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Tonic regulation of vascular permeability

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Abstract

Our major theme is that the layered structure of the endothelial barrier requires continuous activation of signaling pathways regulated by S1P and intracellular cAMP. These pathways modulate the adherens junction, continuity of tight junction strands, and the balance of synthesis and degradation of glycocalyx components. We evaluate recent evidence that baseline permeability is maintained by constant activity of mechanisms involving the small GTPases Rap1 and Rac1. In the basal state, the barrier is compromised when activities of the small GTPases are reduced by low S1P supply or delivery. With inflammatory stimulus, increased permeability can be understood in part as the action of signaling to reduce Rap1 and Rac1 activation. With the hypothesis that microvessel permeability and selectivity under both normal and inflammatory conditions are regulated by mechanisms that are continuously active it follows that when S1P or intracellular cAMP are elevated at the time of inflammatory stimulus, they can buffer changes induced by inflammatory agents and maintain normal barrier stability. When endothelium is exposed to inflammatory conditions and subsequently exposed to elevated S1P or intracellular cAMP, the same processes restore the functional barrier by first reestablishing the adherens junction, then modulating tight junctions and glycocalyx. In more extreme inflammatory conditions, loss of the inhibitory actions of Rac1 dependent mechanisms may promote expression of more inflammatory endothelial phenotypes by contributing to the up-regulation of RhoA dependent contractile mechanisms and the sustained loss of surface glycocalyx allowing access of inflammatory cells to the endothelium.

Keywords

Permeability; endothelium; S1P; cAMP; Rap1; Rac1; glycocalyx

1. INTRODUCTION

Mechanisms that maintain the normal endothelial barrier are the primary focus of this review. We also examine how these mechanisms can be modified during physiological and pathophysiological increases in permeability. One particular focus is a dynamic view of the regulation of the endothelial barrier. We highlight recent observations that the low

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permeability state of normal microvessels requires the continuous activity of mechanisms to maintain the barrier. A second focus is on the three dimensional structure of the functional barrier. This is because blood-to-tissue gradients are determined not only by the stability of the intercellular junction which is essential to prevent inflammatory gap formation, but also by the pattern of strands of tight junction proteins within the intercellular cleft, and by the endothelial surface glycocalyx. All three structures are best viewed as undergoing continuous modulation. Mechanisms known to attenuate gap formation also contribute to the stability of the glycocalyx and the organization of the tight junctions. This organization of the review is based on our working hypothesis that the permeability and selectivity of microvessels under both normal and inflammatory conditions are regulated by mechanisms that are continuously active to maintain the barrier. Continuous activation of key mechanisms in the normal state is in contrast to the idea that agents that stabilize the endothelial barrier act primarily to increase cell-cell adhesion and attenuate endothelial contraction to reduce inflammatory gap formation. The two mechanisms are not mutually exclusive. The latter more generally accepted view might apply to barriers in which adhesion is already compromised and contractile mechanisms up-regulated.

Our overall approach is to evaluate two mechanisms that stabilize the endothelial barrier by modulating one or more the following determinants of normal low permeability: stability of the peripheral actin band and associated components of the endothelial cytoskeleton, adhesion between endothelial cells at the level of the adherens junction, the continuity of the tight junction strands, cell-matrix adhesion, and the membrane associated components of the endothelial glycocalyx. One is the ligation by sphingosine-1-phosphate (S1P) of endothelial S1P1 receptors to activate a range of endothelial functions including the maintenance of a basal permeability tone, as well as a rapid attenuation of inflammatory responses. Most of the latter actions have been identified only over the past decade and the role of S1P, carried in plasma by albumin and high density lipoprotein (HDL), as a modulator of permeability is an area of active research (Hla et al., 2008, McVerry and Garcia, 2005, Wang and Dudek, 2009). The second mechanism is the much better understood action of intracellular cAMP to attenuate acute increases in permeability by modulation of endothelial cell contraction and adhesion (Surapisitchat and Beavo, 2011, van Nieuw Amerongen and van Hinsbergh, 2002, Vandenbroucke et al., 2008). Some of the most important new information about the cAMP dependent mechanisms is the shift in understanding of their role from modulation of endothelial actin-myosin contraction to the modulation of the endothelial actin cytoskeleton and cell-cell adhesion (Spindler et al., 2010, Komarova and Malik, 2010, Adamson et al., 2008, Cullere et al., 2005). Furthermore, the balance between adhesion and contraction appears to be regulated at least in part by cross talk between small GTPases in signaling pathways activated by S1P and those modulated by cAMP.

Although most of this review focuses on the regulation of molecular mechanisms determining adhesion and the stability of the cytoskeleton, it is important to emphasize that the functional properties of the endothelial barrier can not be accounted for in terms of changes in cell-cell or cell-matrix adhesion or the glycocalyx alone. For example, a detailed understanding of the adherens junctions is not sufficient to explain the effective plasma oncotic pressure differences that regulate transvascular fluid exchange according to the classic Starling principle. This is because the glycocalyx is the principal barrier to plasma

protein exchange and the oncotic pressure difference across this barrier depends not only on a stable adherens junction but also on the arrangement of the proteins forming the tight junction strands. To help bridge between investigations at the molecular level and those in intact microvascular beds we briefly review current attempts to account for measured permeability properties in intact microvessels and microvascular beds in the next section (Michel and Curry, 1999, Weinbaum et al., 2007, Levick and Michel, 2010). The reader is also referred to two recent reviews from our laboratory for additional detailed discussion of the structure and function of the glycocalyx, and linkages between investigations of the endothelial barrier at the cellular, single vessel, and whole organ levels (Curry and Adamson, 2012, Curry and Adamson, 2010).

2. DETERMINANTS OF NORMAL PERMEABILITY: MOLECULAR INSIGHTS INTO THE GLYCOCALYX-JUNCTION-BREAK MODEL

2.1. The intercellular cleft and adherens junctions

The principal pathway for most water and water soluble solute in vessels with normal baseline is through junctions between the cells forming the endothelial layer. It is now understood that both the number (density) and size of the equivalent pores in classical pore theory of capillary permeability are determined by molecular assemblages within, or closely associated with the intercellular cleft (proteins forming the adherens and tight junction, a luminal surface glycocalyx, and cellular microdomains around the cleft exit) (Levick and Michel, 2010, Spindler et al., 2010, Tarbell, 2010, VanTeeffelen et al., 2010, Curry and Adamson, 2012). The intercellular cleft formed by the juxtaposition of adjacent endothelial cell membranes usually has a complex geometry as two (and often more) cells interdigitate. The simplest description of the intercellular exchange pathway is as a deep gorge or, more commonly, as a narrow cleft with a remarkably constant spacing between the adjacent endothelial cell membranes (width near 22 nm) around the complete perimeter of cells. The cleft has a variable depth depending on the extent of overlap of adjacent cells (ranging from less than 100 nm in thin regions where adjacent cells simply abut, to long highly tortuous pathways formed as a region of one cell is overlaid by projections of the edge of an adjacent cell. Increases in cleft depth increase the diffusion distance between luminal and abluminal surface of the endothelial barrier. It is noted however that the common use of changes in electrical resistance as an index of barrier permeability can be a poor indicator of changes in permeability to larger solutes because the resistance to diffusion along the wide region of the junction may be a small fraction of the total resistance to their transvascular exchange (e.g. through the glycocalyx). It follows that changes in cleft depth, regulated by the mobility of the endothelial cells, must be distinguished from changes in the organization of intercellular junctions that determine the barriers (Xu et al., 2007).

The regular spacing close to 22 nm along the cleft length is accounted for by the uniform distribution of adherens junctional proteins, principally vascular endothelial cadherin (VE-cadherin or cadherin 5), whose extracellular homophilic domains span the space between adjacent cells to stabilize the adherens junction (other adhesion proteins may also be present including, nectins and platelet endothelial adhesion molecule-1 (PECAM)) (Dejana et al., 2009, Komarova and Malik, 2010, Vestweber, 2008, Ebnet et al., 2008, Privratsky et al.,

2010). The mechanisms involve calcium-dependent and calcium-independent binding within the intercellular space as well as protein assemblages regulating the anchoring of the adhesion molecules to the peripheral actin band. The detailed reviews cited above appropriately emphasize the key role of mechanisms regulating the adherens junctions to determine barrier stability by resisting inflammatory gap formation, however much less attention has been given to regulation of the organization of the tight junction proteins into strands within the adherens junction and the surface glycocalyx which are also key determinants to normal barrier function.

2.2. The tight junctions

Since the time of the Alfred Benzon Symposium held in Copenhagen in 1969 it has been recognized that less than 10% of the perimeter of the intercellular cleft must be open (i.e. forming a continuous pathway from blood to tissue) to account for the measured permeability properties of the microvasculature in organs such as skeletal muscle, lung, and heart (Lassen and Trap-Jensen, 1970). This is less than 0.1% of the total endothelial surface area and corresponds to the area for exchange through water filled pores in the classical pore theory of capillary permeability (Michel, 1997, Michel and Curry, 1999). In the region of well formed tight junction strands, adjacent cell membranes are much closer than 22 nm for long segments of the intercellular cleft thereby effectively closing off the cleft for paracellular exchange. These occlusions are often branched and have several strands in series in central nervous system blood vessels (forming part of the blood-brain-barrier) and in tight epithelia (Schulzke et al., 2012). However, in peripheral microvasculature the strands are generally single or with a few branches and often but not always found near the luminal opening of the cleft. The extracellular membrane-bound components of the tight junctions are formed by proteins from three families, claudins, the occludin family (including tricellulin) and junction adhesion molecules (JAMs) (Ebnet, 2008, Schulzke et al., 2012, Bazzoni, 2003). The present understanding is that the permeability of the barrier to ions in tight epithelial is conferred by the claudins (Colegio et al., 2003, Morita et al., 1999); although recognized for nearly 20 years as a component of the tight junction, the function of occludin is not yet clear (Furuse et al., 1993). These proteins are anchored to the peripheral actin cytoskeleton by an array of scaffolding and regulatory proteins, the best known of which include the catenins and the ZO proteins (Dejana et al., 2009, Stevenson et al., 1986). The presence of these organized continuous strands explains why experiments with electron dense tracers in intact microvessels to evaluate the patency of the inter-endothelial cleft usually showed no penetration of the tracer through the cleft in the majority of cross sections examined in electron microscopy (EM) studies. As explained below when up to 90% of the perimeter of the endothelial cells is made effectively impermeable to water and most solutes by tight junction strands, the regions of the cleft that must be the focus of attention are those where the continuity of the tight junction strands is disrupted. This highlights a fundamental sampling problems with attempts to relate ultrastructure or cytochemistry of junctions to barrier properties.

Observations at the level of light microscopy cannot detect changes in the size and distribution of infrequent breaks in the junction strand, and other structures that may modulate normal permeability. While acknowledging a role for tight junction proteins as

sites of cell-cell adhesion (so called “kissing points” between adjacent cells), some reviewers question their role as a critical determinant of permeability properties, except in barriers such as the brain (Komarova and Malik, 2010, Vestweber, 2008). While there is support for the idea that stable adherens junctions are a prerequisite to establish tight junction strands, the number, distribution, and continuity of the tight junction strands are a critical determinant of normal vascular permeability and must be evaluated using appropriate electron microscopic methods. Currently, the best approach available to evaluate tight junction strand geometry as it determines vascular permeability is serial sectioning to enable 3D reconstruction of regions of closed and open cleft. Measurements from single EM sections are of limited value because even under the best conditions only one in 10 to one in 20 sections can be expected to reveal a region along a junctional strand that is effectively open for exchange. Serial sections to overcome this problem were first attempted in microvessels in cardiac muscle where the low frequency of real breaks was confirmed (Bundgaard, 1984, Michel and Curry, 1999, Crone and Levitt, 1984). Extension of this to detect molecular sized pores in the tight junction using ultrathin serial sections (about 12 nm thick) was attempted in a small sample without conclusive results (Bundgaard, 1984). Serial sectioning has been much more successfully applied in individually perfused microvessels of mesentery (rat and frog) where the baseline permeability is determined by discontinuities in the junctional strand much longer than the thickness of a typical EM section (50–70 nm). In venular microvessels of rat mesentery with a hydraulic conductivity of 0.5×10^{-7} cm/(sec cmH₂O) (about twice the average in skeletal muscle) infrequent breaks in the continuity of junctional strands (average 315 nm long) and separated by an average distance of 3.6 μ m along the strand (equivalent figures in the frog vessels are 150 nm breaks separated by 4.4 μ m) (Adamson et al., 2004, Adamson and Michel, 1993). The size and frequency of junction strand breaks is consistent with the estimate that less than 10% of the junction need to be open to account for the permeability of these vessels. To incorporate these observations into models of transvascular exchange, detailed 3D models of diffusion and water flow have been developed to enable quantitative predictions of permeability and to enable the contribution of steric exclusion and resistance to diffusion of the molecular structures such as the endothelial glycocalyx to be evaluated (Hu and Weinbaum, 1999, Levick and Michel, 2010, Michel, 1997, Weinbaum, 1998, Weinbaum et al., 2007).

2.3. The glycocalyx as a molecular sieve

The observation that the membrane spacing at sites of long breaks in the junction strand is not reduced but remains close to 22 nm means that the breaks do not form the size limiting structures within the endothelial barrier. Nevertheless these vessels remain highly selective to plasma protein (exerting more than 90% of their expected colloid osmotic pressure). Detailed modeling experiments to evaluate the possibility that cross bridging structures formed by adherens junction (molecules that maintain the normal 22 nm spacing, distributed throughout the cleft) have failed to provide a consistent description of the barrier properties (including water flow across the wall, the osmotic gradients between blood and tissue, and the permeability of solutes up to the size of albumin) (Hu and Weinbaum, 1999, Michel, 1997, Weinbaum et al., 1992). On the other hand the inner portion of the endothelial glycocalyx, with a quasi-ordered structure (maintained by anchoring to the peripheral actin band) can form a molecular filter (Michel, 1997, Weinbaum, 1998, Curry and Adamson,

2012). Fibers 10–12 nm in radius, extending up to 200–400 nm from the surface, and arranged in a hexagonal array not only account for the size selectivity of the barrier, but have resistance to water and solute diffusion sufficient to maintain gradients of albumin concentration and colloid osmotic pressure that balance microvessel hydrostatic forces (the Starling balance) (Weinbaum et al., 2007, Arkill et al., 2011, Squire et al., 2001). The same structure also is found on the luminal surface of fenestrae, and determines the selectivity of fenestrated vessels in kidney, synovial joints, and others (Levick and Michel, 2010). The inner ordered region of the glycocalyx is part of a more extensive endothelial surface layer formed as hyaluronan, glycosaminoglycans (GAGs), and plasma proteins bind to proteoglycans at the entrance to the cleft region (Curry and Adamson, 2012, Tarbell, 2010, Levick and Michel, 2010). The glycocalyx structure is determined by the balance of synthesis and secretion of the glycocalyx components and the sloughing and degradation of these components by enzymes such as membrane bound proteases (Lipowsky, 2012).

2.4. The working model of a glycocalyx-junction-break endothelial barrier in continuous endothelium

Figure 1 shows a 3 dimensional layered structured of the endothelial barrier. The specifics in the figure are based on measurements in venular microvessels of rat mesentery but, as detailed below, the fundamental principles apply to microvessels with continuous endothelium, especially those with a limited number of junctional strands. They also apply in fenestrated microvessels (Levick and Michel, 2010). Mesenteric microvessels are used as a model system because the permeability, selectivity, and ultrastructure of the microvessel wall can be measured over a range of well-control experimental conditions. In this review we draw from investigations in this experimental model to provide representative descriptions of normal ultrastructure, baseline permeability, responses to acute and sustained inflammation, changes in ultrastructure after inflammation, and changes in phenotype after inflammation. In general these data suggest that observations of rat venular microvessels are more representative of the functional properties of other uninjured venular microvessels with continuous endothelium than many cultured endothelial cells (Adamson et al., 2010). Approaches used in these vessels can be extended most directly by applying updated imaging methods in organs such as skin and muscle, particularly in mouse models (Kim et al., 2009), and more labor intensive tissue sampling methods in other organs (Curry et al., 2010, Lin et al., 2012, Lin et al., 2011). A small number of observations have been made on cultured endothelial cells derived from rat mesenteric microvessels (Baumer et al., 2008a) but in general experiments in cultured monolayers are carried out on endothelial cells from different sources (human umbilical vein, bovine aorta, mouse myocardial tissue, human and bovine pulmonary artery, rat fat pad, and human dermis). In many of these models of barrier function, the role of the endothelial glycocalyx and the organization of junction strands are not reported. However, further understanding of the role of molecular insights from in vitro investigations require continued efforts to design experiments to evaluate the contribution of these mechanisms in whole microvascular beds. Some of these experiments are discussed throughout this review.

Experiments on individually perfused microvessels have provided new insights into the normal function of the endothelial barrier, particularly with respect to transvascular fluid

exchange determined by the Starling Principle (Levick and Michel, 2010). One prediction of the model is that the colloid osmotic pressure difference that opposes filtration is exerted across the endothelial glycocalyx, not across the whole endothelial barrier. This is because, during filtration, the plasma protein concentration in the cleft beneath the glycocalyx is determined primarily by the plasma ultrafiltrate crossing the glycocalyx. The plasma protein concentration in this ultrafiltrate is low, and, as it crosses through the cleft, is kept lower than the average tissue plasma protein concentration because the ultrafiltrate is funneled into the infrequent breaks in the junction strands. The water velocity at these sites is close to the diffusion velocity of protein, thereby limiting diffusion of tissue protein back along the cleft. The net result is that the oncotic pressure difference opposing filtration is larger than that expected from the average plasma to tissue colloid osmotic pressure difference, and changes in plasma oncotic pressure are more important determinants of fluid balance than tissue osmotic pressure. This balance requires that the complete barrier remains intact.

A second prediction of models like Figure 1 is that there is always slow net filtration all along all microvessels in a microvascular bed. Steady reabsorption in the venular microvessels is not possible without additional mechanisms to modify tissue protein concentration, such as transepithelial fluid reabsorption (Levick and Michel, 2010), or possibly a tissue sink such as activated lymphatic drainage. This is because tissue protein accumulation in the cleft during transient reabsorption will always dissipate colloid osmotic differences favoring the reabsorption. Note that a model of the endothelial barrier formed only by an adherens-type junction does not account for these important functional properties. The latter describes only symmetric blood to tissue colloid osmotic pressure differences. The model also indicates how fluid exchange is compromised even when the adherens junctions remain intact. Damage to the glycocalyx, leading to increased plasma protein leakage favors edema formation, and loss of glycocalyx exposes the endothelial cells to inflammatory cells. As a result of this model there is now re-examination of some of the long standing controversies in fluid therapy (crystalloids alone vs. crystalloids containing oncotic agents) guided by understanding of the revised Starling Principle (Aman et al., 2012, Woodcock and Woodcock, 2012a, Woodcock and Woodcock, 2012b).

We note that emphasis on the intercellular pathway does not ignore contributions from water transport across the cell membranes due to transient changes in plasma osmolarity, or uptake and transport of plasma proteins via vesicles. In particular vesicle transport may contribute to normal blood-to-tissue exchange in endothelial barriers where junctional exchange of plasma proteins is less than in venular microvessels and baseline permeabilities to albumin fall close to 10^{-8} cm/sec. The contribution of such pathways is overwhelmed when the paracellular pathway becomes leaky. However we note that mechanisms discussed in this review such as reorganization of the peripheral actin band and degradation of the glycocalyx will modify exchange through any of the forms of vesicle pathway that have been proposed (vesicle shuttling, exchange of vesicle contents, or fusion to form transcellular shunts (Nagy et al., 2008).

3. MOLECULAR MECHANISMS THAT MODULATE THE PERMEABILITY BARRIER

3.1. Regulation of the barrier: The importance of the initial state of the endothelial barrier

The permeability barrier in Figure 1B is compromised when gaps up to 1 μm long form along the intercellular junction as adjacent cells separate at localized regions after exposure to inflammatory mediators *in vivo* (Adamson et al., 2003, McDonald et al., 1999) or in cultured monolayers (Stevens et al., 2000). Gaps also disrupt the glycocalyx in the region of the junction. Although there is evidence that some glycocalyx material may deposit in gaps during injury (Clough et al., 1988), a stable glycocalyx in the region of gaps is not likely to reform at the cleft entrance until the gaps close and the adherens junction is stable. It is therefore appropriate to consider the stability of the junctions as a starting point to understand regulation of the barrier. When gaps form there are breaks in both the adherens and tight junctions seen as discontinuities in the immunofluorescence of the VE-cadherin and occludin (Adamson et al., 2010). Equally conspicuous are numerous spikes also containing VE-cadherin but turned at angles close to 90° to the line of contact between the endothelial cells. These represent VE-cadherin linked to actin that has been organized into numerous filipodia extending from adjacent endothelial cells (Hoelzle and Svitkina, 2012). Because these structures are similar to those described during the formation of new junctions between adjacent endothelial cells as they form a monolayer, they have been hypothesized to be part of a mechanism to reclose the gaps, and restore the junctions (McDonald et al., 1999).

The presence of such structures is characteristic of an endothelial barrier that has undergone some injury or exposure to sustained inflammatory conditions. Importantly, they are present in many cultured endothelial cells monolayers under “control” conditions. However, they are relatively rare in the endothelium of intact microvessels with no prior exposure to inflammatory conditions, and stable baseline permeability (for example, individually perfused rat mesenteric microvessels) but are quite prominent four days after these vessels have been exposed to inflammation (Yuan and He, 2012). The presence of even a few of these spikes is characteristic of an endothelial barrier which is structurally weaker than a normal barrier, as measured by increased response to inflammatory agents (Yuan and He, 2012) and in the case of cultured endothelial cell monolayers a baseline permeability to albumin that is 1–2 orders of magnitude higher than *in vivo* vascular barriers including those of skeletal muscle, skin, lung, and mesentery (Michel and Curry, 1999).

It follows that the initial state of the endothelial barrier must be taken into account when evaluating mechanisms that regulate permeability. We have previously stressed this idea based on the observation that RhoA dependent contractile mechanisms contribute to increased permeability in intact microvessels (single perfused microvessels and a mouse skin wound model) only after the vessels are subjected to injury (skin wound) or inflammatory conditions (Curry and Adamson, 2010). Waschke has noted that activation of RhoA dependent mechanisms occur in endothelial cells that undergo changes in cell shape and orientation after exposure to inflammatory conditions (Spindler et al., 2010). These observations led to the suggestion that endothelial cells in culture that also have up-regulated

contractile responses are better models of endothelial barriers exposed to chronic inflammatory conditions than normal vessels that have not been exposed to such disturbances. We extend this idea in this review focusing on the regulation of adherens junctions, tight junctions, and the glycocalyx.

3.2. Overview of signaling pathways

In this section we briefly review the mechanisms regulating the stability of the barrier as deduced mainly from investigations of endothelial cells in culture and describe some of the limitations of this current understanding when applied to intact microvessels. The two parts of Figure 2 summarize key signaling pathways activated by S1P (Fig 2A) and cAMP (Fig 2B) known to contribute to the regulation of the permeability barrier (Spindler et al., 2010, Wang and Dudek, 2009). The action of the small GTPases Rac1 and Rap1 are understood to increase adhesion between adjacent endothelial cells and to stabilize the peripheral actin band. When the development of tension contributes to gap formation, the small GTPase Rho A regulates actin-myosin force generation and this is modulated by the PKA dependent action of cAMP to attenuate MLCK. A full description is beyond the scope this review, but some of the most important changes include the following. Activation of Rac1 or Rap1 dependent pathways are associated with reduced stress fibers and increased peripheral band actin, as well as the peripheral localization of the actin binding protein cortactin, and non-muscle myosin light chain kinase (Dudek et al., 2004, Garcia et al., 2001, Schlegel et al., 2008). The prevalence of cell-cell and cell-substrate junctions is also rapidly increased. Proteins of the adherens complex including VE-cadherin, α - and β -catenin are enhanced at the cell periphery following activation (Lee et al., 2006, Mehta et al., 2005). Also, components of tight junctions including ZO-1, occludin, and claudin-5 become more localized and the number of tight junction “kissing points” has been shown to increase (Baumer et al., 2008b, Lee et al., 2006). Localization of focal adhesion associated proteins such as paxillin and focal adhesion kinase (FAK) following S1P (Shikata et al., 2003) or specific activation of the epac/Rap1 pathway (Lorenowicz et al., 2008) has also been associated with improved endothelial barrier function. These changes to the cytoskeleton and adhesion complexes are nearly universally associated with improved barrier function. Understanding these pathways is an area of active research. In the following we focus on those aspects of the Rac1 and Rap1 dependent pathways that are beginning to be linked to maintaining the integrity of the intact barrier as shown in Figure 1.

3.3. Mechanisms that protect the endothelial barrier: current understanding mainly from cell culture

A standard interpretation of the contribution of the S1P and cAMP dependent mechanisms in Figure 2 to the regulation of vascular permeability is that elevated levels of circulating S1P or increased intracellular cAMP increase the stability of the intercellular junctions and cell-matrix adhesion. This is a reasonable description for barriers that have some of the characteristics of a weakened barrier. Thus after a period of pretreatment with elevated S1P or conditions that raise intracellular cAMP, an increase in some or all of the factors modulating cell adhesion (stabilization of the cortical actin network, increased cell-cell and cell-matrix adhesion) as well as a reduction of baseline permeability have been described in cultured endothelial cell monolayers. Furthermore when exposed to inflammatory mediators,

the extent of gap formation and increased permeability is reduced relative to untreated controls. We note however that the levels of S1P to which cultured cells are exposed prior to such pretreatment has not been reported for previous studies. As described below normal endothelial barriers are exposed to a constant supply of S1P from red blood cells, so the absence of this source of S1P further complicates a description of the initial state of the barrier.

In spite of this relatively straight-forward account of mechanisms that may improve some barriers, the way in which they may apply in normal vessels is much less well understood. For example, in contrast to changes observed in culture, exposure of the rat venular microvessels to increased S1P does not change the distribution of key components of the intercellular junctions including VE-cadherin and occludin (Adamson et al., 2010, Minnear et al., 2005). Further, for these vessels with baseline permeabilities to water and albumin within the normal range, (e.g. mean L_p of 0.5×10^{-7} cm/(sec cmH₂O)), such pretreatment does not significantly reduce baseline permeability. The same results are found using the cAMP analog O-Me-cAMP to activate the epac/Rac1/Rap1 pathway (Adamson et al., 2008). These results suggest the organization of cortical actin network, cell–cell adhesion, and cell-matrix adhesion, characteristic of the normal low permeability state, are close to their maximum stable state. This suggests that, under the conditions of these experiments, at least some of the signaling pathways needed to establish the permeability barrier are already activated to maintain a steady state of low permeability. In particular, given that the small GTPase Rac1 is a point of convergence of signaling cascades for both S1P and the PKA independent pathways (epac/Rap1), this steady state model of baseline permeability control raises the question whether Rac1 or Rap1 dependent processes are continuously activated to maintain baseline permeability. Additional questions then arise about the role of these pathways to attenuate inflammatory mechanisms. For example, instead of acting to strengthen weakened junctions, increased levels of S1P or cAMP in the presence of an inflammatory agent may maintain Rac1 activation and junction stability close to normal rather than act via sequential weakening and restoration. In other words the barrier may be maintained in the presence of elevated S1P or increased intracellular cAMP by offsetting the tendency of inflammatory mechanisms to weaken the junctions. The topics reviewed below are selected to point to directions for future investigations based on these possibilities.

4. S1P MODULATION OF BARRIER PERMEABILITY IN INTACT MICROVESSELS

4.1. Stability of junctions in vessels with normal permeability

In this section we review new evidence that the stable junctional structure in Figure 1B requires continuous activation of signaling pathways regulated by S1P. Figure 3A shows experiments using engineered mice in which sphingosine kinase 1 (SphK1) genes were excised from hematopoietic cells (including erythrocytes and platelets). Sphingosine kinases are key enzymes leading to the intracellular production of S1P (Fyrst and Saba, 2010). S1P was not detectable (i.e., less than 50 nM) in mice with specific SphK1 knockout. (Camerer et al., 2009, Pappu et al., 2007). In these mice lacking circulating S1P (plasma S1P-less mice) the lungs accumulated albumin at three times the rate of the wild type control mice,

and changes in skin thickness demonstrated evidence of edema. Furthermore, microvessels in the lung and skin of the plasma S1P-less mice were more susceptible to inflammatory agents (Camerer et al., 2009). The stability of the endothelial barrier was restored when wild-type erythrocytes, a major source of circulating S1P, were added back to the circulation. These are the most direct observations to date in intact animals showing that conditions that may compromise S1P availability to endothelial cells predispose the barrier to inflammation and increased basal permeability.

Figures 3B and 3C shows experiments in individual venular microvessels with an artificial perfusate (mammalian Ringer with 10 mg/ml serum albumin) to control the availability of S1P to the endothelial barrier (Curry et al., 2012). When S1P was present at concentrations found in normal plasma, the permeability of the microvessels was stable for many hours. In contrast, when known sources of S1P were removed (no erythrocytes in the perfusate, and with the use of albumin from which fatty acids had been stripped to remove S1P bound to albumin) the normal stable permeability of the vessel was not maintained. In this case there is a large increase in permeability reflecting, at least in part, the significant reduction in S1P available at the endothelial cells under the conditions of these experiments where the microvessel lumen is continuously perfused to remove any locally generated S1P. Replenishment of sources of S1P (red cells, red cell conditioned medium, or exogenous S1P) returned the permeability to control levels. Furthermore, the presence of an S1P1 receptor antagonist (W146) increased permeability to the same extent as conditions with severely depleted S1P as expected if the S1P1 receptor was the principal endothelial receptor modulating the barrier stability as reported by others (Zhang et al., 2010).

The results in Figure 3B & C conform to the hypothesis that the normal status of an endothelial barrier represents a balance between active mechanisms to maintain the barrier and those that compromise the barrier. On the other hand, the differences in the magnitude of the increases in permeability when S1P is depleted under different experimental conditions (whole animal in Fig 3A; individually perfused microvessel in Figure 3B) show that S1P acting via S1P1 receptors on endothelial cells is not necessarily the only S1P dependent mechanism acting in the microvasculature to modify changes in permeability. In addition to the integrity of the junctions which is the focus of Figure 2, the change in permeability may also be determined by the integrity of the endothelial glycocalyx as well as a range of local immune, hemodynamic, and metabolic demands. These include changes in local shear (Chiu and Chien, 2011, Tarbell, 2010, Melchior and Frangos, 2010) immune cell interactions with the vessel wall (Chase et al., 2012, Rodrigues and Granger, 2010, Williams et al., 2011), the release of S1P from other cells in a microvascular unit including endothelial cells (Venkataraman et al., 2008) and platelets when they are activated (Stokes and Granger, 2012), and release of pro- and anti-inflammatory agents by vascular smooth muscle, mast cells, and other inflammatory cells (Eringa et al., 2012). We are only at the beginning of such studies, and a full coverage is beyond the scope of this review. Here we focus on the contribution of S1P1 dependent mechanisms acting directly or indirectly on the endothelium.

Camerer and colleagues described two models that may account for their observations in Figures 3A and 3B (Camerer et al., 2009). In their tonic activation model, S1P within the

blood vessel lumen continuously interacts with the S1P1 receptor on the endothelium, providing a constant signal that stabilizes cell spreading and cell-cell junctions and sets a threshold that determines responses to leak-inducing agents. The nature of the signal distal to S1P1 ligation was not discussed in detail, but constant Rac1 activation, as suggested above, would be one possibility. In a separate dynamic signaling model they suggested there are qualitative or quantitative differences in S1P1 receptor function at the luminal versus abluminal plasma membrane. It is assumed these enable the endothelial cells to detect S1P from plasma entering the subendothelial space. They suggest that the abluminal S1P1 receptors act as a leak detector mechanism to provide negative feedback to close intercellular gaps. Camerer and colleagues advanced this model to account for junctions opened in response to leak-inducing agents, but it would also apply in the normal state that was determined by dynamic balance of junction formation, transient leakage, and rapid recovery. At this stage there is no definitive evidence for or against each model. It is noted however that the normal state of Rac1 activation relative to the control of permeability is not known and Rac1 regulation is known to be complex (Castro-Castro et al., 2011, Symons, 2011). Thus more detailed investigations of regulation of pathways downstream from the G-protein coupled receptor S1P1 and the activation of Rac1 are needed. On the other hand lumen to tissue gradients of S1P control the trafficking of inflammatory cells from the circulation; the idea that similar gradients might play a role in a dynamic model of normal permeability regulation is novel. It is of course likely that different versions of these possible mechanisms may apply under different experimental conditions, particularly depending on the initial state of the barrier, the endothelial cell phenotype, and the range of other cAMP and S1P dependent mechanisms contributing to barrier instability.

4.3. S1P modulation of acute inflammatory responses

It is well established that increased levels of exogenous S1P attenuate the response of intact endothelial barriers to inflammatory agents such as Bk, PAF, thrombin, LPS and mechanical ventilation stress (Adamson et al., 2010, Garcia et al., 2001, Minnear et al., 2005, Peng et al., 2004). These observations show that the levels of S1P that maintain normal permeability in intact microvessels and organs as described above (Fig 3) are not sufficient to fully protect the endothelial barrier when exposed to inflammatory agents. This means that the effective balance described by either the constant activation model or the dynamic model is shifted towards mechanisms that weaken cell-cell adhesion and open junctions. The way S1P dependent mechanisms in Figure 2A attenuate the resulting increase in permeability depends on the state of the junctions at the time when S1P is increased. For example when S1P is elevated after the junctions forming the vascular barrier in intact microvascular beds such as the lung have been weakened by exposure to inflammatory agents, S1P dependent mechanisms appear to strengthen adhesion and restore the barrier in the same way that exogenous S1P strengthens cultured endothelial monolayers where junctions already display evidence of weakness (Garcia et al., 2001). On the other hand the idea that a primary action of elevated S1P is to always strengthen junctions does not appear sufficient to account for the action of S1P to protect the barrier in vessels with normal junctions.

As described above, after exposure of normal venular microvessels (periods of 15–30 min) to increased levels of S1P (1–5 μ M in excess of amounts available from endogenous sources

such as erythrocytes, normally close to 0.3 μM) there are minimal changes in the normal barrier of intact microvessels. The distributions of VE-cadherin and the tight junction associated protein occludin quantified from light microscopy are not changed in ways that indicate further strengthening of the barrier (Adamson et al., 2010). Although such preconditioning attenuated increased permeability in these vessels, it was not clear from such experiments that S1P could act to maintain normal permeability if present only during exposure to an inflammatory agent. Figure 4 illustrates experiments to test the hypothesis that the primary action of S1P to attenuate acute increases in permeability takes place during exposure to the inflammatory agents (Adamson et al., 2012). Notably, S1P is as effective to attenuate an inflammatory response when added at the same time as the inflammatory agent bradykinin as when the vessel is pre-incubated with S1P. That no pretreatment is required for maximum attenuation (on average about two thirds of the peak inflammatory response after 1–2 minutes is attenuated by S1P) suggests that the tendency for endothelial gap formation is arrested very early when elevated S1P is present. These observations point to the need for detailed analysis of the dynamics of Rac1 activation and gap formation in intact vessels, which are not currently available. Measurements in cultured endothelial cells provide a guide to the direction for future experiments. In human dermal endothelial cells the time to peak Rac1 activation after exposure to elevated S1P in cultured cells is rapid, reaching a maximum within 1–2 minutes (Fig 4C) (Adamson et al., 2012). This is the same time period as the initial increase in cytoplasmic calcium, a common trigger for a cascade of inflammatory mechanisms that lead to gaps between adjacent endothelial cells. These include reduction in Rac1 activity itself (e.g. by interfering with GDP/GTP exchange factors (GEFs) specific for Rac1 such as Tiam1), and indirect reduction of effectiveness of many of the Rac1 dependent mechanisms in Figure 2 by activation of Src, PKC etc (Komarova and Malik, 2010).

In summary, on the basis of previous investigations of S1P to attenuate increased permeability and the newer information from experiments such as those in Figures 3 and 4, a qualitative description of the actions of S1P to regulate endothelial barrier includes: (1) a threshold level of S1P is required to maintain normal permeability and this threshold is sustained or exceeded in the normal vasculature by the S1P derived from red blood cells (Fig 3); (2) the susceptibility of the endothelial barrier to inflammatory agents depends upon the level of S1P, susceptibility is increased when S1P reduced, and vice versa (Fig 3, Fig 4); (3) the normal plasma levels of S1P are not sufficient to fully protect the barrier against inflammatory stimulation, but it may contribute to recovery after the peak response (Figure 4A) as permeability returns towards control in the presence of the inflammatory agent without elevated S1P; (4) S1P must be continuously present at elevated concentrations to attenuate inflammatory increases; (5) any effect of preconditioning is rapidly lost when the barrier is exposed to inflammatory condition at the time S1P is removed. Much more quantitative information about levels of S1P associated with these responses is required (including the free and bound forms) but the simplest hypothesis to account for these observations is that at least one of the roles of S1P is to regulate the stability of the peripheral actin network and its link to the adherens junction under both normal and elevated vascular permeability via signaling pathways that converges on Rac1 activation.

Further the regulation of the barrier in both normal and high permeability states is determined by the constant activity of Rac1 dependent pathways.

As suggested below these are not the only actions of SIP to modulate parts of the barrier, but it should be possible to begin to test predictions of the hypothesis in some current cultured endothelial models. As is usually the case these would also direct later more technically difficult experiments in intact microvessels. Required are measurements of the time course of Rac1 activation under conditions where adherens junctions have been strengthened under baseline conditions and where SIP levels are carefully measured and controlled, then during exposure to inflammatory agents such as Bk, PAF etc. that have been investigated in intact microvascular beds. Parallel monitoring of cAMP levels to evaluate possible changes in epac/Rap1/Rac1 pathways, and measurements of the reorganization of fluorescently labeled actin in the peripheral actin band may provide dynamic information.

4.4. Additional evaluations of Rac1 dependent mechanisms

The role of Rac1 as a control point for some of the key mechanisms regulating normal and elevated barrier permeability is consistent with the observation that use of a bacterial toxin to specifically inhibit Rac1 (*C. sordelii* lethal toxin, LT) induces a permeability increase in rat venular microvessels similar to the results when SIP is absent (Waschke et al., 2004). Also, a less specific bacterial toxin (toxin B from *C. difficile*, a major source of infection in hospitals) inhibits Rac1, Cdc42, and RhoA, and increases permeability of both venular and arterial barriers (Adamson et al., 2002). Investigators have also used a pharmacological Rac1 inhibitor (NSC23766) to distinguish Rac1 dependent pathways from the action of other GTPases. In human dermal endothelial cells NSC23766 caused inflammatory gap formation under basal conditions (Spindler et al., 2011). The contribution of multiple factors regulating Rac1, the GEFs upstream from Rac1, and the downstream targets obviously make the actual control mechanisms far more complicated, but these are gradually being elucidated (Komarova and Malik, 2010). An emerging idea is that Rac1 activation also contributes to basal permeability by modulation of RhoA activity and the stability of the glycocalyx; these are discussed below.

We note that under some circumstances Rac1 activation is associated with processes that increase permeability. In one instance vascular endothelial growth factor (VEGF) receptor 2 activation stimulates Rac1 activation and results in the internalization of VE-cadherin and attendant loss of endothelial barrier function (Gavard and Gutkind, 2006). Rac1 also has been implicated in the generation of reactive oxygen species and diminished VE-cadherin mediated cell-cell adhesion, and may thereby induce increased permeability in some situations (van Wetering et al., 2002).

4.5. Cross talk between the small GTPases Rac1 and Rho

One mechanism whereby activated Rac1 can contribute to the maintenance of normal permeability is the suppression of the contractile mechanisms dependent on the small GTPase RhoA. Specifically, Rac1 normally inhibits the activity of the small GTPase RhoA, via p190RhoGAP (Komarova and Malik, 2010). The magnitude and time course of increased RhoA activity when Rac1 activation is reduced is not known. Because RhoA

inhibition does not contribute significantly to the hyperpermeability due to inflammatory agents in normal vessels (Adamson et al., 2003), it is unlikely that Rac1 modulation of RhoA is active in the inflammatory response to normal vessels with no prior exposure to inflammatory agents. However this mechanism may contribute to the RhoA dependent increase in permeability often described in cultured endothelial monolayers particularly those activated by thrombin (Komarova and Malik, 2010), and in endothelial barriers of microvessels exposed to inflammatory agents or an aseptic skin wound (Curry et al., 2003, Kim et al., 2009).

4.6. S1P modulation of the glycocalyx in normal vessels

The normal distribution of the components of the endothelial surface and thickness of the glycocalyx layer depends on the balance between active synthesis and assembly on one hand and degradation by digestive enzymes and proteinases. A wide range of conditions leads to degradation of the glycocalyx (Lipowsky, 2012). This degradation increases permeability not only because of reduced resistance to macromolecules to enter the open region of the intercellular cleft, but also exposes receptors on the cell surface and increases penetration of circulating macromolecules and inflammatory cells to the endothelial surface (Becker et al., 2010, Pahakis et al., 2007, VanTeeffelen et al., 2010). S1P can promote the activity of endothelial surface matrix metalloproteinases (MMPs) to increase the rate of endothelial cell migration (Kang et al., 2011, Langlois et al., 2004). However, recent evidence indicates that S1P ligation of S1P1 on endothelial cells suppresses activity of MMPs and thereby promotes retention of the membrane anchored syndecan and associated chondroitin sulfate and heparin sulfate, essential components of the endothelial glycocalyx (Zeng et al., 2013). As noted in section 2.4 above the integrity of the endothelial glycocalyx is an essential component of endothelial barrier function. While S1P interaction with MMPs is complex, it is possible that tonic suppression of MMP activation may contribute to normal glycocalyx stability. An important question for further study is how S1P plays a role in suppressing MMP activity in the endothelial glycocalyx. Recently it has been recognized that albumin and HDL play key roles in the delivery of S1P from sites of synthesis to the endothelial surface. Thus S1P bound to albumin may account, at least in part, for the well known observations that albumin stabilizes the endothelial barrier and the glycocalyx in particular (Michel et al., 1985).

4.7. Modulation of S1P supply: a new aspect of the role of S1P as a determinant of normal permeability

The recent observations that a threshold level of circulating S1P is required to maintain normal vascular permeability indicate that S1P dependent mechanisms regulating the endothelial barrier include not only the down-stream effector sites summarized in Figure 2, but also the proximal mechanisms that determine the level of Rac1 activation on a moment to moment basis. For S1P these include the availability of S1P, its rate of synthesis, degradation, and delivery via binding to plasma proteins, and the distribution of S1P1 receptors and other receptors stimulated by S1P, some of which appear to be pro-inflammatory (Rosen et al., 2008). S1P is synthesized in many cell types including RBCs after uptake of sphingosine and is released via novel ATP-dependent transporters (Kobayashi et al., 2009).

The release of S1P from RBCs into plasma is a regulated process triggered by interaction of RBCs with HDL and serum albumin (Bode et al., 2010). S1P is carried in plasma bound to albumin (30%), HDL (60%), and other plasma proteins (10%). The total plasma concentration is in the range 300–500 nM, but the free concentration only a few percent of the total (Lucke and Levkau, 2010, Ohkawa et al., 2008). This concentration represents a balance between synthesis, storage, and release primarily from erythrocytes (when platelets are not activated) and degradation by S1P lyase and S1P phosphatase. A constant supply of S1P is required to maintain the circulating concentration (half-life 15 min). That a stable low permeability state is compromised when the supply of S1P is limited (e.g. in S1Pless mice, Figure 3) or when the S1P1 receptor is antagonised indicates that some capillary leak syndromes may be caused in part by a failure of RBCs to maintain a normal supply of S1P in microvessels. We have suggested that the conditions under which there is reduced S1P production and release include those where RBCs are damaged due to genetic defects, such as in sickle cell disease and spherocytosis (Curry et al., 2012). It is also possible that some forms of infectious disease such as malaria may modify S1P production from RBCs. For example, children with cerebral malaria have diminished plasma S1P (Finney et al., 2011). We also note that many experimental models of the endothelial barrier that involve artificial perfusates and endothelial cell culture do not have RBCs present. Thus the contribution of S1P from other sources such as the endothelial cells themselves under different culture and perfusion conditions, and the potential role of S1P bound to commercial supplies of albumin and components of culture media should be evaluated as part of future investigations.

While platelets and platelet derived medium can be significant sources of S1P or other mediators that modulate vascular permeability (Minnear et al., 2001, Schaphorst et al., 2003), the relative contributions from erythrocytes and platelets to circulating S1P under normal conditions remain under investigation. There is clear evidence that S1P generation, storage, and release differs in red cells in comparison to platelets (Ito et al., 2007). For example, release of mediators, including S1P, from platelets often requires activation by other mediators or exposure to injury. Under activated conditions platelets may potentiate inflammatory responses, especially those by leukocytes (Stokes and Granger, 2012). In contrast, red cells appear not to require stimulation for S1P release; nor do they directly potentiate an inflammatory response. Thus when evaluating the contribution of platelets relative to red cells in the regulation of vascular permeability, it is important to distinguish between normal and injury or inflammatory conditions.

5. cAMP DEPENDENT MECHANISMS REGULATING BARRIER FUNCTION

5.1. Overview

A central idea linking S1P modulation of normal and increased vascular permeability and elevated cAMP modulation of permeability is that the initial conditions of the endothelial barrier determine the subsequent modulation of the barrier. A second idea linking modulation of the permeability barrier by S1P and cAMP is the extent to which these pathways converge on Rac activation. A third theme is that the barrier in Figure 1 involves not only the stability of the adherens junction, but also the organization of the tight junction, and the glycocalyx (discussed above). These topics are developed below with particular

emphasis on similarities and differences between S1P dependent mechanisms and both PKA independent (epac/Rap1 pathways) and the PKA dependent pathways in the regulation of normal permeability and inflammatory responses.

5.2. cAMP dependent regulation of baseline permeability

While the roles of cAMP dependent pathways have been described in detail with respect to the modulation of increased permeability, their roles in baseline permeability are less well understood. Ideally experiments on intact microvessels and cultured endothelial monolayers would be treated similarly in the same laboratory to directly compare responses of cultured endothelium with that of intact microvasculature. Of course, this is not always possible and the alternative has been to design experiments in intact microvessels which closely parallel experiments that previously have been carried out on cultured endothelial cell monolayers. In the latter experiments, use of the cAMP analog O-Me-cAMP to preferentially activate the PKA independent epac/Rap1/Rac1 pathway significantly improved the barrier function as measured by increased electrical resistance (Baumer et al., 2008b) or decreased permeability to 70kD fluorescently labeled dextran (Cullere et al., 2005). The improved barrier function was associated with increased immunolabeling of VE-cadherin and claudin 5, linearization of their distribution along the cell border, and increased activation of Rac1 by close to 150% (Baumer et al., 2008b). Similarly, increased Rap1 activation, increased continuity of staining of the tight junction molecules, and increased association of the cytoskeletal linker protein AF-6 along cell-cell contacts were also reported (Cullere et al., 2005). By contrast in venular microvessels of rat mesentery, O-Me-cAMP induced no change in permeability as measured by L_p and no significant change in the distribution of VE-cadherin or occludin (Adamson et al., 2008). However, pretreatment with O-Me-cAMP significantly attenuated increased permeability induced by agents in both barriers. Independent activation of Rac1 by CNF-1 (cytotoxic necrotizing factor 1 from *E. coli*) produced similar results (Waschke et al., 2006). These observations parallel those described above for the action of S1P on endothelial cells in culture and the action of S1P on the microvessels.

The questions that arise from these observations include: (1) Does elevated cAMP act to strengthen weakened junctions only via an epac/Rap1 pathway similar to that described above for S1P, (2) do normal levels of intracellular cAMP and epac/Rap1 activation also contribute to the maintenance of baseline permeability as described for baseline S1P levels, and (3) in addition to the actions of elevated cAMP to strengthen weakened junctions when they are present, does elevated cAMP act to maintain near normal junction structure? These questions focus on PKA independent pathways. Equally important are questions of the relative contributions of PKA dependent pathways.

5.3. Rap1 activation in baseline permeability control

The relative importance of baseline intracellular cAMP and any associated epac1/Rap1/Rac1 activation to baseline permeability regulation in intact microvessels cannot currently be evaluated in ways that have become possible for S1P because conditions where their contribution can be independently evaluated have not been described in detail. Preliminary reports from mice with the epac1 knockout describe a small increase in baseline permeability in skin and muscle (Karlsen et al., 2012). Quantitative comparison with

S1Pless mice is not available, but these results suggest a role for the epac/Rap1 pathway in the maintenance of basal permeability assuming the epac1 null mice have normal plasma. A similar conclusion can be drawn from the two-fold increase in L_p of rat venular microvessels in which stimulation of β -agonist receptors was inhibited by propranolol (Spindler and Waschke, 2011). This is clearly an area for further research.

While the epac1/Rap1/Rac1 mechanism has been most extensively investigated, recent studies emphasize that additional pathways involving Rap1 in endothelial regulation remain to be described. In cultured endothelial cells PDZ-GEF, a GEF for Rap1, activates Rap1 and promotes stability of endothelial cell-cell junctions to maintain basal permeability separate from but in concert with epac1 (Pannekoek et al., 2011). In another study different Rap1-dependent downstream modulation has been described. The protein Krev Interaction Trapped gene 1 (KRIT1) was reported to be a Rap1-specific effector, which localizes to cell-cell junctions where it promotes junction stability by suppressing RhoA activity, thus enhancing barrier function via a Rap1/RhoA crosstalk mechanism (Liu et al., 2011).

5.4. Additional cAMP regulation of tight junctions under baseline conditions via both epac/Rap1 and PKA dependent pathways

Although pretreatment of intact microvessels with the epac specific analog of cAMP (O-Me-cAMP) to activate PKA independent pathways does not significantly alter the distribution of junction proteins or baseline permeability, experimental conditions to activate both PKA independent (epac/Rap1) and PKA dependent pathways (e.g. elevation of cAMP by β -agonists, or use of the phosphodiesterase IV inhibitor rolipram, alone or in combination with forskolin to activate adenylate cyclase) results in a steady fall in baseline permeability over a period of 20–40 minutes (Adamson et al., 2008, Adamson et al., 1998, Spindler et al., 2011). While some of the additional reduction in baseline permeability due to cross talk between PKA and Rho GTPases regulated independently of Rap1 (Spindler et al., 2010) cannot be ruled out, the slow reduction in permeability over 20–40 minutes *in vivo* can be indirectly attributed to synthesis and organization of new tight junction proteins and the formation of additional tight junction strands (e.g. claudin or occludin). Specifically, frog mesenteric microvessels treated with rolipram and forskolin to elevate cAMP demonstrated close to a doubling of the number of tight junction strands within inter-endothelial clefts (Adamson et al., 1998). This observation is consistent with the observed reduction in baseline L_p and is also consistent with the idea that the extent of tight junction strands determines the paracellular exchange area discussed in section 2.2 above. A similar reduction in baseline permeability has been reported in mammalian vessels after exposure to isoproterenol or rolipram and forskolin, but detailed ultrastructure in these vessels after such pretreatment has not been carried out (Adamson et al., 2008).

These observations are important because new tight junction strands significantly reduce the effectiveness of the small fraction of the cleft that is open for exchange as illustrated in Figure 1. Not only will they increase the tortuosity of the exchange pathways through the junctions as suggested by Wissig (Wissig and Williams, 1978), but increased number of junction proteins may also reduce the size and frequency of breaks in the existing strands. These changes in baseline permeability also may have clinical applications even in the

absence of inflammatory conditions. For example, pretreatment of animals with β -agonists results in increased retention of intravenous fluid without modifying renal fluid output (Kinsky et al., 2008, Vane et al., 2004). Also, direct measurement of vascular permeability to albumin in the skin and muscle of mice pretreated with rolipram and forskolin to increase intracellular cAMP demonstrated reduced blood-to-tissue exchange of protein, sufficient to overcome the physiological actions of atrial natriuretic peptide to shift plasma protein from the vascular to the extravascular space (Lin et al., 2011). The result was retention of a plasma volume expanded state in these mice (Lin et al., 2012). Strategies based on these observations may improve fluid retention in the vascular space where appropriate before and during surgery.

5.5. Contractile mechanisms: the importance of phenotype

Before we discuss the role of cAMP dependent pathways to attenuate increased permeability, it is useful to introduce the idea of endothelial cell phenotype plasticity as it effects endothelial barrier modulation. The idea is well recognized for endothelial cells from different segments of the same microvascular bed (arterial vs. venular), and from different organs (lung v skin and muscle) (Stevens et al., 2001). Gender also influences phenotype (Huxley and Wang, 2010). Less well recognized are changes in endothelial phenotype after exposure of endothelial cells to injury or inflammatory conditions. Such phenotype changes may be responsible in part for differences in expression levels and composition of junction proteins in cell culture models (Wolburg et al., 1994) and junction properties in intact microvessels with no prior exposure to injury. The examples below also indicate that prior exposure to inflammatory conditions can result in a more pro-inflammatory phenotype with up-regulated contractile machinery compared with a more stable phenotype where cell–cell adhesion dominates.

A specific example is that venular microvessels in rat and the microvessels in mouse muscle and skin exposed within 1 hour of making a sterile skin wound do not increase permeability after acute exposure to thrombin, even though they respond to a range of other inflammatory agents (Curry et al., 2003, Kim et al., 2009, Yuan and He, 2012). However, these same microvessels do respond to thrombin with increased permeability 24 hours after exposure to inflammatory conditions (Kim et al., 2009). Because the signaling pathways distal to thrombin receptor ligation activate RhoA dependent contraction (Komarova and Malik, 2010), and the thrombin induced increased permeability response in skin and muscle is attenuated by the Rho kinase inhibitor, Y27632, it is reasonable to assume that RhoA dependent contraction is up-regulated in the microvessels 24 hours after inflammatory stimulation. Likewise, the robust response of many cultured endothelial monolayers to thrombin may indicate that the cells forming these barriers express a more contractile phenotype. The observations are important because some of the strongest evidence that cAMP acts via modulation of contractile mechanisms to reduce inflammatory responses comes from experiments using thrombin to increase permeability, or vessels that have been subjected to severe injury (Reynoso et al., 2007).

5.6. cAMP dependent modulation of increased permeability

With the exception of mechanisms linked to generation of cAMP via cytoplasmic adenylate cyclase (as described in lung endothelium (Sayner et al., 2006), it is universally accepted that elevated cAMP in endothelial cells (in regions close to the cell membrane) protects the barrier from increased permeability induced by a wide range of inflammatory agents (Komarova and Malik, 2010, Shen et al., 2010, Spindler et al., 2010). Until relatively recently, the most common mechanism to account for the increase in permeability in response to inflammatory agents was a calcium dependent increase in actin-myosin tension development. Increased peripheral tension above that required to resist shear force is assumed sufficient to overcome adhesive forces between adjacent endothelial cells resulting in the development of gaps between cells. The action of cAMP to attenuate gap formation was understood in terms of PKA dependent mechanisms acting on endothelial contractile machinery. While an understanding of cAMP regulation of gap formation in terms of reduced tension remains of fundamental importance for the barriers where RhoA dependent contractile mechanisms are up-regulated, the detailed investigations outline above in cultured monolayers demonstrated that inflammatory mechanisms were associated with reduced Rac1 activation, and less stable junctions as measured by decreased immunolabeling of VE-cadherin and claudin 5, loss of their linear distribution along the cell borders, and reduced association of the cytoskeletal linker proteins with cell-cell contacts. Parallel observations in both cultured mouse myocardial endothelial cells and in rat venular microvessels highlight the importance of changes in junction adhesion rather than increased tension. Insights derived from microvessel studies included the observation that inhibition of contractile machinery (MLCK; myosin ATPase, Rho kinase) did not attenuate increased permeability when Rac1 was inhibited (Adamson et al., 2002, Waschke et al., 2004, Spindler et al., 2010). These observations were extended to show that inhibition of contractile machinery did not inhibit the large inflammatory permeability increase in venular microvessels caused by PAF or bradykinin. On the other hand increased permeability was significantly attenuated by exposure to the conditions that stimulated Rac1 activation (Waschke et al., 2006).

It follows that under conditions where active contractile forces are not a major factor in inflammatory gap formation, the primary action of elevated cAMP is to oppose the tendency of inflammatory agents to weaken junction formation. A direct application of this idea is that elevated cAMP offsets the reduction of intracellular cAMP induced by inflammatory agents. Support for this idea is found in both intact microvessels and in cultured monolayers. Enzyme-linked immunosorbent assays (ELISA) demonstrated both reduction in cAMP levels in intact microvessels after exposure to inflammatory conditions, and restoration of levels to near normal in the presence of rolipram and forskolin (Schlegel et al., 2009). Recent direct measurements of intracellular cAMP using dual wavelength imaging show there can be a rapid fall in intracellular cAMP within minutes of exposure to inflammatory agent, likely resulting in reduced Rap1/Rac1 activation and reduced effectiveness of mechanisms that maintain the barrier (Baumer et al., 2009). As suggested above the actions of cAMP which act via Rap1/Rac1 pathways to maintain normal permeability operate in parallel to those of S1P, with Rac1 as a point of convergence of the signaling pathways.

In spite of the effectiveness of the Rap1/Rac1 pathway to attenuate increased permeability, it is notable that conditions that stimulate only PKA independent targets do not attenuate increased permeability as effectively as conditions likely to stimulate both PKA dependent and PKA independent pathways (Adamson et al., 2008). This suggests that the most effective cAMP dependent mechanism stabilizes not only the adherens junctions and associated peripheral actin binding sites, but also the organization of the actin network and modulation of the turnover of proteins comprising tight junctions as described above in the baseline state (Taddei et al., 2008).

5.7. Comparison of S1P and cAMP dependent mechanisms

To compare the role of cAMP in the regulation of both normal and elevated barrier function in microvessels with the action of S1P we note the following similarities and contrasts. With respect to baseline permeability: (1) experimental conditions that raise intracellular cAMP lower baseline permeability over periods of tens of minutes when normal levels of S1P are present (e.g. S1P supplied by red cells). Elevated S1P has no such effect and higher levels even tend to increase permeability; (2) in the presence of normal perfusate levels of S1P, experimental conditions that activate only epac/Rap1/Rac1 pathways do not lower normal permeability; (3) there is only preliminary data on the effect of reduced activation of cAMP dependent mechanisms in normal vessels, all in the presence of normal S1P, but the available data indicate a small increase in permeability (epac knockout; propranolol to reduce activation of endogenous beta adrenergic agents). Taken together these observations are consistent with actions of cAMP dependent mechanisms on baseline permeability to modulate additional stability of junctions and tight junction strands when the adherens junctions and the cortical actin network are well established by normal S1P levels. The interactions of S1P dependent pathways and cAMP dependent pathways in the regulation of normal permeability are an important area for further research. One example of significant gaps in our knowledge is that action of increased cAMP to restore the barrier under conditions such as those in Figure 4 where permeability is increased by reducing S1P have not been investigated. It is expected that increased cAMP, acting via epac/Rap1/Rac1 would be as effective to stabilize the barrier as restoration of S1P.

As with S1P, the resting levels intracellular cAMP are not sufficient to fully attenuate experimental models of acute inflammation. Thus with respect to inflammatory responses: (1) experimental conditions that increase cAMP to activate both PKA dependent and PKA independent pathways before exposure to inflammatory agents produce the most effective reduction of the inflammatory responses; (2) similar preconditioning conditions that activate epac/Rap1 are as effective to attenuate increased permeability as S1P, but both are less effective than increased cAMP; (3) the only data available which addresses the kinetics of “on” and “off” responses such as those described for S1P in Figure 4 (Adamson et al., 2012) use rolipram and forskolin to increase intracellular cAMP. When there is no pretreatment, rolipram and forskolin attenuate increased permeability, but less effectively than with pretreatment. Taken together, these observations are another way of demonstrating that cAMP (acting via epac/Rap1) and S1P share a common pathway which activates Rac1 and the Rac1 dependent pathways to stabilize the actin network and the adherens junctions, and

to offset the inflammatory mechanism by maintaining the cortical actin network and adherens junction.

These observations highlight the need for new investigations of the relative effectiveness of cAMP dependent epac/Rap1/Rac1 activation versus S1P activation of Rac1 to maintain permeability under conditions of elevated permeability. This is an important area for further research, especially because modulation of intracellular cAMP using phosphodiesterases (Schick et al., 2012) inhibitors specific for conditions in endothelium, and the use of S1P and S1P analogs continue to be areas of focus for development of anti-inflammatory strategies. Finally we note the role of Rac1 as a modulator of Rho dependent contractile mechanisms highlights the important of continued investigations of the way S1P dependent processes and both PKA dependent and PKA independent mechanisms modulate tension development in microvessels, particularly when inflammation is sustained.

6. SUMMARY

Figure 5 summarizes major themes of this review including the idea that the layered structure in the endothelial barrier formed by the luminal surface glycocalyx, the complex region of overlay between adjacent cells, and the branching network of tight junction strands (Figure 1B) requires continuous activation of signaling pathways regulated by S1P and intracellular cAMP (Figure 2). The downstream targets of these signaling pathways modulate not only the adherens junction region of cell overlap, but also the continuity of the junction strands and the balance of synthesis and degradation of the glycocalyx components. In particular, baseline permeability is maintained by constant activity of mechanisms involving the small GTPases including Rap1 and Rac1. In the absence of inflammatory agents, the barrier can be compromised when baseline levels of activation of the small GTPases are reduced by a failure to maintain the normal S1P supply or delivery. In the presence of inflammatory agent, increased permeability can be understood, at least in part, as the action of inflammatory agents to reduce Rap1 and Rac1 activation and reduce the effectiveness of their down stream targets. When S1P or intracellular cAMP levels are elevated at the time of exposure to inflammatory agent, they buffer changes induced by inflammatory agents and maintain junction stability at near normal levels. When the barriers in Figure 1 are exposed to inflammatory stimuli and subsequently exposed to elevated S1P or intracellular cAMP, the same processes restore the adhesion by first re-establishing the adherens junction, then modulating both tight junctions and glycocalyx stability. In more extreme inflammatory conditions, loss of the inhibitory actions of Rac dependent mechanisms may promote expression of more inflammatory endothelial phenotypes by contributing to the upregulation of RhoA dependent contractile mechanisms and the sustained loss of surface glycocalyx allowing increased binding of inflammatory cells to the endothelium.

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Figure 1. A cartoon of the layered structure of the glycocalyx-junction-break model of capillary permeability

A. A simplified *en face* view of an interendothelial cleft with a single break in the junction strand is shown. An ordered matrix representing the endothelial glycocalyx on the endothelial cell surface forms a molecular filter determined by the spacing between the fibers. Plasma protein entry into the matrix is significantly restricted so that the plasma protein in the plasma ultrafiltrate is low. Filtered fluid is funneled through the break in the junction strand so that the water velocity at the break is increased. The result is a protected region beneath the glycocalyx where the concentration of plasma proteins is determined by the ultrafiltrate emerging from the glycocalyx and by the reduced back diffusion of interstitial proteins. The resulting osmotic pressure differences for plasma proteins across the glycocalyx is larger than that from blood to tissue ($\pi_c - \pi_t$). The arrangement provides a very efficient safety factor against edema whereby increases and decreases in filtration rate lead to rapid readjustments of the Starling forces all along the capillary. **B.** illustrates key determinants of the the hydraulic conductivity and solute permeability coefficients of the barrier: L_c available cleft length per unit area of vessel wall [$0.5 \text{ cell perimeter } (p_c) / \text{cell area } (A_c)$]; L_f , depth of the glycocalyx; L_1 , depth of the tight junction strand from luminal cleft entrance; L_2 , distance from tight junction strand to the abluminal cleft exit; L , mean distance from lumen to interstitium through the cleft ($= L_1 + L_2$); $2h$ mean spacing between adjacent endothelial cells in the adherens junction, $2d$, mean length of breaks in the junctional strand; $2D$, mean spacing between irregular breaks in the junctional strand. Adapted from Adamson et al., 2004, and Curry, 2005.

Figure 2. The side by side juxtapositioning of summary diagrams from recent reviews highlighting the actions of S1P to activate Rac1 (on left) and cAMP to activate Rac1 mainly via an epac/Rap1 pathway (on right) to regulate the stability of inter-endothelial junctions

A. Regulation of vascular permeability by S1P/S1P1 signaling involves ligation of S1P to the S1P1 receptor to stimulate Rac1 activation via the G_i-dependent recruitment of PI3 kinase and Tiam1 to a common site. Activation of Rac1 induces a series of profound events including adherens junction and tight junction assembly, cytoskeletal reorganization, and formation of focal adhesions that combine to enhance vascular barrier function. Other S1P dependent mechanisms such as an increase in intracellular Ca²⁺ concentration via a G_i–PLC pathway and transactivation of S1P1 signaling by other barrier-enhancing agents are beyond the scope of this review. **B.** The schematic shows endothelial barrier regulation by cAMP dependent mechanisms. Intracellular cAMP is increased after ligation of β₂-adrenergic receptors to activate adenylyl cyclases (AC), via stimulatory G proteins (G_s), or by forskolin to activate AC, and rolipram to inhibit phosphodiesterase 4. Activating cAMP increases Rac1 activity mainly via exchange factor directly activated by cAMP (epac)-mediated modulation of guanine nucleotide exchange factors (GEF). cAMP via Rap1 and Rac1 modulates endothelial barrier properties as the result of increased adhesion of intercellular junction molecules, tethering of junctional complexes to the actin cytoskeleton, or strengthening of the cortical actin cytoskeleton. TJ, tight junction; AJ, adherens junction; S1P1, Sphingosine-1-phosphate receptor 1; PI3K, Phosphoinositide 3-kinase; Tiam 1, T-lymphoma invasion and metastasis gene 1; Rac1, Rho family GTPase Rac1; PAK1, p21-activated protein kinase 1; LIMK, LIM kinase; PLC, Phospholipase C; ZO-1, Zona occluden protein-1; nmMLCK, non-muscle myosin light chain kinase; VE-Cad, Vascular endothelial cadherin; α-Cat, α-Catenin; β-Cat, β-Catenin; Vin, Vinculin; Pax, Paxillin; FAK, focal adhesion kinase. Used by permission from Spindler et al., 2010 and Wang and Dudek, 2009.

Figure 3. Increased permeability under conditions of low S1P

A. Plasma S1Pless mice exhibit increased basal vascular leak. Evans blue (1 mg/100 µl saline) was injected i.v., and 30 minutes later, mice were perfused with saline via the right ventricle, lungs were removed and photographed, and Evans blue content was determined. Left: Representative control (bottom) and pS1Pless lungs. Right: Evans blue quantitation. Each point represents data for a separate mouse. The horizontal bars denote the mean. **B.** Plasma S1Pless mice show increased paw edema in response to inflammatory agents. Histamine (60 µg) or serotonin (not shown) were injected into the hindpaws of pS1Pless mice and their control littermates. The contralateral paw was injected with vehicle. (Agent-injected paw thickness)/(vehicle-injected paw thickness) was determined at the indicated times and expressed as percent increase. Data are mean $\pm$ SEM. Note that responses to leak-inducing agents were higher in pS1Pless mice. **C.** S1P1 receptor-specific antagonist W-146 reversibly inhibits effect of RBC-conditioned (RBC-cond) perfusate on permeability to serum albumin (P_s^{BSA}) in single perfused rat mesenteric vessels. Representative data show the stabilizing effect of RBC-conditioned perfusate on P_s^{BSA} and that P_s^{BSA} rises rapidly when the vessel is reperfused with perfusate additionally containing W-146 (10 µM), an S1P1-specific antagonist. The modulating effect of RBC-conditioned perfusate is returned during the subsequent recannulation in the absence of W-146. **D.** Inhibition of receptor S1P1 using W-146 in presence of red blood cells (RBCs) reversibly increases hydraulic conductivity (L_p) in rat mesenteric vessels. Representative data show that the stable L_p measured in the presence of RBCs (RBCs1) is increased when the antagonist (W-146) to the principal S1P receptor in these microvessels (S1P1) is present with the RBCs. Normal permeability is restored when the W-146 is removed and the vessel is perfused again with the RBCs alone (RBCs2). Adapted with permission from Camerer et al., 2009 and Curry et al., 2012.

Figure 4. S1P efficacy depends strongly on timing of application

A. Representative data show that S1P given only as pretreatment yielded no inhibitory action toward Bk despite 30 min of S1P pretreatment. **B.** After a 30 min period of perfusion with vehicle solution only each vessel was tested with Bk in the presence of S1P (concurrent-only treatment group). S1P applied concurrent-only with Bk strongly inhibited the Bk response. **C.** Averaged values of groups; number of vessels in each group shown at base of bars. S1P 1 μM and Bk 10 nM in each expt. Values are mean $\pm$ SEM; @ $p < 0.05$; unpaired t-test. **D.** Activation of Rac by S1P in cultured human microvascular endothelial cells (HMVECd). Activation by S1P is indicated by solid line. Values are mean $\pm$ SEM; minimum number of independent observations in each group was 4. Vehicle treatment shows control activation level. **E.** Rac activation by S1P (1 μM) was about 200% of control when present for 1, 2, or 3 min (filled columns). Off response shown by exposing cells to S1P for 1 min and then switching to vehicle for 1 or 2 min (open columns); the Rac activation returns toward control levels and was significantly different from continuous S1P (*, $p < 0.05$, one-tailed Student's t-test, $n=4$ in each group). Modified from Adamson et al, 2010.

Figure 5. A schematic of mechanisms that maintain the normal permeability of the endothelial barrier and how increased permeability can be understood in terms of the modulation of these processes

This figure overlays a simplified representation of key pathways activated by S1P and intracellular cAMP, described as modulators of increased permeability on the schematic of the three-dimensional layered structure in the endothelial barrier. This is to emphasize that downstream targets of the signaling pathways in Figure 2 modulate all three components of the barrier under normal conditions: the adherens region of cell overlap to maintain cell-cell adhesion, the continuity of the junction strands to seal off most of the paracellular cleft, and the balance of synthesis and degradation of the glycocalyx components to provide a molecular sieve at the cell surface and cleft entrance. In the absence of inflammatory conditions that activate platelets, red cells are a major source of circulating S1P which is carried to the endothelium bound to albumin and HDL. Reduced effectiveness of any one of the components results in increased susceptibility of the endothelial barrier to inflammation. S1P, sphingosine-1-phosphate; RBC, red blood cells; albumin, circulating serum albumin; HDL, high density lipoprotein; PKA, protein kinase A.