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Review

Blood-brain barrier tight junction permeability and ischemic stroke

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ABSTRACT

The blood-brain barrier (BBB) is formed by the endothelial cells of cerebral microvessels, providing a dynamic interface between the peripheral circulation and the central nervous system. The tight junctions (TJs) between the endothelial cells serve to restrict blood-borne substances from entering the brain. Under ischemic stroke conditions decreased BBB TJ integrity results in increased paracellular permeability, directly contributing to cerebral vasogenic edema, hemorrhagic transformation, and increased mortality. This loss of TJ integrity occurs in a phasic manner, which is contingent on several interdependent mechanisms (ionic dysregulation, inflammation, oxidative and nitrosative stress, enzymatic activity, and angiogenesis). Understanding the inter-relation of these mechanisms is critical for the development of new therapies. This review focuses on those aspects of ischemic stroke impacting BBB TJ integrity and the principle regulatory pathways, respective to the phases of paracellular permeability.

Contents

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Introduction

The blood-brain barrier (BBB) is a diffusion barrier, consisting of an interdependent network of cells designed to segregate the central nervous system (CNS) from the systemic circulation. One of the primary responsibilities of the BBB is the strict regulation of paracellular permeability. In this regard, the endothelial tight junctions (TJs) of the capillary are the primary mediators, limiting paracellular movement of solutes, ions, and water. These TJs are regulated via highly specialized proteins, which can be modulated by numerous intracellular and extracellular signaling pathways. Although the physiological properties of the TJs have been intensely investigated over the past few decades, less is known concerning their regulation under pathological conditions.

During ischemic stroke and subsequent reperfusion, the TJs of the BBB are disrupted, resulting in the increase of vascular derived substances into the brain. However, such disruption is not a singular event. TJ permeability occurs in phases, and although these phases are interdependent they are mediated through different mechanisms. As such, through delineation of these processes we will not only gain a greater understanding of TJ alteration and regulation, but also gain valuable insight with regard to therapeutic applications.

This review focuses on the emerging concepts surrounding BBB TJs with regard to ischemic stroke. It will begin with addressing the functional anatomy of the BBB, followed by clarification of the endothelial TJs and associated regulatory proteins. It will then focus on the ischemia and reperfusion (I/R) dependent phases of BBB paracellular permeability. Next it will highlight the molecular pathways and principle mediators involved in BBB TJ changes, correlated to the time-course of I/R events. Lastly, it will address the clinical and drug development implications.

Functional anatomy

The BBB has long been described as the gate-keeper of the CNS, maintaining the fragile homeostasis of the brain. The unique functionality and morphology of the BBB is attributed to multiple factors. In addition to endothelial cells, the BBB is composed of pericytes, astrocytes, neurons, and extracellular-matrix (ECM), which have been collectively redefined as the neurovascular-unit (NVU) (Fig. 1A). The individual components of the NVU work in concert to regulate microvascular permeability, ion gradients, nutrient uptake, toxin removal, and cerebral hemodynamics. Likewise, a breakdown in any of the individual components may contribute to BBB dysfunction.

Endothelial cells

The first-line of the defense between the systemic circulation and the brain is the endothelium. The endothelial cells of the BBB are distinguished from peripheral endothelial cells by their lack of fenestrations, minimal pinocytotic activity, and the presence of TJs (Hawkins and Davis, 2005). These characteristics are the historic basis for the endothelium being defined as the primary barrier for drug transport into the brain. Additionally, there exists an increased mitochondrial content (Oldendorf et al., 1977), required for the multiple energy dependent processes involved in nutrient support and protection of the brain. Endothelial cells also provide a metabolic barrier, expressing a number of enzymes capable of degrading both harmful and therapeutic molecules (Witt et al., 2001).

Extracellular-matrix

Endothelial cells and pericytes are surrounded by the ECM, which serves several functions, including support and anchorage for cells via adhesion receptors, separation of cells from one another, and regulating intercellular communication. Endothelial cells, pericytes, and astrocytes express the integrin and dytoglycan families of matrix adhesion receptors, which adhere to the ECM and serve to mediate NVU function. The ECM is composed of structural proteins (i.e. collagen type-IV, laminin, fibronectin, elastin, trombospondin, and various proteoglycans), which are susceptible to enzymatic degradation. Degradation of the ECM and adhesion receptor alterations are associated with increased BBB paracellular permeability during ischemic stroke (del Zoppo and Milner, 2006; Wang and Shuaib, 2007). Disruption of the ECM is also necessary for growth factor-mediated angiogenesis and vascular remodeling (Zhao et al., 2006).

Pericytes

In the CNS, pericytes cover a significant portion of the abluminal endothelial surface and contribute to the stability of the microvessels (von Tell et al., 2006). In general, the recruitment and interaction of pericytes with the endothelium is essential for the formation, maturation, and maintenance of the BBB. Lack of pericytes has been identified with endothelial hyperplasia, increased capillary diameter, and changes in TJ proteins (Hellstrom et al., 2001). Furthermore, pericytes release angiogenic factors, regulating microvascular permeability and remodeling (Armulik et al., 2005). It also appears that CNS pericytes can communicate directly to endothelial cells through invaginations referred to as “peg-socket” contacts (Armulik et al., 2005). In this manner a single pericyte can be in contact with several endothelial cells, allowing for an additional layer of communication. Evidence has also shown that pericytes have contractile properties capable of regulating capillary blood-flow, impacting cerebrovascular autoregulation (Bandopadhyay et al., 2001; Peppiatt et al., 2006).

Astrocytes

Astrocyte glial cells are localized between the neurons, pericytes, and endothelial cells, communicating through numerous foot-processes. Studies have consistently shown that astrocytes are necessary for maintenance and maturation of the BBB (Abbott et al., 2006). Astrocyte end-feet contacts have also been shown to mediate regional cerebral blood-flow (rCBF) (Koehler et al., 2006). Several glial-produced factors, including transforming growth factor-β, basic fibroblast growth factor, glial-derived neurotrophic factor, and angiopoetin-1, support TJ formation and/or BBB phenotype of endothelial cells (Abbott et al., 2006). Astrocyte–endothelial cell interactions have also been shown to be essential in regulating brain water content and electrolyte balance under normal and pathological conditions. In this regard, astrocyte end-feet show unique features, including a high density of aquaporin-4 (AQ-4) water channels. These

AQ-4 channels are associated with the rapid water uptake during ischemic stroke and cytotoxic edema (Kleffner et al., 2008).

Neurons

To date, there is limited understanding with regard to the neuronal contributions to endothelial TJ regulation. Yet, given the metabolic demands of nervous tissue, as well as the direct correlation between brain activity and rCBF, it is apparent that a strong interaction exists between neurons and vascular function. Neuronal-NVU communication is believed to be routed primarily through astrocytes (Koehler et al., 2006), although direct neuronal contact with the endothelium has also been implicated (Hamel, 2006). Two-way communication between glial cells and neurons has been identified. Glial cells have shown to regulate synaptic transmission (Newman, 2003), as well as neuronal firing thresholds and plasticity (Nedergaard et al., 2003). Astrocytes may also regulate synaptogenesis. Neurons co-cultured with astrocytes have been shown to develop approximately seven-fold more synapses, with enhanced synaptic efficacy (Pfrieger and Barres, 1997; Ullian et al., 2001). In turn, evidence has identified modulation of microvascular endothelium and/or associated astrocytes through GABA-ergic (Rancillac et al., 2006; Vaucher et al., 2000), cholinergic (Hamel, 2004; Tong and Hamel, 1999), serotonergic (Cohen et al., 1996), and noradrenergic neurons (Cohen et al., 1997). The implication of a dynamic equilibrium existing between neurons and the other components of the NVU has significant repercussions with regard to ischemic stroke treatment and recovery.

Tight junctions

Much of what is currently understood regarding endothelial TJs has been derived from epithelial cell examinations, owing to the significant degree of structural and functional similarities. Nevertheless, BBB endothelial TJs hold many unique attributes, which may be more appropriately correlated with the endothelium of other systems when evaluating dynamic regulation and paracellular permeability. Thus, it should be understood that much of the current theory regarding BBB TJ regulation is based on data from different cells lines, research approaches/techniques, researcher interpretation, and the multiple variables associated with respective pathophysiological events.

TJs are the most apical structure within the intercellular cleft, limiting the paracellular flux of hydrophilic molecules across the BBB. TJs, along with adherens junctions, form a circumferential zipper-like seal between adjacent endothelial cells, maintaining distinct tissue spaces through separation of the luminal side from the abluminal side of the plasma membrane (Fig. 1B). Under normal physiological conditions, substances with a molecular weight greater than 180 Da do not gain access through the TJs (Mitic and Anderson, 1998). Yet, the sealing properties of TJs can vary between brain endothelial cells of different locations. For example, as endothelial capillaries proceed to post-capillary venules a reduced sealing capacity is observed (Simionescu et al., 1976).

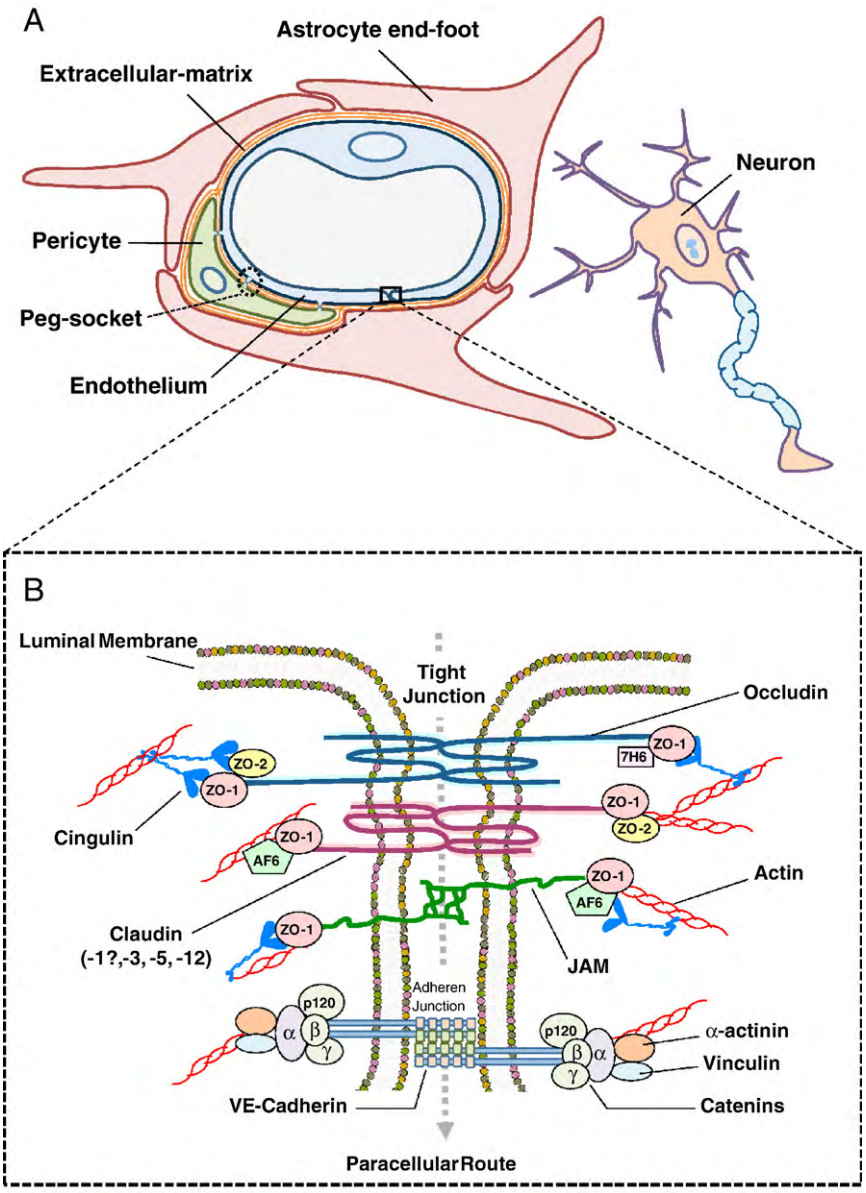
The preservation of the TJ is governed by three essential transmembrane proteins: claudins, occludin, and junction adhesion molecules (JAMs). The TJ proteins can exist in various isoforms and phosphorylation states, respective to their tissue origin and regulatory activity. The cytoplasmic regions of these transmembrane proteins are attached to intracellular scaffolding proteins, which in turn are anchored to the actin cytoskeleton. Generally, movement of these proteins away from the cellular borders or decreases in their expression at the TJ cleft indicates a loss of junctional integrity and increased paracellular permeability.

TJ protein regulation is dependent upon cellular localization, post-translational modification, and protein–protein interactions. Although alterations in specific phosphorylatable residues (i.e. serine, threonine, tyrosine) of the TJ proteins have been correlated to changes in protein interactions and functional state of the TJ complex, current understanding indicates greater complexity. Merely identifying the predominate residue or change in residue ratios on specific TJ proteins cannot independently define junctional integrity or a specific protein's contribution to TJ assembly/disassembly. Even more critical in regards to understanding TJ permeability during pathophysiological events, is the identification of the principle molecular pathways involved. The dynamic nature of BBB TJs implicates significant time-dependent inter-regulation of such pathways.

Occludin

Occludin is a tetraspanning membrane protein found in high concentrations at BBB TJs (Fig. 1C). It is a ~60–65 kDa phosphoprotein that forms dimers which interact homophilically, having two extracellular loops separated by a short cytosolic loop with both amino- and carboxy-terminal domains within the cytosol (Feldman et al., 2005). Although, occludin has been shown not to be required for the formation of TJs (Saitou et al., 1998), evidence consistently identifies occludin as a critical regulatory protein. With this understanding, occludin may act as a primary shock-absorber, mediating TJ responses to acute changes in vascular dynamics (e.g. rCBF, inflammation). The presence of occludin in the membrane is correlated with increased electrical resistance across the membrane and decreased paracellular permeability (Balda et al., 1996). The carboxy-terminus portion, that encodes for a putative coiled-coiled domain, can bind with several proteins that influence its regulatory actions (e.g. protein kinase-C (PKC), c-Yes, connexin-26, and p85 (regulatory subunit of PI3-kinase)) (Nusrat et al., 2000). Occludin's carboxy-terminal binds to zonula occludens (ZO-1, ZO-2, and ZO-3), which in turn binds to the actin cytoskeleton, localizing it to the cellular membrane (Fanning et al., 1998; Furuse et al., 1994; Haskins et al., 1998). The phosphorylation state of occludin has been proposed to regulate its association within the cell membrane at the TJ, dependent on the form and localization of the residue (Feldman et al., 2005). Heavily phosphorylated occludin (i.e. high molecular weight form), has been shown to be concentrated at the cellular membrane, and identified with an intact TJ (Sakakibara et al., 1997; Wong, 1997). Whereas, the less phosphorylated form of occludin (i.e. low molecular weight form) has been identified with the cytoplasmic fraction (Sakakibara et al., 1997; Wong, 1997), and may potentially serve as a pool of reserve protein. Occludin localized within the membrane has been shown to correlate with serine and threonine phosphorylation (Andreeva et al., 2001). In contrast, tyrosine phosphorylation of occludin has been identified with its disassociation from intracellular proteins (ZO-1, ZO-2, and ZO-3) and increased TJ permeability (Kago et al., 2006; Kale et al., 2003; Rao et al., 2002). Nevertheless, many discrepancies in phosphorylation activities arise with different cell types, stimuli, and means of assessment. There is also evidence that trafficking of occludin, as well as claudin-5, to the tight junctional cleft, may be mediated through protein oligomerization at the level of the plasma membrane (Blasig et al., 2006; McCaffrey et al., 2008; McCaffrey et al., 2007). Such mediation would in theory allow for a more fluid control of the TJs. How phosphorylation processes may contribute to such protein oligomerization remains unclear.

Fig. 1. Blood-brain barrier (BBB) neurovascular-unit (NVU). (A) Transverse-section representation of the NVU. The circumference of the capillary lumen is surrounded by endothelial cells, which are connected via tight junctions (TJs). Endothelial cells and pericytes are ensheathed by a common basement membrane. Astrocyte end-feet surround endothelial cells and pericytes, with neuronal signaling also mediating capillary function. (B) Schematic blow-up of the inter-endothelial TJs and integral proteins involved in paracellular regulation, as defined in the text. (C) Immunofluorescent image of a fixed rat brain microvessel stained for occludin (×60), identifying TJ contacts.


A
Astrocyte end-foot
Extracellular-matrix
Neuron
Pericyte
Peg-socket
Endothelium
B
Luminal Membrane
Tight Junction
Occludin
ZO-2
ZO-1
7H6
ZO-1
Cingulin
ZO-1
ZO-2
AF6
ZO-1
Actin
AF6
Claudin (-1?,-3,-5,-12)
ZO-1
JAM
Adheren Junction
p120
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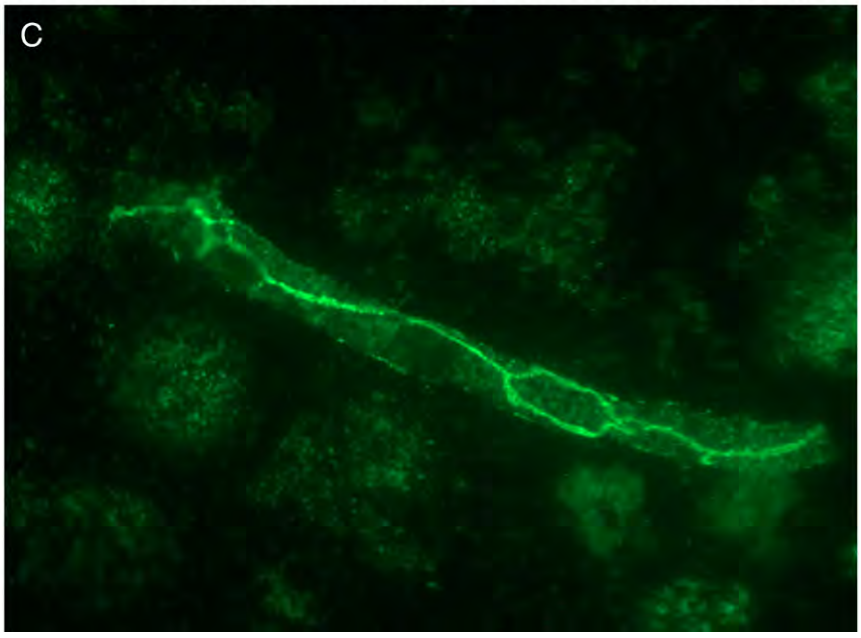
Vinculin
VE-Cadherin
Catenins
Paracellular Route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Claudins

Claudins are a family of proteins with at least 24 members, which contribute to the formation of the TJs. Claudins have two extracellular loops that interlink with claudins of adjacent endothelial cells, forming the primary seal of the TJs (Piontek et al., 2008). Compared to occludin, claudins are smaller (20–24 kDa) and display no sequence homology with occludin. The two internal loops of the claudins have been shown to bind to ZO-1, ZO-2, and ZO-3 via their carboxy-terminals (Itoh et al., 1999; Ruffer and Gerke, 2004). Of the claudin family, claudins-3, -5, and -12 have been identified to be present within BBB endothelial cells (Hawkins and Davis, 2005). Claudin-1 has also been shown to be present, however variability appears to exist between *in vitro* vs. *in vivo* assessments and species evaluated (Witt et al., 2003; Wolburg et al., 2003), thus remaining controversial. Evidence indicates that claudin-5 is specifically involved in the active regulation of small molecule paracellular permeability at the BBB. Drugs that increase claudin-5 expression have been shown to increase transendothelial resistance and decrease BBB permeability (Honda et al., 2006). Furthermore, mice lacking the claudin-5 gene, show a loss of BBB integrity, to where molecules of less than 800 Da had an increased brain uptake (Nitta et al., 2003). Phosphorylation pathways may regulate claudin-5 activity at the TJs. Phosphorylation of claudin-5 at Thr207, via the protein kinase-A (PKA) (Soma et al., 2004), as well as through Rho kinase activation (Yamamoto et al., 2008), has been identified with an increased TJ permeability.

Junctional adhesion molecules

JAMs are a family of immunoglobulin superfamily proteins (~40 kDa) that localize within the intercellular cleft of TJs, yet are also expressed on leukocytes and platelets. JAMs participate in the assembly and maintenance of the TJs, signaling of cytoskeletal-associated proteins, and leukocyte diapedesis (Weber et al., 2007). Several JAM proteins have been identified: JAM-A (a.k.a. JAM, JAM-1, F11R), JAM-B (a.k.a. JAM-2, human-JAM-2, mouse-JAM-3, VE-JAM), JAM-C (a.k.a. JAM-3, human-JAM-3, mouse-JAM-2), and most recently JAM-4 and JAML (JAM-like, a.k.a. AMICA1). JAMs have a single transmembrane domain and their extracellular segment has two immunoglobulin-like loops that are formed by disulfide bonds. Evidence indicates that JAMs interact between cells in a homophilic and heterophilic manner (Weber et al., 2007). JAMs primary intracellular binding partners include ZO-1, afadin (AF-6), partitioning defective protein-3 (PAR-3) and multi-PDZ-protein-1 (MUPP-1) (Ebnet et al., 2003). JAMs-A, -B, and -C have been shown in endothelial cells, with JAM-A shown to be highly expressed in the cerebrovasculature. Homophilic JAM-A interactions have been shown to stabilize cellular junctions and decrease paracellular permeability (Liu et al., 2000; Mandell et al., 2004). Additionally, loss of BBB TJ integrity correlates with decreased JAM-A expression (Yeung et al., 2008).

Cytoplasmic accessory proteins

Cytoplasmic proteins involved in BBB TJ formation and regulation include ZO-1, ZO-2, cingulin, 7H6, and AF-6 (Hawkins and Davis, 2005), although others may likely exist. ZO-1 (220 kDa) and ZO-2 (160 kDa) are phosphoproteins and members of the membrane-associated guanylate kinase-like family of proteins, which are capable of forming heterodimeric complexes with one another. ZOs contain three PDZ domains (PDZ1, PDZ2, and PDZ3), one SH3 domain, and one guanylyl-kinase-like domain. These domains act as protein binding molecules, organizing proteins at the plasma membrane. ZO-1 interacts with both ZO-2 and ZO-3, via the PDZ domains (Wittchen et al., 1999), although ZO-3 has not been verified in BBB endothelium. ZO-2 may function redundantly with ZO-1, replacing it and facilitating formation of a morphologically competent TJ (Umeda et al., 2004). The proline-rich carboxy-terminus of the ZOs mediates binding to actin *in vitro*, and is believed to serve as the link to the actin cytoskeleton (Fanning et al., 1998; Wittchen et al., 1999). In this manner, ZO proteins serve as recognition proteins for TJ placement and act to connect and anchor the transmembrane proteins to the actin cytoskeleton. It has also been hypothesized that ZOs are responsible for recruitment of the transmembrane TJ proteins to their final destination within the apical portion of the cellular membrane (Bazzoni and Dejana, 2004; Tsukita et al., 2001).

Several other TJ accessory proteins have been identified, yet their structural and regulatory roles have yet to be clearly elucidated with regard to endothelial cells of the BBB. Cingulin (140–160 kDa) is a phosphoprotein localized to the cytoplasmic surface of the TJs, and has been shown to bind to the ZO proteins, myosin, JAM-A, and AF6 (Bazzoni et al., 2000; Cordenonsi et al., 1999), implicating cingulin as an important scaffolding protein of the TJs. Cingulin has also been suggested to transduce the mechanical force generated by the contraction of the actin–myosin cytoskeleton, regulating TJ permeability (Cordenonsi et al., 1999). 7H6 (155 kDa) is a phosphoprotein that reversibly dissociates from the TJ under conditions of adenosine triphosphate (ATP) depletion, associated with increased paracellular permeability (Satoh et al., 1996; Zhong et al., 1994). The AF-6 (180 kDa) protein participates in the regulations of tight junctions, via direct interaction with ZO-1 (Yamamoto et al., 1999). To date, several other accessory proteins found in epithelial and peripheral endothelial cell TJs have been implicated as potential mediators of paracellular regulation (e.g. junction-associated coiled-coil protein (JACOP), calcium-dependent serine protein kinase (CASK), regulator of G-protein signaling-5 (RG-5)) (Hawkins and Davis, 2005; Zlokovic, 2008), yet confirmation of their existence or activity within BBB endothelial cells is presently lacking.

Actin

While not traditionally defined as a TJ protein, actin (42 kDa) plays an active role in TJ regulation, and as such is an integral part of the TJ complex. Studies have demonstrated an essential role of actin fibers in the stabilization of the BBB TJ (Lai et al., 2005). Unsequestered globular actin polymerizes into a filamentous-actin (F-actin) form, via an ATP-favored process (Atkinson et al., 2004). The dynamic actin filaments provide the cytoskeletal infrastructure necessary for maintenance of cell morphology and function. A prominent band of F-actin is localized at the apical portion of the cell, anchoring the spatial orientation of the junctional proteins. The typical morphological pattern of F-actin associated with elevated endothelial TJ permeability is increased stress fiber density in conjunction with a reduction or loss of actin banding. Actin redistribution and polymerization to form stress fibers has been shown with inflammatory agents, oxidative stress, and neutrophils, in association with increased BBB TJ permeability (Hixenbaugh et al., 1997; Korthuis et al., 1991; Lum and Roebuck, 2001).

Adherens junctions

The adherens junctions (AJ) form a continuous belt localized near the apical end of the junctional cleft, just below the TJ. While the TJs are identified as the primary paracellular barrier, AJs appear to play a key role in the localization and stabilization of the TJs (Dejana et al., 2008). The AJs assemble via homophilic interactions between calcium-dependent cadherins. The extracellular binding domain of cadherins is itself thought to be insufficient to promote formation of junctions. The cytoplasmic domains of the cadherins bind to the plaque proteins β-catenin, γ-catenin, and p120-catenin, which are linked to the actin cytoskeleton via α-catenin (Dejana et al., 2008). However, some question remains as to the presence of γ-catenin at the human BBB (Vorbrodt and Dobrogowska, 2004). Additionally, p120-catenin which was first identified as a substrate for Src-tyrosine

receptor kinase (Reynolds et al., 1989), has been subsequently shown to be a key component in the stabilization of E-cadherin at the plasma membrane (Hartsock and Nelson, 2008). Other key AJ components have been demonstrated in rat BBB microvessels, including vinculin and α-actinin (Ballabh et al., 2004). Although the impact of AJ on BBB paracellular permeability during pathological events remains to be elucidated, AJs have been shown to interact with the vascular endothelial growth factor receptor-2 (VEGFR-2) (Lampugnani et al., 2006), implicating their importance during angiogenic processes. Interestingly, recent evidence has also shown vascular endothelial (VE)-cadherin mediated upregulation of claudin-5 (Taddei et al., 2008), suggesting a direct regulation of TJ integrity by AJ proteins.

Plasma membrane

Although often overlooked, the plasma membrane is critical for the trafficking, positioning, and inter-regulation of the TJ proteins. The TJ proteins are associated with cholesterol-enriched regions of the plasma membrane (Kachar and Reese, 1982; Nusrat et al., 2000), with TJ permeability capable of being modulated via cholesterol (Lambert et al., 2005; Stankewich et al., 1996). Distinctive sphingolipid- and cholesterol-enriched regions of the plasma membrane (i.e. lipid-rafts) have become recognized as critical sites of protein and lipid trafficking, as well as signal transduction (Helms and Zurzolo, 2004; McCaffrey et al., 2007; Pike, 2005). The tightly packed lipids within the lipid-rafts promote protein oligomerization (Latif et al., 2007). Recent advancements in membrane fractionation of the lipid-raft domain have identified oligomeric occludin and claudin-5 within rat brain microvessels, associated with the tight junctional cleft (McCaffrey et al., 2007). This work suggested that the oligomerization of occludin involved disulfide-bond formation within the transmembrane regions, and that the assembly of the TJ oligomeric protein complex is facilitated by an oligomeric caveolin scaffold (McCaffrey et al., 2007). In a subsequent examination, increased BBB paracellular permeability via peripheral inflammation was associated with the disruption of the occludin disulfide-bonded oligomeric assembly (McCaffrey et al., 2008). These data emphasize the importance of the plasma membrane in the regulation of BBB TJ permeability.

Ischemic stroke impact on BBB permeability and tight junction regulation

Stroke ultimately involves the destruction and/or dysfunction of brain cells, leading to clinically definable neurological deficits. Ischemic stroke consists of two distinctive periods of pathological impact, ischemia and reperfusion. Both ischemia and reperfusion can be further delineated into a series of interdependent biochemical and cellular events that evolve over minutes to days. With this understanding, BBB TJ alterations can be divided into time-dependent phases, based on states of paracellular permeability over the time-course of I/R. Furthermore, delineating the phasic divisions allows for more appropriate reference and categorization of *in vitro* observations.

Ischemia

The ischemic phase of stroke is denoted by a loss of rCBF and increased vascular resistance owing to mechanical plugging of a vessel via a thrombus or emboli, resulting in loss of oxygen and nutrients to the surrounding tissue. The endothelium at the epicenter of an infarct, representing the core ischemic zone, sustains the greatest degree of insult and likelihood for TJ dysregulation and disassembly. Nevertheless, measurable changes in BBB TJ permeability during focal ischemia may not occur immediately, requiring hours of continuous reduction in blood-flow to induce an observable increase in paracellular permeability (Betz, 1996). The TJs within the core zone will have a differing time-frame of response in relation to the endothelium of the surrounding tissue (i.e. ischemic penumbra). Therefore, when evaluating the impact of an I/R event, it is with the understanding that the effected endothelial cells, as well as other components of the NVU, with seemingly marginal spatial separation may likely be undergoing a series of different molecular/cellular events, phasic responses, and degrees of TJ permeability.

Loss of blood supply to the brain brings about a cascade of events throughout the infarcted region including: depletion of ATP, excitotoxic glutamate efflux (neuronal component), ionic imbalance (e.g. increased intracellular calcium), loss of metabolic function with increased acidosis, oxidative stress, and activation of inflammatory processes. These mechanisms demonstrate over-lapping and redundant features. Within minutes to hours of ischemic onset, the internal capillary diameter shrinks, due to endothelial swelling. Lactacidosis, caused by an intracellular accumulation of lactic acid due to anaerobic metabolism, directly contributes to swelling of endothelial cells, neurons, and astrocytes. Furthermore, induction of proteases (i.e. tissue plasminogen activator (tPA), matrix-metalloproteinases (MMPs), cathepsins, and heparanases) contribute to BBB ECM degradation. Such enzyme induction may further perpetuate BBB TJ permeability and anoikis (i.e. apoptosis induced by detachment of cells from the ECM) through integrin mediated mechanisms (Grossmann, 2002; Mannello et al., 2005).

Other activities with the onset of ischemia are strongly associated with downstream endothelial responses. Within minutes of the occlusion, there is an increased expression of early response genes (e.g. c-*jun*, c-*fos*), later (hours) followed by an increase in heat shock genes (e.g. Hsp70, Hsp72) (Hoehn et al., 2001; Weinstein et al., 2004). Within the penumbra, apoptotic pathways are induced through both caspase-dependent (ATP-dependent) and caspase-independent mechanisms. The human brain expresses several proteins (e.g. caspases-1,3,8,9, death receptors, apoptotic protease-activating factor-1 (Apaf-1), p53), capable of inducing apoptosis in the I/R brain (Lo et al., 2003). Proinflammatory cytokines (e.g. interleukin-1, tissue necrosis factor-α (TNFα)) are induced, followed by chemokines (e.g. monocyte chemoattractant protein-1 (MCP-1), cytokine-induced neutrophil chemoattractant (CINC)) associated with an activated endothelium (Huang et al., 2006). As the expression of cytokines and adhesion molecules precedes leukocyte infiltration of the ischemic brain, these cytokines are primary initiators of endothelial inflammatory activation and subsequent leukocyte extravasation. Leukocytes and reactive microglia are recruited to the ischemic brain further enhancing inflammatory activity and toxic free radical production (Huang et al., 2006; Lo et al., 2003). Although microvascular leukocyte accumulation is generally associated with the delayed responses for the purposes of local debris removal and scar formation in post-infarcted regions, it has also been shown to occur as early as 30 min after permanent middle cerebral artery occlusion (MCAO) in rats (Dereski et al., 1993; Huang et al., 2006).

Beyond specific mediators, addressed subsequently, some general understanding of the TJ protein activity during the ischemic period can be deduced. *In vitro* hypoxic conditions have shown to alter the localization of claudin-5 in the plasma membrane and the expression of claudin-5 protein in bEND.3 cells, accompanied by a decrease in transendothelial electrical resistance (Koto et al., 2007). Claudin-5 expression, in *ex vivo* retinal microvascular endothelial cells, has also been shown to be reduced under hypoxic conditions (Koto et al., 2007). *Ex vivo* examination of cerebral ischemia identified a decrease in occludin and ZO-1 after microsphere-induced cerebral embolism (Kago et al., 2006). In another *in vitro* assessment, hypoxia increased paracellular permeability along with the disruption of occludin, ZO-1, and ZO-2 membrane localization (Mark and Davis, 2002). Thus, the observed increases in paracellular permeability generally correlate with the loss of TJ protein localization and/or expression along the cellular membrane. Additionally, localization of ZO-1 and ZO-2 expression has been shown to shift to the nucleus during *in vitro*

hypoxia, concurrent with increased paracellular permeability (Fischer et al., 2004). Interestingly, ZO-1 has been reported to appear in the nucleus during cellular proliferation (Gottardi et al., 1996) and calcium depletion (Riesen et al., 2002), and binds to the adherens junction protein β-catenin during early stages of junctional development (Rajasekaran et al., 1996). In non-hypoxia based experiments, ZO-2 has been shown to shuttle from its cytoplasmic domain to the nucleus (Islas et al., 2002), where it associates with transcription factors Jun, Fos, and C/EBP (Betanzos et al., 2004). Furthermore, recent evidence has shown nuclear ZO-2 to alter gene expression and stability of epithelial and endothelial junctions, with associated increased proliferative activity (Traweger et al., 2008). Although speculative at this time, such migration during I/R may contribute to the initiation and/or progression of downstream angiogenesis, given the association of ZOs with cellular proliferation and junctional development.

Reperfusion

Reperfusion is denoted by the reestablishment of CBF to the ischemic and hypoperfused brain. Although reperfusion is absolutely necessary for tissue survival, it also contributes to additional tissue damage, and the potential for hemorrhagic transformation (i.e. phenomenon in which blood vessels weakened by ischemic stroke rupture to cause brain hemorrhage). Upon reperfusion, three phases of increased BBB TJ opening/paracellular permeability may occur. There is an initial reperfusion permeability associated with acute elevations in rCBF, which is then followed by a "biphasic" permeability response (Fig. 2). The multi-phasic nature of this permeability is dependent upon multiple factors, including the duration of ischemia, degree of reperfusion, and form of animal stroke model used for assessment.

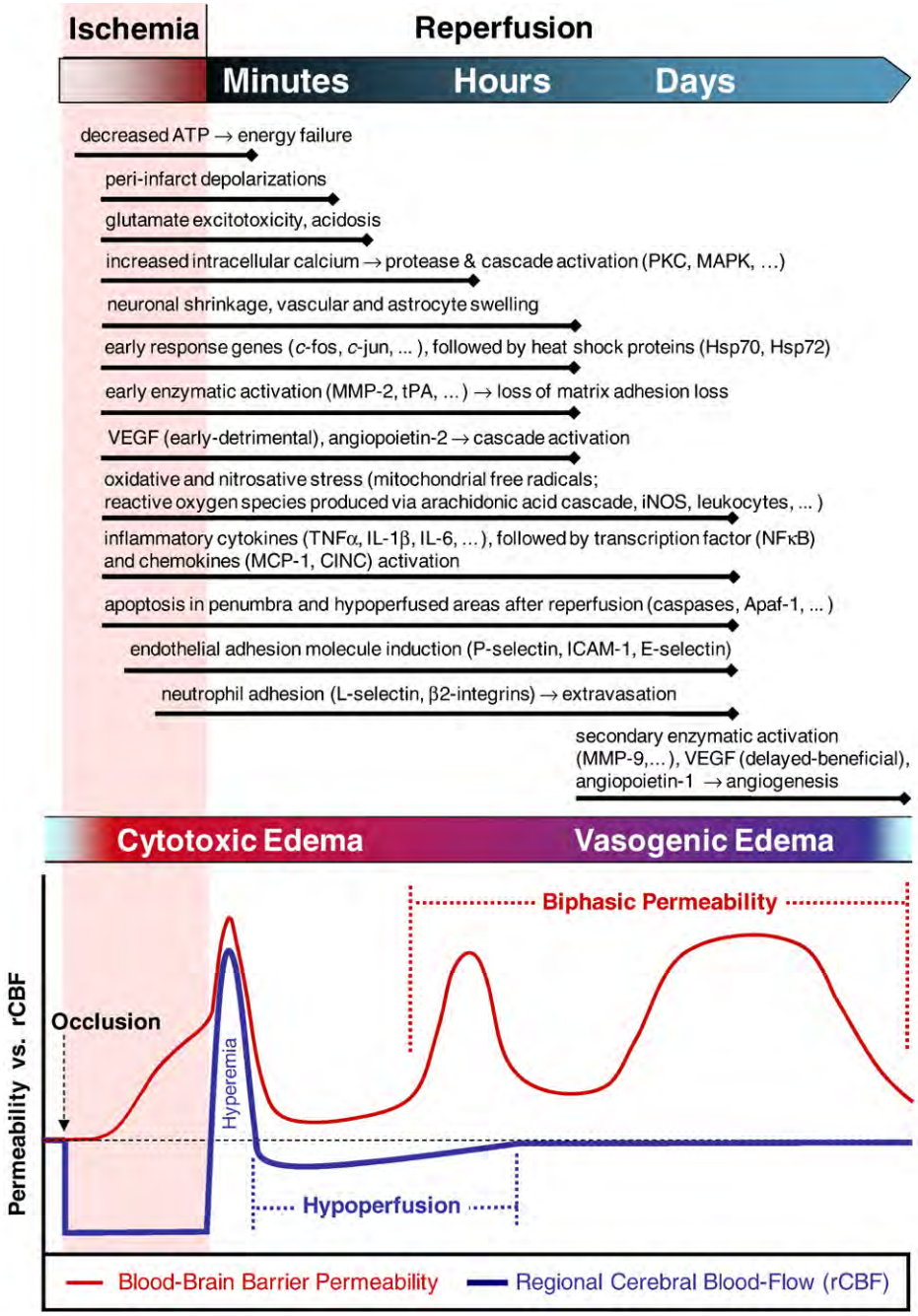

Fig. 2. Schematic of blood-brain barrier phasic events associated with cerebral ischemia and reperfusion time-course, as defined in the text. Variability occurs in time-frame of mediators identified, dependent upon tissue distance from ischemic core and duration of ischemic insult.

One of the initial studies showing this multi-phasic I/R permeability, using a cat MCAO model, identified the initial hyperemia phase followed by a "refractory" period, then a biphasic increase in paracellular permeability at 5 and 72 h respectively (Kuroiwa et al., 1985). In a model of global ischemia, the multi-phasic response was dependent upon the brain region and the severity of insult; with an acute increase in permeability immediately upon reperfusion, followed by a later biphasic increase (Preston et al., 1993). The full biphasic response only occurred when the ischemic insult time was extended or when coupled with hyperthermia (Preston et al., 1993). Other animal studies have consistently identified such phases, with the time-profiles dependent upon the severity and form of ischemic insult (Belayev et al., 1996; Huang et al., 1999; Kuroiwa et al., 1985; Preston et al., 1993; Rosenberg et al., 1998; Witt et al., 2008). Neutrophil extravasation time-frame is also dependent upon severity of insult. Peak neutrophil infiltration has been shown to occur at 6 and 48 h following a transient MCAO (2 h), while 12 and 72 h after a permanent MCAO (Zhang et al., 1994). The correlation between neutrophil infiltration into the brain parenchyma and the overlying time-frame of the biphasic paracellular permeability responses, underscores the inflammatory component in I/R related BBB alterations.

Reactive hyperemia and loss of cerebral autoregulation upon initial reperfusion account for the acute opening of the BBB TJs. Thus, this acute phase is passively dependent on perfusion, and is often concurrent with a sharp increase in blood-pressure (Spengos et al., 2006). While disassembly of the TJs during hyperemia would also be passive in nature, the manner of TJ reassembly is less clear. The TJ reassembly would primarily encompass endothelial, pericyte, and ECM interactions, accompanied by reestablishment of autoregulatory responses. Cerebral pericytes, containing contractile proteins, have the potential to regulate CBF (Bandopadhyay et al., 2001; Peppiat et al., 2006). Thus pericytes would theoretically act to reestablish the TJs through contractile actions upon the capillary. The ECM, acting to support and anchor the capillary endothelium, contains collagen organized for enhanced mechanical resistance. As such, the ECM would allow for an elastic-type rebound of the capillary. Beyond such mechanical means of reducing paracellular permeability, inter-endothelial TJ proteins would still need to appropriately realign and reestablish connectivity. The overall impact of acute hyperemia on subsequent phases of paracellular permeability has not been determined.

Following the initial hyperemia, hypoperfusion of the ischemic area occurs (i.e. no-reflow effect), resulting in a deficiency of nutritional support necessary for a sustainable recovery of the tissue. This hypoperfusion has been attributed to multiple factors, including continued cerebral metabolic depression, microvascular obstruction, occlusion via endothelial and astrocytic end-feet swelling, and the formation of endothelial microvilli (Iadecola, 1998). Hypoperfusion may also enhance neutrophil adhesion and subsequent inflammatory activity within the most susceptible tissues, as well as directly contribute to the next period of increased BBB paracellular permeability (i.e. first phase of the biphasic permeability, at ~3–8 h post-reperfusion). The first phase of the biphasic permeability has been attributed to increased inflammatory and oxidative stress on the BBB, in conjunction with enzymatic degradation of the ECM (Heo et al., 2005; Wang and Shuaib, 2007). Furthermore, the no-reflow effect appears to be more significant with extended periods of ischemia or if the ischemia is associated with venous obstruction (Hossmann, 1993). As increasing the time period of ischemia correlates with increased edema and potentiation of the final phase of the biphasic permeability (Preston et al., 1993), this would implicate the no-reflow effect as a mediator of both the initial and final period of the biphasic TJ permeability. Although direct evaluation of the no-reflow effect on BBB TJ proteins has not been conducted, the numerous inflammatory and oxidative processes corresponding to this period greatly impact TJ regulation.

Angiogenesis and increased vasogenic edema coincide with the final phase of the biphasic BBB TJ permeability,~18–96 h after reperfusion, dependent on ischemic severity and brain region evaluated (Belayev et al., 1996; Huang et al., 1999; Kuroiwa et al., 1985; Preston et al., 1993; Rosenberg et al., 1998) However, neurovascular remodeling may continue weeks after an I/R event (Strbian et al., 2008; Zhao et al., 2006). Upstream inflammatory activity has also been shown to be a contributor to this final phase, as mitigating inflammatory processes has been shown to reduce BBB TJ permeability (Dimitrijevic et al., 2007; Yang et al., 1998). During this final biphasic period, the TJs go through a regulated period of disassembly and assembly. Reperfusion disassembly and assembly exist as a ratio over time, correlated to increased and decreased paracellular permeability, respectively. In theory, assembly of the TJs may consist of reassembly between existing endothelial cells or new-assembly in conjunction with new cell growth. While TJ reassembly predominates after the initial hyperemia and the initial period of biphasic permeability; both reassembly and new-assembly would occur during the final phase of the biphasic permeability, in coordination with angiogenesis. As such, the processes involved in TJ assembly, as well as disassembly, may themselves be further delineated (Table 1).

It should also be emphasized that the periods between the phasic increases in permeability are not necessarily a reestablishment of normal TJ integrity, as they do not appear to reach true basal levels of paracellular impermeability. Furthermore, the decline in BBB permeability between the initial and final biphasic periods brings about additional questions. As the enzymatic degradation of the ECM appears to be a primary mediator of the initial biphasic period, one would not expect the subsequent decline in BBB permeability; as the ECM would still be in the process of degradation even if endogenous levels of enzymes decreased. Additionally, although inflammatory and oxidative processes are strongly implicated with TJ opening during the initial biphasic response, both continue after the subsequent decline

Table 1
Proposed ischemia–reperfusion phase responses of blood-brain barrier tight junctions (TJs)

	Response	Mode	Overlying mediator
Ischemic phase	Disassembly	Secondary	Ischemia (multi-factorial)
Reperfusion			
Hyperemia phase (min)	Disassembly	Secondary	Perfusion pressure
	Reassembly	Secondary–primary	Perfusion pressure — rebound
Biphasic — initial period (~3–8 h)	Disassembly	Secondary–primary	Inflammatory/oxidative/enzymatic
	Reassembly	Secondary–primary	Inflammatory/oxidative/enzymatic — rebound
Biphasic — final period (~18–96 h)*	Disassembly	Secondary–primary	Angiogenesis — initiation
	Reassembly and new-assembly	Secondary–primary	Angiogenesis — stabilization

Primary mode indicates an endothelial derived induction of TJ-response; whereas, secondary mode indicates non-endothelial derived induction of TJ-response. Disassembly, reassembly, and new-assembly may be regulated through different molecular pathways and TJ protein responses.

* Indicates the potential for an extended time-frame of TJ permeability and vascular remodeling, into weeks.

in BBB permeability. Whether the reduction in permeability between the biphasic periods is via a temporary reestablishment/reassembly of the TJs, shift in cellular swelling, or other mechanisms, has not been fully elucidated.

Edema

Edema is one of the primary causes of clinical deterioration, and a leading cause of death subsequent to I/R (Bounds et al., 1981; Davalos et al., 1999). There are two major types of edema associated with I/R, cytotoxic and vasogenic. Cytotoxic edema occurs soon after ischemic onset, and is caused by translocation of interstitial water into the intracellular compartment, in association with ionic and metabolic dysregulation. Sodium entry into the cell exceeds potassium loss, resulting in a net increase in cell ions and water (Betz et al., 1989; Young et al., 1987). All brain cells take in fluid and swell; however, there is not a significant increase in overall brain volume due to the corresponding reduction in extracellular space (Heo et al., 2005). Ultrastructural evidence has shown that astrocyte end-feet swell within 5 min after energy supply depletion (Dodson et al., 1977). Astrocyte swelling appears to persist 24 h after cerebral ischemia, followed by necrosis (Garcia et al., 1994). Neurons appear to swell later and shrink earlier than astrocytes (Heo et al., 2005). In a study by Garcia et al., following an MCAO in rats the first significant increase in necrotic neurons (15%) was observed within the territory of the occluded artery after 6 h, with most neurons (65%) becoming necrotic at 12 h (Garcia et al., 1995b). Endothelial cells first show nuclear swelling, with moderate swelling in cytoplasm within the first 2 h (Garcia et al., 1994). Furthermore, astrocyte swelling contributes to the detachment of the end-feet from the endothelium (del Zoppo and Hallenbeck, 2000; Kimelberg, 2005). Loss of integrin adhesion molecule expression, including $\alpha1\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ on the endothelium and $\alpha6\beta4$ on astrocytes, corresponds with detachment of astrocytic end-feet 2 h after an MCAO (Tagaya et al., 2001). Although cytotoxic edema is generally considered to be independent of BBB TJ alterations, such loss of the endothelium adhesion to the astrocytic end-feet may itself be sufficient to induce TJ dysregulation.

In contrast to cytotoxic edema, vasogenic edema is directly associated with alterations of the BBB TJs, with increasing permeability to macromolecules allowing fluid movement from intravascular to extravascular spaces (Heo et al., 2005). Vasogenic edema increases overall brain volume and has a propensity to appear in white matter over gray matter, owing to the greater compliance of the extracellular space within white matter (Ayata and Ropper, 2002). In humans, vasogenic edema usually peaks at 2–5 days after ischemic stroke (Heo et al., 2005; Schlaug et al., 1997), which correlates well to rodent ischemic stroke modeling. Yet, it should be emphasized that the human brain has a much greater proportion of white matter, as compared to rodent brains (Miller et al., 1980; Underhill et al., 2002), and white matter involvement in stroke often varies along these lines. Although inflammatory and oxidative processes contribute to this period of vasogenic edema, it should again be noted that the increased paracellular permeability is principally a default of the angiogenesis. As the brain is striving to recover and remodel its vascular network, BBB TJs are in the midst of disassembly and assembly. Yet the remodeling process does not occur in a Petri-dish, rather it does so under fluctuating perfusion pressures and significant shear stresses pulling on the endothelium and enhancing TJ opening.

Pathways of mediation

Although several factors have been identified in the regulation of BBB TJ permeability, no single molecular/cellular pathway independently predominates over the course of an I/R event. This is to be expected, given the complexity of events surrounding the different phases of TJ permeability. Nevertheless, a certain inter-relation of mechanisms directing these alterations has been identified.

Phosphorylation and permeability

Phosphorylation is a major regulatory mechanism for both transmembrane and accessory TJ proteins, and as such may provide the means for understanding the regulated aspects of BBB paracellular permeability during I/R. Multiple phosphorylation processes have been implicated, dependent upon the criteria examined and timeframe of events. With this consideration, it is first necessary to address the principle intracellular mediators involved in these processes.

The actin–myosin interaction has been identified in endothelial cell paracellular permeability, primarily governed by phosphorylation status of the myosin light chain (MLC). The calcium/calmodulin-dependent MLC-kinase (MLCK) phosphorylates the MLC, resulting in an actin–myosin contraction (Garcia et al., 1995a; Goeckeler and Wysolmerski, 1995). Constitutive MLCK phosphorylation of the MLC in epithelial cells has been shown to increase TJ permeability accompanied by cellular redistribution of F-actin, ZO-1 and occludin (Shen et al., 2006). Inhibition of the MLCK has been shown to prevent hypoxia-induced disruption and permeability in bovine brain microvascular endothelial cells (BMECs) (Kuhlmann et al., 2007). Furthermore, MLCK-dependent MLC phosphorylation and cytoskeletal contraction plays an important role in inflammatory associated permeability. The phosphorylation of MLC has been identified in endothelial barrier modulation via histamine, cytokines, oxygen radicals, thrombin, and neutrophils (Tiruppathi et al., 2002), all of which are critical mediators of ischemic stroke outcomes.

Despite a limited effect on basal barrier properties, protein kinase-C (PKC) may be a key regulator of BBB permeability under I/R associated stress conditions (Fleegal et al., 2005). PKC inhibitors have been shown to reduce endothelial cell permeability induced through multiple mediators (e.g. thrombin, bradykinin, VEGF, hydrogen peroxide, platelet-activating factor, and neutrophils) (Harhaj and Antonetti, 2004; Yuan, 2002). PKC has been reported to activate endothelial contraction by inducing MLC phosphorylation, actin polymerization, and activation of actin-binding proteins and intermediate filaments (Garcia et al., 1995a; Stasek et al., 1992). The endothelial contractile response has also been shown to occur via PKC-dependent activation of the Rho pathway (Mehta et al., 2001).

The calcium-dependent PKC isoforms (conventional: α, βI, βII, and γ) are likely candidates for the alteration of TJs, although other PKC isoforms (ε, ζ, λ, θ, and δ) are also implicated in the regulation of endothelial TJs. Increased expression of membrane bound PKCα, βI, and ε have been shown with hypoxia/aglycemia in BMECs (Yang et al., 2006), and increased expression of PKC βII and γ during hypoxia coincide with increased paracellular permeability in rat brain endothelial cells (Fleegal et al., 2005). Other studies have shown increased endothelial permeability resulting from hyperglycemia, ischemia, angiogenesis, or inflammatory stimulation through a PKCα dependent pathway (Aiello et al., 1997; Hempel et al., 1999; Mehta, 2001; Sandoval et al., 2001). PKCα has also been implicated in the disassembly of the adherens junctions, via VE-cadherin, resulting in increased paracellular permeability (Sandoval et al., 2001), supporting the hypothesis that adherens junctions may mediate tight junctional integrity. PKC (principally PKCβ) has also been implicated downstream of VEGF-receptor activation in association with angiogenic permeability (Harhaj and Antonetti, 2004). Lastly, increases in the expression of the calcium-dependent isozymes PKC-βII and PKC-γ, along with atypical PKC-ζ, can regulate nitric oxide synthase (NOS) (Banan et al., 2003), suggesting that PKC mediated changes of the TJs may be through nitric oxide (NO) activity.

Mitogen-activated protein kinases (MAPKs) are a family of serine–threonine kinases. MAPK pathways have been shown to be activated by a wide variety of different stimuli, including hormones, growth factors that act through receptor tyrosine kinases, MMPs, inflammatory

cytokines of the TNF family, and ischemic injury (Kyriakis and Avruch, 2001; Rosenberg, 2002). The most studied MAPKs in mammalian cells tend to be extracellular signal-related kinases (ERK 1/2), c-*jun*, N-terminal kinases (JNK) and p38-protein (Yuan, 2002). ERK 1/2 is closely associated with it role in cell growth, while p38 MAPK plays a significant role in cellular responses to stress and injury (Kyriakis and Avruch, 2001; Pearson et al., 2001), both of which may mediate permeability. Inhibition of ERK 1/2 after VEGF stimulation has been shown to block the VEGF-mediated increase in microvascular permeability (Lal et al., 2001). In addition to growth factors, several inflammatory agonists, including histamine, thrombin, hydrogen peroxide, and elevated intracellular calcium are able to phosphorylate ERK 1/2 (Fleming et al., 1995; Kevil et al., 2000; Verin et al., 2000; Wheeler-Jones and Pearson, 1995).

Increased levels of intracellular cyclic adenosine monophosphate (cAMP) are consistently associated with increased endothelial TJ integrity (Deli et al., 1995a; Hurst and Clark, 1998; Ishizaki et al., 2003). *In vitro* examinations of hypoxia/reoxygenation induced increases in microvascular permeability have shown to be reversed by cAMP (Seibert et al., 1992). The action of cAMP is most likely mediated through PKA. PKA has been reported to induce stabilization of cytoskeletal filaments (Hastie et al., 1997), dephosphorylation of MLC (Moy et al., 1993), dissociation of F-actin from myosin (Langeler and van Hinsbergh, 1991), and strengthening of cell-matrix adhesions (Lampugnani et al., 1990). PKA is also associated with inhibition of platelet aggregation and leukocyte adhesion (Granger and Kubes, 1994), which may provide an avenue for the mitigation of I/R inflammatory-induced BBB permeability.

Protein kinase-G (PKG) is a serine/threonine-specific protein kinase that is activated by cyclic guanosine monophosphate (cGMP). The cGMP-PKG pathway appears to have a variable impact on endothelial paracellular permeability. Examinations using endothelial cells from large vessels or non-exchange microvessels have shown that increased cGMP by guanylate cyclase activators or NO donors decreased endothelial permeability (Draijer et al., 1995a; Draijer et al., 1995b; Westendorp et al., 1994). Yet, several *in vitro* assessments, using microvessel endothelial cells, and *in vivo* studies have identified a NO-cGMP dependent increase in paracellular permeability in response to various factors associated with I/R, including bradykinin, histamine, NO, TNFα, platelet-activating factor, and VEGF (Fischer et al., 2004; Wong et al., 2004; Yuan, 2002). Additionally, in a brain endothelial cell model assessing hypoxic stress, increased paracellular permeability that was associated with a shift in ZO-1 and ZO-2 cellular localization from the cytoplasm to the nucleus was mitigated by inhibiting PKG (Fischer et al., 2002). It has been proposed that PKG and PKA act in a reciprocal manner in the mediation of microvascular permeability; with PKA playing a dominant role in the maintenance of basal barrier integrity, as well as countering PKG responses to inflammation (Yuan, 2002).

Protein tyrosine kinases (PTKs) are intracellular signal transduction molecules, as well as regulators of endothelial paracellular permeability. Tyrosine kinase agonists and tyrosine phosphatase inhibitors have been shown to affect phospho-tyrosine levels of ZO-1 and ZO-2, which correlate with increased permeability and redistribution of tight junctional components in epithelial and endothelial cells (Anderson and Van Itallie, 1995; Staddon et al., 1995; Takeda and Tsukita, 1995). Tyrosine phosphorylation of occludin has been shown to reduce its ability to bind to ZO-1, 2, and 3, resulting in increased paracellular permeability (Kago et al., 2006; Kale et al., 2003; Rao et al., 2002). Additionally, tyrosine phosphorylation promotes the dissociation of adherens junction proteins from their cytoskeletal anchors (Dejana et al., 2008), which may also contribute to TJ permeability.

PTKs may be divided into receptor mediated and non-receptor mediated forms. Receptor tyrosine kinases (e.g. VEGF-receptor family) are high affinity cell surface receptors for many growth factors, cytokines, and hormones. Receptor tyrosine kinases can activate several effector proteins and other kinases including PI3-kinase, phospholipase-Cγ (PLCγ), GTPase activating proteins, Src family protein tyrosine kinases, and the phosphoinositide-metabolizing enzymes (Bogatcheva et al., 2002; Schlessinger, 2000). Of the non-receptor tyrosine kinases, the Src family of kinases appears to have a critical regulatory role with regard to TJ permeability, especially as a downstream mediator of VEGF-induced angiogenesis (Eliceiri et al., 1999; Paul et al., 2001). Src has also been shown to mediate the increased endothelial permeability responses to TNF, IL-1β, and reactive oxygen species (ROS) (Akiyama et al., 2004; Kevil et al., 2001; Nwariaku et al., 2002). Additionally, phosphorylation of myosin MLCK by Src has been shown to lead to increased actin–myosin interaction and subsequent increased paracellular permeability (Birukov et al., 2001; Tinsley et al., 2004). Src family members have also been shown to be essential for the recruitment and activation of monocytes, macrophages, neutrophils, and other immune cells (Okutani et al., 2006). Src activation may serve as a common signal in the coordinating junctional disassembly, cytoskeletal contraction, and focal adhesion redistribution in response to inflammatory mediators.

Focal adhesion kinase (FAK) is another non-receptor protein tyrosine kinase, which may increase BBB TJ permeability through its role in endothelial contractile responses and actin polymerization. The attachment of the endothelial cells to the ECM is mediated by focal adhesions composed of transmembrane receptors (i.e. integrins). Evidence has implicated integrin-endothelial cell alterations, via the focal adhesion complex (FAC), in the regulation of endothelial permeability. The FAC-mediated endothelial contractile response has been associated with increased shear stress, growth factors, and inflammatory mediators (Girard and Nerem, 1995; Soldi et al., 1996; Wu et al., 2003). This response can be regulated through FAK. The activity of FAK has been shown to be regulated via Src family tyrosine kinases (Cox et al., 2006), although NO has also been shown to mediate its activity (Goligorsky et al., 1999). While little is known at this time regarding FAK as applied to BBB permeability under I/R stress, the interdependence of the endothelium and ECM implicates FAK as a significant contributor to TJ regulation, especially under conditions of angiogenesis (Avraham et al., 2003; Eliceiri et al., 2002; Okutani et al., 2006).

Calcium dysregulation

Calcium (Ca^{2+}) overload is primarily affiliated with neuronal excitotoxicity. Nevertheless, elevations of intracellular calcium also contribute significantly to I/R associated BBB alterations. Calcium dysregulation is concurrent with ischemia related energy loss, which carries over into the initial reperfusion period. As a critical second messenger, calcium regulates a variety of cellular functions, through multiple mediators (e.g. PKC, MAPK, phospholipase-A2). Calcium mediated activity can be delineated into rapid events, via direct protein interactions and protease activation, and slower events occurring via calcium regulated signaling cascades and activation of transcription factors (Brown and Davis, 2002; Seta et al., 2004).

Both ischemia and *in vitro* hypoxia have been shown to increase intracellular calcium concentration in endothelial cells, concurrent with the loss of ATP-dependent calcium-efflux mechanisms and intracellular calcium sequestration by the sarcoplasmic–endoplasmic reticulum calcium-ATPase (Brown and Davis, 2002; Tiruppathi et al., 2002). Additionally, inflammatory stimuli (e.g. thrombin, histamine) have been shown to increase intracellular calcium concentration in endothelial cells (Tiruppathi et al., 2002), and thus may contribute to secondary calcium fluctuations during reperfusion. Oxidative processes also contribute to intracellular calcium dysregulation. Reoxygenation stress in BMECs has been shown to impair intracellular calcium mobilization, partially due to superoxide anion generation via mitochondrial electron transport (Kimura et al., 2000).

One of the principle effects of increased intracellular calcium in endothelial cells is the activation of calcium/calmodulin-dependent MLCK (Garcia et al., 1995a; Goeckeler and Wysolmerski, 1995), inducing actin reorganization, alterations in cell shape, and increased paracellular permeability (Dudek and Garcia, 2001; Lum and Malik, 1996). This increased permeability has been linked to downstream PKCα dependent signaling pathways, mediating contraction of the actin–myosin cytoskeleton and alterations in the adherens junctions (Tiruppathi et al., 2002). Additionally, inhibition of hypoxia-induced increased paracellular permeability by nifedipine (L-type calcium channel blocker) has been identified to be mediated via PKCα (Hempel et al., 1999). BMECs undergoing hypoxia coupled with aglycemia were shown to have a rapid increase in TJ permeability, directly linked to increased intracellular calcium levels (Abbruscato and Davis, 1999; Brown and Davis, 2005; Park et al., 1999). Additionally, treatment with a receptor-operated/store calcium channel blocker has been shown to reduce BBB paracellular permeability associated with hypoxia/aglycemia, which correlated with partial protection of occludin membrane localization (Brown and Davis, 2005).

Other calcium mediated processes could also impact BBB integrity. Enhanced activation of phospholipase-A2 by calcium leads to release of free fatty acids, including arachidonic acid from membrane phospholipids. Such actions may have direct effects on the BBB endothelial membrane, as well as on TJ protein stability within the membrane. Lastly, calcium-dependent calmodulin-dependent kinase can activate transcription factors, such as cAMP response element binding protein (CREB) and c-*fos* (Cruzalegui and Bading, 2000), which mediate numerous downstream events affiliated with endothelial regulation.

Inflammation

Inflammation associated with I/R involves the induction of cytokines and adhesion molecules at the level of the endothelium, in coordination with the activation and migration of neutrophils and microglia. TNFα and IL-1β initiate the I/R inflammatory response, as these cytokines have been identified in the CSF of stroke patients (Feuerstein et al., 1994) and in animal brains after global and focal ischemia (Tarkowski et al., 1997). Microvascular endothelial cells exposed to TNFα, IL-1β, and IL-6 have shown increased paracellular permeability, which could be abolished by cyclooxygenase inhibition (Candelario-Jalil et al., 2007; de Vries et al., 1996), directly implicating the arachidonic acid cascade in I/R increased BBB permeability. Furthermore, upregulation of TNFα expression by neurons and astrocytes in ischemic regions has been shown to precede BBB permeability (Hosomi et al., 2005; Yang et al., 1999). Independent of hypoxia, exposure of TNFα to the luminal membrane of BMECs has been shown to result in F-actin stress fiber formation and increased paracellular permeability (Deli et al., 1995b).

Cytokines also stimulate the production and release of chemokines chemoattractant proteins MCP-1 (a.k.a. CCL-2) and CINC (member of IL-8 family) (Huang et al., 2006). MCP-1 is a major factor associated with leukocyte infiltration into the brain parenchyma in a variety of neuropathologic and inflammatory conditions, including stroke. An *in vitro* examination identified a biphasic increase in permeability during post-hypoxic reoxygenation, which coincided with increased secretion of MCP-1 by both astrocytes and brain endothelial cells (Dimitrijevic et al., 2006). The biphasic increase in permeability was associated with a redistribution of occludin, ZO-1, ZO-2, and claudin-5 (Dimitrijevic et al., 2006). In a subsequent study, MCP-1 receptor knock-out mice showed a decrease in BBB permeability, infarct size, brain edema, leukocyte infiltration, and inflammatory mediator expression after I/R (Dimitrijevic et al., 2007). In another examination, MCP-1 treated BMECs increased paracellular permeability, as well as a redistribution and decreased expression of occludin, ZO-1, VE-cadherin, and β-catenin (Song et al., 2007). Interestingly, the observed changes were in coordination with a decreased expression of the lipid-raft associated scaffolding protein caveolin-1 (Song et al., 2007), implicating MCP-1 action at the level of the plasma membrane.

Subsequent to cytokine/chemokine action, there is an induction of endothelial (P-selectin, intercellular adhesion molecule-1 (ICAM-1), E-selectin) and leukocyte (L-selectin, β2-integrin) adhesion molecules (del Zoppo et al., 2000). TNFα has been shown to increase the expression of the ICAM-1 at the surface of cultured endothelial cells (VandenBerg et al., 2004; Wong et al., 2007). Such induction of ICAM-1 can be mediated through TNFα activation of nuclear factor-κ B (NFκB) (Mattson et al., 2000; Wosik et al., 2007). As a primary early response transcription factor, NFκB has been hypothesized to be a critical mediator of downstream TJ alterations (Brown et al., 2003). In human pulmonary artery endothelial cells, PKCζ has shown to play a regulatory role in TNFα induced ICAM-1 transcription, via NFκB activation (Rahman et al., 2000). Additionally, Src activation may be a critical intermediary of the inflammatory response associated with TJ alterations. While TNFα activation of Src has yet to be fully evaluated in brain endothelial cells in association with I/R, TNFα activation of PKC-dependent Src has been identified with the downstream activation of NFκB in epithelial cells (Huang et al., 2003; Okutani et al., 2006).

IL-1 is also associated with induction of endothelial cell adhesion molecules expression during I/R. In a study of chronic IL-1 administration, peak IL-1 expression levels were associated with an increased recruitment of neutrophils, vasodilatation, and increased BBB paracellular permeability (Ferrari et al., 2004). In another study, the IL-1 receptor antagonist IL-1ra significantly reduced infarct volume and BBB permeability following 24 h of reperfusion in mice (Yang et al., 1998). IL-1β induced neutrophil adhesion and increased BBB paracellular permeability has also been identified with the loss of occludin and ZO-1 at the junction (Bolton et al., 1998).

Neutrophils and mononuclear phagocytes recruited to the I/R brain contribute to the induction of iNOS, free radicals, MMPs, cyclooxygenase-2, VEGF, and other mediators (Heo et al., 2005; Rosell et al., 2008). Upon leukocyte adhesion to the endothelium, leukocytic β2-integrin signaling triggers the release of heparin-binding protein, inducing calcium-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial cell monolayers (Gautam et al., 2001). Additionally, ICAM-1 cross-linking has been shown to induce calcium-PKC mediated tyrosine phosphorylation of actin-associated proteins associated with cytoskeletal rearrangement (Etienne-Manneville et al., 2000). Lastly, activated neutrophils have been shown to cause a concentration-dependent increase in FAK tyrosine phosphorylation with a time-course that correlated with increased paracellular permeability (Guo et al., 2005).

Of the TJ proteins, only the JAMs have direct inflammatory-related activities. During the transmigration of neutrophils in the activated endothelium, JAM-A has been shown to redistribute around the site of penetration (Shaw et al., 2004), and has been identified as a mediator of TJ opening and resealing in coordination with diapedesis (Weber et al., 2007). TNFα has been shown to stimulate the redistribution of JAM-A from the intercellular junctions to the apical surface *in vitro* (Ostermann et al., 2002; Ozaki et al., 1999). Anti-JAM antibody has been shown to reduce leukocyte transmigration across endothelial cells *in vitro* and *in vivo* (Del Maschio et al., 1999; Martin-Padura et al., 1998). Interestingly, JAM-A and JAM-C have also been shown to be involved in endothelial angiogenesis (Cooke et al., 2006; Lamagna et al., 2005), with JAM-A identified as a critical mediator of integrin-specific endothelial migration (Cooke et al., 2006). To date, there remains a clear deficit with regard to investigations of BBB JAMs in association with I/R. Nevertheless, there is every indication that JAMs have a significant impact on BBB paracellular permeability and may likely serve as a means to regulate the ischemic stroke inflammatory response.

Oxidative and nitrosative stress

Oxidative stress is a significant contributor to BBB damage and vasogenic edema, particularly during reperfusion (Heo et al., 2005). Superoxide and hydroxyl radicals are ROS that can rapidly overwhelm endogenous scavenging mechanisms, damaging cellular macromolecules (lipids, proteins, and nucleic acids). Oxidants are also mediators in signaling involving mitochondria, DNA repair enzymes, and transcription factors that may lead to apoptosis during reperfusion. Mitochondria are heavily implicated in the formation of ROS, as excessive superoxide production during electron transport and inhibition of electron transport mechanisms by free radicals, leads to further generation of oxygen radicals (Chan, 2001). Mitochondrial oxygen radical production can be stimulated by elevated intracellular calcium, sodium, and adenosine diphosphate levels in ischemic cells. Enzymatic conversions, such cyclooxygenase-dependent conversion of arachidonic acid to prostanoids, can also produce oxygen radicals.

Evidence indicates that oxidative stress disrupts endothelial TJs, resulting in increased paracellular permeability (Rao et al., 2002; Schreibelt et al., 2007; Sheth et al., 2003). Oxidative stress-induced endothelial cell permeability has been shown to be associated with tyrosine phosphorylation of occludin and ZO-1, mediated through Src (Rao et al., 2002). Increased endothelial permeability in response to oxidative stressors has also been reported to induce F-actin redistribution and stress fiber formation (Lee et al., 2004; Liu and Sundqvist, 1995). An examination assessing ROS impact on BMECs, identified RhoA acting upstream of PI3K, which in turn activated PKB/Akt and increased permeability (Schreibelt et al., 2007). This pathway resulted in F-actin stress fiber formation, as well as a decline in claudin-5 and occludin expression and cellular redistribution, which could be blocked through inhibition of PI3K or PKB/Akt (Schreibelt et al., 2007). Furthermore, inhibiting PKB activity prevented ROS-induced monocyte migration across the endothelial monolayer (Schreibelt et al., 2007). In another examination, an increased paracellular permeability in BMECs by hydrogen peroxide correlated to changes in the junctional localization of occludin, ZO-1, and ZO-2 (Fischer et al., 2005). The changes were prevented by blocking the p44/p42 MAPK activation, implicating the MAPK pathway in oxidative dysregulation of the TJs (Fischer et al., 2005).

Oxidative stress also contributes to the activation of enzymes, impacting BBB permeability. Oxidative stress-related genes such as the lipoxygenases can be particularly damaging because of their lipid-oxidizing properties. The 12/15-lipoxygenase (12/15-LOX, encoded by the ALOX15 gene), the dominant isoform in the brain, has been identified to damage mitochondria (Kuhn et al., 2002). A recent MCAO mouse examination showed a partial prevention of claudin-5 degradation in the peri-infarct region with 12/15-lipoxygenase inhibition (Jin et al., 2008).

NO is unique in that it is not only involved in intracellular signaling, but is also a contributor to the production of ROS. With this understanding, NO has been identified to have both protective and deleterious effects on cerebral tissues during I/R, dependent on amount produced and the specific enzyme involved in its production (Dalkara et al., 1998; Zhang et al., 1995). There are three distinct known isoforms of NOS, endothelial-NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). While eNOS and nNOS are both calcium-dependent, iNOS is a calcium-independent enzyme. Constitutive forms of NOS (i.e. eNOS and nNOS) generally produce a small amount of NO (nM) under basal conditions (Kuo and Schroeder, 1995). In contrast, iNOS is responsible for synthesis of NO in the μM–mM range, in response to stimuli such as lipopolysaccharides and cytokines (Kirk et al., 1990).

While eNOS production of NO has been shown to be beneficial to cerebral tissue associated with MCAO examinations (Huang et al., 1996; Huang et al., 1994), NO production via nNOS and iNOS has been linked to deleterious effects. Increased expression of nNOS has been shown in the core ischemic zone and penumbra 24–48 h following permanent MCAO (Leker et al., 2001). Inhibition of nNOS, by nNOS antiserum, has been shown to improve sensory motor function, decrease edema formation, and reduce paracellular permeability following traumatic brain injury (Sharma et al., 2006). Additionally, mice lacking the nNOS gene have been shown to have significantly less infarct volume, neurological deficits, and brain water volume than their wild-type counterparts following I/R (Hara et al., 1996; Huang et al., 1994). Inhibition of iNOS has also been shown to have beneficial effects following cerebral ischemia. In an MCAO study, mice lacking the iNOS gene displayed less neurological deficits and infarct volume when compared to their wild-type littermates (Iadecola et al., 1997), with iNOS protein and catalytic activity peaking at 48 h (Iadecola et al., 1995). The mechanism of the deleterious effects is likely linked to the generation of the ROS peroxynitrite (Forman et al., 1998; Kumura et al., 1996). Peroxynitrite formation can lead to nitrotyrosination of proteins, which can then lead to compromised cellular function and viability (Radi, 2004). It has also been suggested that decreased endothelial ATP concentration, via the inhibition of glyceraldehyde-3-phosphate dehydrogenase activity by NO may induce TJ permeability (Heo et al., 2005). Lastly, NO has been identified to activate MMP-9 (Gu et al., 2002), with NO-sensitive transcription factors (i.e. NFκB, activated protein-1) also being transcription factors for MMP-9 (Gum et al., 1996).

Enzymatic activity

Although many proteases, including heparanases and cathepsins, are activated during ischemic stroke, tPA and MMPs have become the primary focus of vascular alterations during ischemic stroke. The rationale for this focus is that tPA and MMPs are the major enzyme systems involved in ECM degradation. In this regard, these enzymes are strongly correlated with hemorrhagic transformation and vasogenic edema. This is of further importance, as tPA is also used as a first-line therapy for clot lysis.

As a serine protease, tPA activates plasminogen into plasmin. Plasmin is a fibrinolytic enzyme capable of rapidly degrading fibrin-based blood clots. Increased endogenous tPA levels are associated with early cerebral ischemia, with elevated activity shown as early as 1 h after MCAO, concurrent with increased paracellular permeability (Yepes et al., 2003). Evidence strongly supports the tPA induction of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) during I/R (Sumii and Lo, 2002; Tsuji et al., 2005; Yang et al., 2007). Induction of MMPs via tPA has been implicated to work through low-density lipoprotein receptor-related protein (LRP) activation (Wang et al., 2003). LRP, which has been shown to bind tPA (Zhuo et al., 2000), is associated with numerous downstream signaling processes (Herz and Strickland, 2001). Furthermore, tPA-LRP induced increase in BBB permeability has been linked to a loss of astrocyte adhesion to the ECM (An et al., in press; Polavarapu et al., 2007).

MMPs are a family of zinc endopeptidases that have been identified as mediators of I/R outcomes associated with BBB TJ disruption. MMP levels have been shown to be elevated in the plasma and brains of stroke patients, associated with hemorrhagic transformation (Clark et al., 1997; Montaner et al., 2001b). MMP-2 and MMP-9 have been the focus of several studies of cerebral ischemia due to their substrate specificity for fibronectin, laminin, and collagen. Both MMP-2 and MMP-9 exist in "pro" inactivated forms, although MMP-2 is constitutively expressed in the brain. Within the CNS, endothelial cells, glia, pericytes, and neurons can generate proMMP-2 and proMMP-9 (del Zoppo et al., 2007). ProMMP-2 has been shown to be activated by membrane-type MMP (MT-MMP) (Sato et al., 1994), in association with tissue inhibitor of metalloprotease-2 (TIMP-2) (Strongin et al., 1995). ProMMP-9 has been shown to be activated by MMP-3 (stromelysin-1) and free radicals during ischemic conditions (Gasche et al., 2001; Jian Liu and Rosenberg, 2005). MMP induction and activation can be further augmented through inflammation (i.e. TNFα, IL1β) (Harkness et al., 2000; Rosenberg et al., 1995), oxidation

(Gasche et al., 2001; Haorah et al., 2007) and nitrosative stress (Akool el et al., 2003; Gu et al., 2002).

Increased MMP-2 activity has been identified with the initiation of ischemia (Chang et al., 2003), and has also shown to strongly correlate with the first period of biphasic opening of the BBB (Rosenberg, 2002; Yang et al., 2007). The second period of biphasic opening has been correlated to increased MMP-9 activity, associated with increased vasogenic edema and hemorrhagic transformation in both humans (Montaner et al., 2001a; Montaner et al., 2001b) and animal models of stroke (Lee et al., 2007; Rosenberg and Yang, 2007). BBB permeability via leukocyte derived MMP-9 has also been identified following I/R (Gidday et al., 2005; Justicia et al., 2003), which correlated with the peak period of post-MCAO neutrophil infiltration (Zhang et al., 1994). Recombinant tPA treatment has also been shown to promote MMP-2 and MMP-9 release from human neutrophils *ex vivo* (Cuadrado et al., 2008). Furthermore, MMPs have been identified to promote neutrophil migration (Van Lint and Libert, 2007), resulting in a positive feedback loop of inflammatory activity.

The downstream effect of MMPs upon the TJ proteins associated with I/R have also been examined. Following transient focal ischemia, MMP-9 knock-out mice displayed significantly reduced BBB disruption and edema, which correlated with reduced degradation of ZO-1 compared to wild-type mice (Asahi et al., 2001). Additionally, a recent examination showed that increased MMP-2 and MMP-9 during transient focal ischemia, resulted in fragmentation/relocalization of claudin-5 and occludin following 3 h reperfusion (Yang et al., 2007). The decreased claudin-5 and occludin expression at 3 h, was shown to be reversed with use of an MMP inhibitor (Yang et al., 2007). Additionally, at 24 h reperfusion confocal micrographs indicated a colocalization of claudin-5 with the glial fibrillary acidic protein (GFAP) reactive astrocytes, within the penumbra region (Yang et al., 2007). Interestingly, claudin-5 has shown to promote activation of proMMP-2, via MT-MMPs (Miyamori et al., 2001). The relocalization of claudin-5 to these astrocytes during reperfusion in combination with its promoter activity of MMP-2, would implicate claudin-5 as an active mediator of ECM degradation and potentially angiogenesis.

Angiogenic growth factors

The vascular endothelium-specific growth factors include members from the VEGF, angiopoietins, and ephrin families, which act in coordination during angiogenic remodeling (Yancopoulos et al., 2000). To date, the majority of growth factor related studies affiliated with I/R and BBB TJ permeability have focused on VEGF and associated intracellular mechanisms (Fig. 3). However, recent investigations also implicate angiopoietin-1 (Ang-1) as a subsequent stabilizer of the BBB TJs. In endothelial cells, VEGF has consistently shown to initiate vessel formation in adult animals during wound healing, but by itself only promotes the formation of leaky and unstable vessels; whereas subsequent Ang-1 administration induces stabilization (Weis and Cheresh, 2005).

VEGF is a highly specific mitogen for vascular endothelial cells by acting on two tyrosine kinase receptors, VEGF receptor-1 (flt-1) and VEGF receptor-2 (flk-1/KDR), with VEGFR-2 considered to be the major mediator of angiogenesis. The VEGF protein has been shown to be upregulated through the activation of hypoxia inducible factor-1α (HIF-1α), the regulatory subunit of transcription factor HIF-1 (Semenza, 2007). HIF-1α activation is principally mediated through increased intracellular hypoxia (Semenza, 2007), although IL-1β

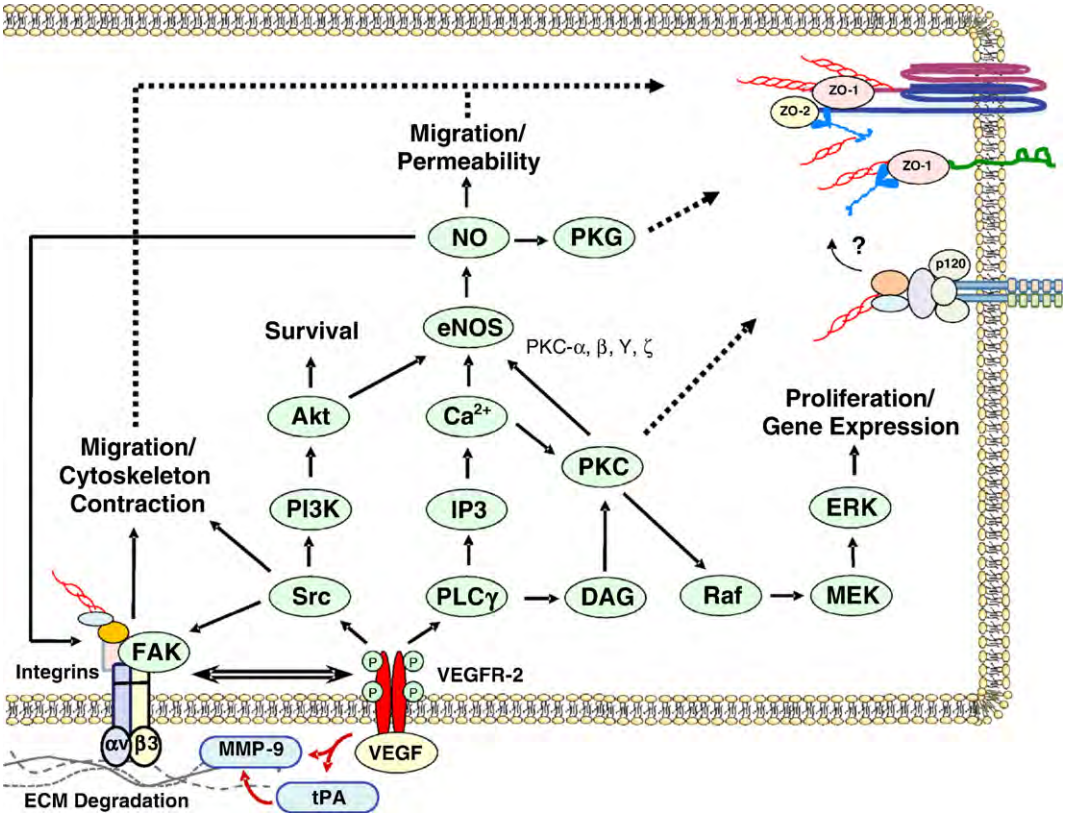

Fig. 3. Schematic of potential pathways by which vascular endothelial growth factor (VEGF) regulates blood-brain barrier tight junction (TJ) disassembly and extracellular-matrix (ECM) degradation, subsequent to ischemic stroke, as defined in the text. VEGF receptor-2 (VEGFR-2) mediates several brain endothelial cell functions, including proliferation, differentiation, permeability, vascular tone, and the production of vasoactive molecules. Upon ligand binding, the receptor tyrosines are autophosphorylated, allowing the receptor to associate with and activate a range of pro-angiogenic signaling molecules and events. A dashed line indicates the VEGF downstream components that may directly mediate TJ protein activity. The double-lined arrow identifies interdependent-synergistic activities between integrins and VEGFR-2. The impact of the adherens junction proteins on the tight junctional complex during angiogenesis has not been elucidated.

mediated activation has also been implicated (Argaw et al., 2006). VEGF expression has been shown to appear as early as 1 h after the onset of ischemia, reaching peak levels around 24 h, and then persisting for ~7 days (Abumiya et al., 1999; Plate et al., 1999). Early intravenous administration of VEGF has also been shown to increase BBB permeability, increasing infarct size and worsening neurological outcome following an ischemic insult (Kaya et al., 2005; Kilic et al., 2006; Zhang et al., 2000). Yet, delayed increases in VEGF (24 h or greater) appear to beneficially enhance angiogenesis (Zhang and Chopp, 2002), neuronal survival (when given ICV or topically) (Hayashi et al., 1998; Kaya et al., 2005), and may even decrease infarct size (Kaya et al., 2005). Thus, the time-table of VEGF action on the BBB endothelium is critical in the determination of detrimental and beneficial effects subsequent to I/R.

VEGF has been shown to increase paracellular permeability, decrease ZO-1 and occludin localization at the TJs, and alter the distribution of actin filaments in BMECs (Fischer et al., 2004; Wang et al., 2001). In the Fischer study, effects of VEGF on the TJ proteins were shown to be dependent on downstream activation of PLCγ, PI3K, and PKG (Fischer et al., 2004). Following an MCAO in mice, VEGF induction of Src-dependent processes were shown to result in increased vascular permeability associated with cerebral edema (Eliceiri et al., 1999; Paul et al., 2001). Using an embolism model of stroke, an *ex vivo* increase in tyrosine phosphorylation of occludin coincided with increased Src activity, in association with a decreased occludin and ZO-1 expression (Kago et al., 2006). A PKC-dependent model of VEGF-mediated TJ disassembly and vascular permeability has also been proposed (Harhaj and Antonetti, 2004). In this model, VEGF activation of VEGFR-2 stimulates PLC-γ activation through Src, with subsequent production of inositol 1, 4, 5-triphosphate (IP3) and activation of conventional and novel PKC isoforms (He et al., 1999; Shen et al., 1999), directly mediating the TJ protein disassembly. Furthermore, NO has been shown to mediate the I/R induced VEGF response, through an eNOS mediated mechanism (Fukumura et al., 2001; Murohara et al., 1998). The VEGFR-2 mediated increases in eNOS expression have been identified to be downstream of the PKC signaling (Shen et al., 1999).

VEGF-receptor activation can also regulate endothelial-ECM interactions subsequent to ischemic stroke. VEGF has been shown to induce expression of integrin αvβ3 following MCAO (Abumiya et al., 1999), with β3 integrin shown to regulate VEGF-induced increases in vascular permeability (Robinson et al., 2004). Inhibition of the integrin αvβ3 prior to ischemic stroke has been shown to reduce infarction volume (Shimamura et al., 2006). Interestingly, the relationship between VEGFR-2 and β3 integrin appears to be synergistic. The activation of VEGFR-2 has been shown to induce β3 integrin tyrosine phosphorylation through Src, which, in turn, is crucial for VEGF-induced tyrosine phosphorylation of VEGFR-2 (Mahabeleshwar et al., 2007). Moreover, Src mediates VEGF-induced β3 integrin activation, ligand binding, β3 integrin-dependent cell adhesion, directional migration of endothelial cells, and initiation of angiogenic programming in endothelial cells (Mahabeleshwar et al., 2007). Lastly, BBB TJ alterations associated with VEGF have been closely correlated with enzymatic activity. VEGF has been shown to increase the expression and release of MMP-2 and MMP-9 (Wang and Keiser, 1998). Inhibition of MMP-9, 7–14 days after transient MCAO was shown to reduce vessel growth, which could be countered via i.c.v. administration of VEGF (Zhang et al., 2000; Zhao et al., 2006; Zlokovic, 2006). VEGFR-2 activation in non-BBB endothelial cells has also been shown to increase tPA expression, which was associated with proteolysis of fibrin matrices (Ratel et al., 2007).

Similar to VEGF, angiopoietins mediate angiogenesis and have been shown to impact BBB permeability. Ang-1 signaling has been shown to mediate angiogenesis via the tyrosine kinase Tie2-receptor, expressed on endothelial cells. While Ang-1 upregulation occurs 2–21 days after ischemia (Beck et al., 2000; Lin et al., 2000), its action is correlated to a reduced endothelial permeability and enhanced stabilization (Gamble et al., 2000). Over-expression of Ang-1 has been shown to prevent plasma leakage in the ischemic brain, as well as decrease ischemic lesion volume (Thurston et al., 2000; Zhang et al., 2002). Ang-1 has also been shown to block VEGF-induced BBB TJ permeability increases, in association with decreased MMP-9 activity (Valable et al., 2005). In retinal microvessel endothelial cells, occludin and ZO-1 expression have been shown to be induced by pericyte derived Ang-1, through tyrosine phosphorylation of Tie-2 (Hori et al., 2004; Wang et al., 2007). Additionally, under hypoxic conditions, Ang-1 partially mitigated the decreased occludin expression (Wang et al., 2007). Recent work has also identified a member of the agiopoietin-related family, angiopoietin-like protein (Angpt)-1 (a.k.a. angiopoietin-related protein, angioarrestin), to decrease BBB paracellular permeability and vasogenic edema after MCAO in mice (Lai et al., 2008). Angiopoietin-2 (Ang-2) has been proposed as the natural antagonist for Ang-1 (Maisonpierre et al., 1997). Focal cerebral ischemia has shown to acutely down-regulate Ang-1, while up-regulating Ang-2 (Beck et al., 2000; Lin et al., 2000). A recent study has shown that when Ang-2 was combined with VEGF in retinal endothelial cells, there was a three-fold increase in permeability over VEGF alone (Peters et al., 2007). Therapeutic strategies aimed at both the VEGF and angiopoietin pathways, strictly regulating the angiogenic processes, may prove to be more effective in mitigating I/R associated TJ permeability and vasogenic edema.

Basic fibroblast growth factor (bFGF) has potent trophic effects on brain neurons, glia, and endothelial cells, with increased expression following MCAO (Speliotes et al., 1996). bFGF is produced primarily by astrocytes and has been shown to bind the FGF receptor-1 on endothelial cells (Sobue et al., 1999). Additionally, bFGF has been shown to activate signaling pathways involved in regulating endothelial cell survival following I/R (Leker et al., 2007; Sobue et al., 1999). In BBB cell culture examinations, bFGF has been shown to decrease paracellular permeability (Bendfeldt et al., 2007; el Hafny et al., 1996). This is consistent with the findings that mice lacking bFGF showed decreased levels of the TJ proteins occludin and ZO-1, while displaying increased paracellular permeability (Reuss et al., 2003). Other trophic factors have been implicated in the I/R response (e.g. neuropilin-1, Ephrin B4, brain-derived neurotrophic factor) (Zhang and Pardridge, 2006; Zhang and Chopp, 2002), and may have singular or synergistic impact on the BBB permeability and recovery; however, their direct actions upon the TJs have yet to be evaluated.

Clinical and drug development implications

As addressed, the I/R induced phases of TJ permeability are the result of multiple interdependent events. Appropriately matching the mechanisms of action with the time-frame of events can allow for more effective hospital treatment and drug development. In this regard, it is necessary to be more critical in our approach to ischemic stroke modeling, especially within the context of drug evaluations. For example, assessment of BBB paracellular permeability as a primary measure of drug efficacy must also take into account downstream angiogenesis. A drug which completely abolishes paracellular permeability may reduce vasogenic edema, but may likewise eliminate the vascular remodeling process critical for long-term brain cell survival. Additionally, both basic-science and clinical drug evaluations often focus on singular parameters. Yet, the magic-bullet approach is not viable for a dynamic pathology in a heterogenous population. It is necessary to work towards an approach in which experimental drugs are designed to complement clinically established therapies and/or used in combination (e.g. neuroprotectants and vascular modulators). Such revision of pharmacologic application not only forces us to take a new look at "failed" drugs, but opens the window on new patent applications. With this understanding, an algorithm for drug development and optimization is a necessary tool (Fig. 4). An effective

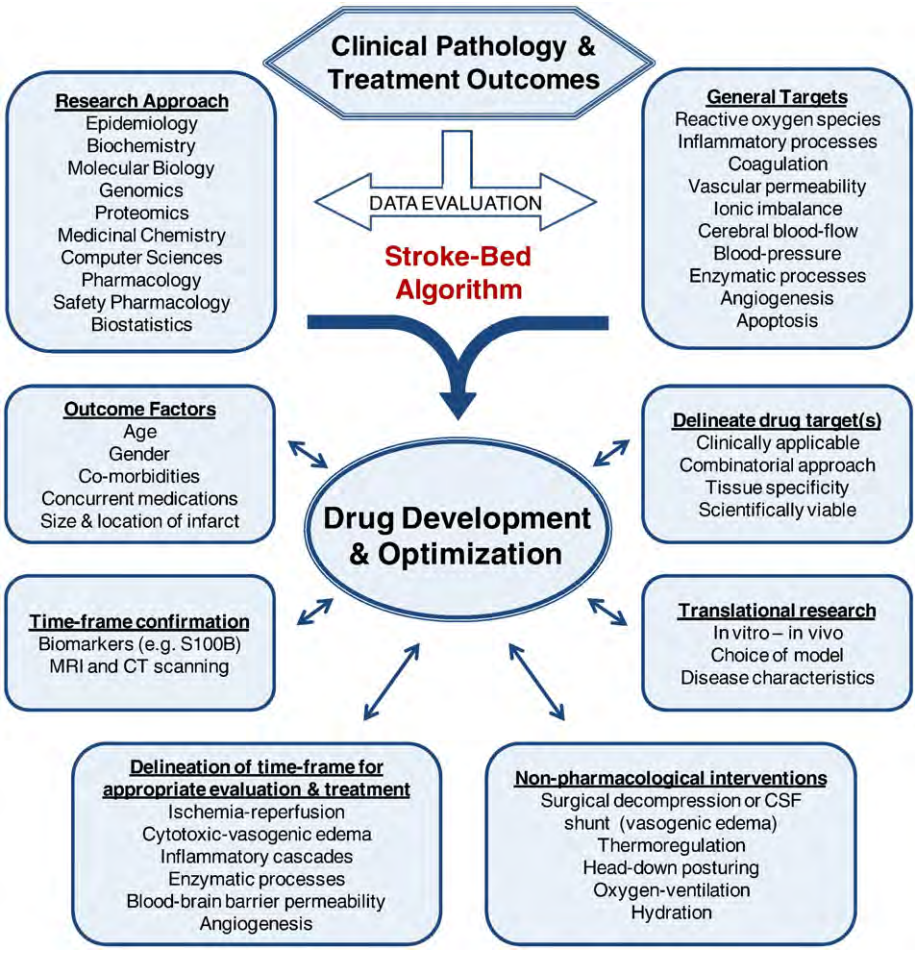

Fig. 4. Proposed algorithm for ischemic stroke drug development and optimization.

algorithm should apply a hierarchy based on the clinical pathology and treatment outcomes observed from the stroke-bed, identifying the appropriate targets, and then (re-)optimization of the pharmacology. Such an algorithm must also take into account multiple variables, including concurrent medications taken by the patient, comorbidities (e.g. diabetes, hypertension), non-pharmacological procedures, and other outcome factors. For example, studies have identified aging to effect TJ protein levels (Mooradian et al., 2003), angiogenesis (Reed and Edelberg, 2004), and BBB integrity during ischemic stroke modeling (Dinapoli et al., 2007). Thus, the loss of BBB plasticity with age is a critical factor that must be taken into account during evaluations of drugs aimed at TJ or vascular function. Nevertheless, the majority of drug examinations for ischemic stroke are still conducted in young healthy animals.

Conclusions

It has become apparent that the processes governing BBB TJ permeability during ischemic stroke are extremely complex. Yet, through delineation of the phases of permeability, we are better able to clarify the molecular mechanisms of TJ regulation. As these phases have many unique characteristics, they provide the opportunity to more accurately tailor time-dependent therapeutics. Furthermore, there are strong implications for BBB TJ proteins acting as primary mediators of angiogenesis and inflammatory processes, rather than simply secondary passive components. Although such non-barrier maintenance activities require further assessment, current data indicates that the TJ proteins are far more dynamic than previously believed.

As we progress in our understanding of the various components of ischemic stroke, the TJs of the BBB will continue to serve as a critical focal point. Nevertheless, many questions remain. What is the impact of age and comorbidities on BBB plasticity? To what extent do the TJ proteins actively mediate angiogenic and inflammatory processes? Can we prophylactically modulate the components of the BBB to maintain healthy brain function? Are there other unexamined intracellular scaffolding proteins at the BBB that contribute to the TJ responses during I/R? Perhaps most importantly, how do we appropriately apply our understanding of BBB regulation to clinically viable outcomes?

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