V divided by the surface area for diffusion becomes $r/2$, so that $P_s = (1/I_f) \times (dI_f/dt)_0 \times (r/2)$. Adapted from (Yuan *et al.*, 1993a; Durán *et al.*, 2008) with permission.

Fig. 3.

Expression of GFP-actin in HUVEC does not affect agonist-induced changes in endothelial barrier function. Panel *A* shows that expression efficiency can be as high as 95% in some areas (scale bar = 100 μm). *B*. Western blot analysis of β-actin (left) and GFP (right) in HUVEC lysates from cells expressing GFP-actin versus controls. The top arrow indicates the GFP-actin band, while the bottom arrow indicates native actin. Panels *C*, *D*, and *E* show GFP-actin (green) along with labeling of F-actin with Alexafluor-594-phalloidin (red) and nuclei with Hoechst 33342 (blue) in paraformaldehyde-fixed cells. Scale bar = 20 μm. Panels *F* and *G* show changes in TER elicited by thrombin (1 U/ml) and S1P (2 μM) in GFP-actin- and mock-transfected HUVEC. Reproduced with permission from (Breslin *et al.*, 2015).

Fig. 4.

Kymograph analysis of lamellipodia characteristics. *A*. To generate a kymograph in a particular area on the edge of a cell, a line perpendicular to the edge was drawn. In the resulting kymograph (line scan), the x-axis represents time and the y-axis represents distance. *B*. Membrane protrusions can be identified in the kymograph and lines are drawn as shown in panel *C* from the starting point to the endpoint of each protrusion. The protrusion velocity, distance, and persistence are determined from these lines as shown in panel *D*. The same type of analysis is used as shown in panel *E* to determine the withdrawal distance, velocity, and time. Reproduced with permission from (Breslin *et al.*, 2015).

Fig. 5.

Assessment of the lateral movement of actin stress fibers by kymograph analysis. *A*. In HUVEC expressing GFP-actin, a line is drawn across the center of a single cell in order to generate a kymograph, as shown in panel *B*. In this kymograph, the x-axis represents distance, and the y-axis represents time. The brighter areas that resemble lines horizontally represent where stress fibers were crossing the plane drawn by the line in panel *B*. In panel *C*, lines were superimposed over these brighter areas and the geometric data was determined using ImageJ. The data collected included the number of fibers present at regular time points, plus the lateral velocity of the stress fibers between each time point. Reproduced with permission from (Breslin *et al.*, 2015).

Fig. 6.

Timing of decreased protrusion frequency of lamellipodia and increased actin stress fiber formation in response to thrombin. **A.** The top left image shows HUVEC expressing GFP-actin. These cells displayed frequent local lamellipodia (small arrows) and actin “clouds” (large arrowhead) during baseline, prior to addition of thrombin. Shortly after thrombin (1 U/ml; 3 min image) was added, a coordinated increase in actin was observed on the edges of cells (thin arrowheads) and on the perimeters of vesicles (very small arrows), after which there was a marked decline in protrusions. Later in the time course (27.5 min image), there was de novo formation of ventral stress fibers (SF), and cortical actin fibers migrated centrally, becoming transverse arc (TA) stress fibers. Opening of a tricellular junction (*) was also apparent. **B.** The protrusion frequency was quantified by counting all local lamellipodia events throughout the time-lapse image set. Thrombin decreased the mean protrusion frequency of local lamellipodia during the first 20 min of the time course. **C.** The

number of actin fibers was also quantified, and these were significantly increased at 30 min after 1 U/ml thrombin addition. * $P < 0.05$ versus the zero-minute time point. The summarized data were collected from 9 different cells from multiple experiments. Reproduced with permission from (Breslin *et al.*, 2015).