CHANGES OF THE BLOOD-BRAIN BARRIER PERMEABILITY, ULTRASTRUCTURE, AND PROTEIN AND GENE EXPRESSIONS IN A RAT MODEL OF CEREBRAL HYPOPERFUSION

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by

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LIST OF ABBREVIATIONS

2D	two-dimensional gel electrophoresis
2D-DIGE	two-dimensional differential gel electrophoresis
2VO	bilateral carotid artery occlusion/two-vessel occlusion
ABC	ATP-binding cassette
ALS	amyotrophic lateral sclerosis
AP	alkaline phosphatase
BAB	blood-arachnoid barrier
BBB	blood-brain barrier
BCSFB	blood-cerebrospinal fluid barrier
BMP	bone morphogenetic protein
bps	base pairs
BSA	bovine serum albumin
CBF	cerebral blood flow
CNS	central nervous system
cDNA	complementary deoxyribonucleic acid
CSF	cerebrospinal fluid
CSFBB	cerebrospinal fluid-brain barrier
EBD	Evans blue dye
eNOS	endothelial nitric oxide synthase
ERH	enhancer rudimentary homolog
FGF	fibroblast growth factor
GLUT1	glucose transporter isoform 1
HMW	high molecular weight

HNRPD	heterogenous nuclear ribonucleoprotein D0
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
IL	interleukin
JAM	junctional adhesion molecule
LC	liquid chromatography
LMW	low molecular weight
MAGUK	membrane-associated guanylate kinases
MDR	multidrug resistance
miRNA	microRNA
MMP	metal metalloproteinases
MRI	magnetic resonance imaging
MS	mass spectrometry
NaF	sodium fluorescein
NO	nitric oxide
NVU	neurovascular unit
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PDGF-β	platelet derived growth factor beta
pI	isoelectric point
qPCR	quantitative polymerase chain reaction
RFU	relative fluorescence unit
ROS	reactive oxygen species
SAGE	serial analysis of gene expression
SD	Sprague-Dawley

siRNA	small interfering RNA
SOD	superoxide dismutase
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TNF	tumor necrosis factor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
w/v	weight/volume
ZO	zonula occludens

LIST OF SYMBOLS

α	alpha
β	beta
®	registered trademark
™	trademark
r^2	coefficient of determination
Δ	delta/differences
p	statistical significance

PERUBAHAN KETELAPAN RINTANG DARAH-OTAK, ULTRASTRUKTUR, DAN EKSPRESI PROTEIN DAN GEN DALAM MODEL HIPOPERFUSI SEREBRUM TIKUS

ABSTRAK

Hipoperfusi serebrum ialah keadaan di mana terdapat pengurangan aliran darah ke otak. Keadaan ini boleh diperhatikan dalam kalangan pesakit yang menghidapi penyakit kardiovaskular, hipertensi dan dalam kalangan penduduk yang semakin tua. Hipoperfusi serebral boleh menyebabkan penyakit neurologi yang lebih teruk termasuk demensia vaskular, penyakit Alzheimer dan sklerosis berbilang. Hipoperfusi serebrum mengaruh kepada kerosakan rintangan darah-otak (BBB) dan kajian terperinci ini telah dilakukan untuk memahami perilaku BBB semasa hipoperfusi serebrum. Dalam kajian ini, model penyumbatan dua pembuluh digunakan untuk mimik keadaan hipoperfusi serebrum dalam tikus. Menggunakan pengesan eksogenus yang mempunyai berat molekul berbeza iaitu pewarna biru Evans (69 kDa) sebagai penanda kebocoran makromolekul dan natrium fluoresein (376 Da) sebagai penanda kebocoran molekul kecil, kebocoran pewarna biru Evans melalui BBB dapat diperhatikan pada fasa awal hipoperfusi serebrum iaitu 1 jam selepas surgeri. Melalui penggunaan mikroskopi elektron pancaran, pembentukan vesikel dan fenestrasi dalam sel endotelium dan pembengkakan hujung kaki astrosit dapat diperhatikan. Ini menunjukkan kerosakan selular yang membawa kepada kebocoran BBB semasa hipoperfusi serebrum. Dengan menggunakan pendekatan proteomik, perubahan dalam ekspresi protein hidratase aconitate, hidratase enoil-CoA, ribonukleoprotein nukleus heterogenen D0 dan β-sinuclein yang terlibat dalam metabolisme tenaga dan proses selular termasuk pembentukan rangka sitoplasma, metabolisme asid nukleik

dan tekanan retikulum endoplasma diperhatikan, menunjukkan gangguan dalam laluan ini yang mungkin menyumbang kepada kegagalan BBB semasa hipoperfusi serebrum. Di samping itu, perubahan dalam ekspresi protein caperon dan protein yang terlibat dalam laluan untuk pembentukan saluran darah baru termasuk protein kalretikulin dan homolog rudimen peningkat mungkin menunjukkan pengaktifan mekanisme pertahanan BBB terhadap kerosakan yang diaruhkan oleh hipoperfusi serebrum. Dengan menggunakan pendekatan genomik, perubahan dalam ekspresi gen yang mengekod untuk protein hidratase aconitate dan homolog rudimen peningkat tidak diperhatikan, menunjukkan perubahan pada tahap translasi tidak diperhatikan di peringkat transkripsi. Oleh itu, adalah baik untuk menyasarkan laluan yang telah dikenalpasti pada peringkat protein untuk membangunkan strategi terapeutik untuk menghalang kegagalan BBB semasa fasa awal hipoperfusi serebrum. Pada keseluruhannya, kajian ini menunjukkan perubahan setempat fungsi BBB dalam keadaan hipoperfusi serebral, dan mencadangkan laluan yang berpotensi untuk menyumbang kepada perubahan fungsi fisiologi BBB. Hasil dapatan kajian ini adalah bernilai untuk merekabentuk strategi terapeutik berkesan untuk penyakit-penyakit neurologikal yang mempunyai prognosis yang sama.

CHANGES OF THE BLOOD-BRAIN BARRIER PERMEABILITY, ULTRASTRUCTURE, AND PROTEIN AND GENE EXPRESSIONS IN A RAT MODEL OF CEREBRAL HYPOPERFUSION

ABSTRACT

Cerebral hypoperfusion is a condition where there is reduced blood flow to the brain. This condition is observed in patients with cardiovascular diseases, hypertension and among the aging population. Cerebral hypoperfusion leads to more severe neurological diseases including vascular dementia, Alzheimer's disease and multiple sclerosis. Cerebral hypoperfusion induces damages to the blood-brain barrier (BBB) and this detailed study was carried out to understand the BBB behavior during cerebral hypoperfusion. In this study, two-vessel occlusion (2VO) model was used to mimic cerebral hypoperfusion in rats. Using exogenous tracers of different molecular weight i.e. Evans blue dye (69 kDa) as a marker for macromolecule leakage, and sodium fluorescein (376 Da) as a marker for small molecule leakage, BBB leakages to Evans blue dye was observed during the early phase of cerebral hypoperfusion, 1-day post-surgery in the brain regions frontal cortex, posterior cortex and thalamus-midbrain. Using transmission electron microscopy, vesicles and formation of fenestration in endothelial cells, and swelling of astrocyte end-feet were observed, indicating cellular damages leading to BBB leakages during cerebral hypoperfusion. Using proteomics approach, decreases in the expression of proteins, including aconitate hydratase, enoyl-CoA hydratase, heterogenous nuclear ribonucleoprotein D0 and β-synuclein, involved in energy metabolism and cellular processes including cytoskeleton formation, nucleic acid metabolism and endoplasmic reticulum stress were observed, indicating possible disruption in these pathways which could

contribute to BBB failure during cerebral hypoperfusion. Besides that, increases in the expression of chaperone protein and proteins that are involved in the pathway for new vessel formation including calreticulin and enhancer rudimentary homolog, may indicate the activation of BBB defensive mechanisms against damages induced by cerebral hypoperfusion. Using genomics approach, changes in the expression of genes coding for the proteins aconitate hydratase and enhancer rudimentary homolog were not observed, indicating changes at the translational level is not observed at the transcriptional level. Therefore, it is good to target the identified pathways at the protein level to develop therapeutic strategies to intervene BBB damages during the early phase of cerebral hypoperfusion. On the whole, this study demonstrates regional alterations of the BBB function in cerebral hypoperfusion, with suggestions of pathways which could potentially contribute to the altered BBB physiology. Findings from this study are valuable for designing effective therapeutic strategies for neurological diseases which shared common prognosis.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

The blood-brain barrier (BBB) is a dynamic structure that regulates entry and exit of substances into and out of the brain (Abbott et al., 2006). It is formed by brain microvascular endothelial cells. The BBB together with astrocytes, pericytes and basement membrane which are in close association with neurons are collectively known as the neurovascular unit (NVU). The components of the NVU are in constant communication via signalling pathways to ensure homeostasis of the brain microenvironment is maintained for optimal functioning of the neuron. The BBB as as component of the NVU therefore prevents any damaging changes in the brain by tightly regulating the movement of substances between the brain and the circulating blood.

Although the BBB is regarded as the brain's first-line of defence against pathological damages, during certain disease state including cerebral hypoperfusion, damages to the BBB incurs leading to severe brain pathologies (Obermeier et al., 2013). Cerebral hypoperfusion is characterized as a reduction of blood flow to the brain thus leading to disruption in steady, constant supply of oxygen and nutrients to the brain. As a result, a cascade of changes occurs in the brain including white matter lesion and neuronal damages which may lead to more severe neurological disorders including Alzheimer's diseases, dementia and multiple sclerosis (Ueno et al., 2002; Garbuzova-Davis et al., 2007; de la Torre, 2012).

To date, data are available to demonstrate openings of the BBB that occurs during cerebral hypoperfusion leading to secondary brain damages (Ueno et al., 2002; Khallout, 2013; Edrissi et al., 2016). However, very little is yet known about the possible mechanisms that may lead to BBB openings during cerebral hypoperfusion. Here, in order to reveal the possible mechanisms that may lead to BBB damages during cerebral hypoperfusion, a rat model that mimics the condition of cerebral hypoperfusion was adopted to investigate the changes that occur to the BBB during cerebral hypoperfusion. Spatiotemporal profiling the BBB permeability to low and high molecular weight tracers, and ultrastructural changes of the BBB were investigated to understand the pattern of the BBB damages during cerebral hypoperfusion. Molecular research methods including comparative total protein profiling and target gene analysis were carried out to reveal possible mechanisms that may lead to BBB damages during cerebral hypoperfusion. **Figure 1.1** outlines research activities conducted in this study.

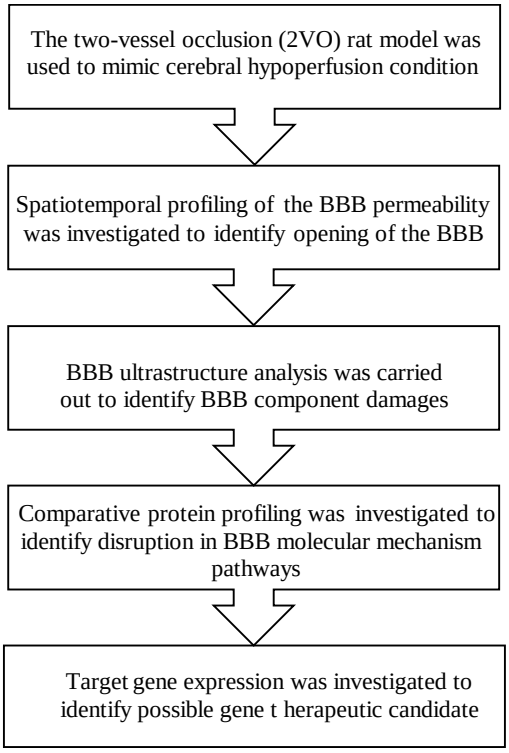

Figure 1.1 Schematic diagram of research activities.

1.2 Research questions

- Will the BBB opens during cerebral hypoperfusion in a rat model?
- What is the time and brain regional patterns of the BBB openings in the brain during cerebral hypoperfusion in rat model?
- Will there be morphological changes to the components of the BBB during cerebral hypoperfusion in rat model?
- What are the changes in the expressions of the BBB proteins during cerebral hypoperfusion in rat model?
- Are there any specific gene changes during cerebral hypoperfusion that may play a role as a therapeutic target for BBB damages during cerebral hypoperfusion in rat model?

1.3 Objectives of the thesis

- To investigate the spatiotemporal profiling of the BBB permeability during cerebral hypoperfusion
- To investigate ultrastructural changes of the BBB during cerebral hypoperfusion
- To investigate the changes in the expression of BBB total proteins during cerebral hypoperfusion
- To investigate the changes in expression of specific genes which may serve as a therapeutic target during cerebral hypoperfusion

1.4 Hypothesis

Cerebral hypoperfusion leads to BBB damages and may cause more severe neurological disorders such as vascular dementia and Alzheimer's disease. During cerebral hypoperfusion, opening of the BBB will be observed in specific regions of

the brain associated with cognitive functions. Damages to components of the BBB including specialized endothelial cells, astrocytes and pericytes may lead to BBB openings during cerebral hypoperfusion. Protein expression levels in the BBB will be affected as results of cerebral hypoperfusion which then affects the regulatory pathways leading to BBB damages. Changes in the protein expressions will be reflected at the genes level.

CHAPTER TWO

LITERATURE REVIEW

2.1 The brain barriers in human

The brain, as the core organ that controls and regulates all bodily functions, requires a constant homeostatic microenvironment for its optimal functioning. Therefore, it is important that the entry and exit of substances into and out of the brain are strictly regulated. As for the purpose, there are four barriers in the brain that formed boundaries between the periphery and the brain namely the blood-brain barrier, the blood-cerebrospinal fluid barrier, the blood-arachnoid barrier and the cerebrospinal fluid-brain barrier (**Figure 2.1**).

2.1.1 Blood-brain barrier

The blood-brain barrier (BBB) is the barrier that is found at the interface between the circulating blood and the brain except at the circumventricular organs including area postrema, median eminence, neurohypophysis, pineal gland, subfornical organ and lamina terminalis (Ballabh et al., 2004). The BBB is made up of specialized endothelial cells of the cerebral microvessels, in close association with other cells of the neurovascular unit (NVU) including astrocytes and pericytes, which together formed a dynamic functioning barrier regulating the movement of substances between the blood and the brain parenchyma.

The barrier characteristics of the BBB include the presence of tight junction proteins between the adjacent endothelial cells which are more extensive compared to endothelial cells in other parts of the body. The specialized endothelial cells are also

characterized to have very little amount of pinocytotic vesicles and fenestrations, which further restricts entry of blood-borne substances into the brain parenchyma.

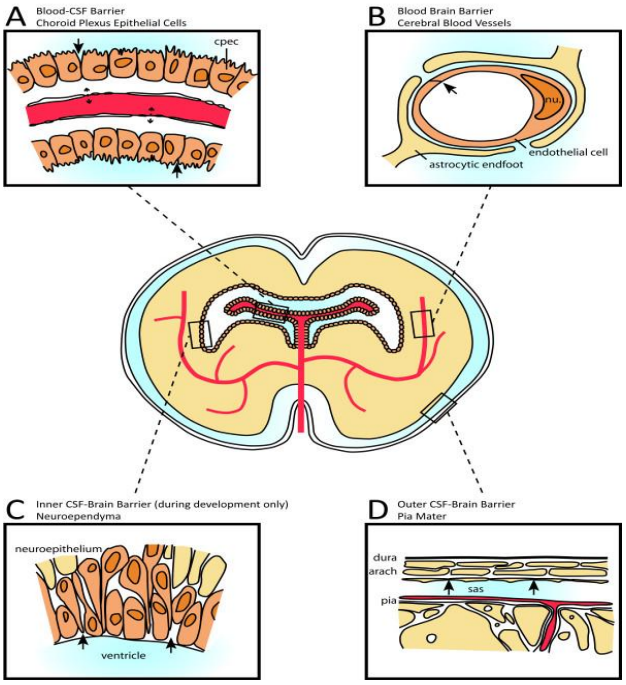

Figure 2.1. The four barriers present in a human brain. A. The blood-CSF barrier is made up of specialized epithelial cells found at the interface between the blood and the CSF at the level of choroid plexus epithelium. B. The blood-brain barrier is made up of specialized endothelial cells found at the interface between the blood and the brain, formed by cerebral microvessel endothelium. C. The CSF-brain barrier is made up of embryo specific neuroependymal cells found at the interface between the CSF and the brain in the ventricles during early developmental stage. D. The blood-arachnoid barrier is made up of arachnoid epithelial cells at the interface between the CSF in the subarachnoid space and the overlaying layer of dura mater. Figure is adapted from Liddelow (2011).

2.1.2 Blood-cerebrospinal fluid barrier

The blood-cerebrospinal fluid barrier (BCSFB) is the barrier found at the interface between the blood and the cerebrospinal fluid (CSF) at the choroid plexus (Laterra et al., 1999). Specialized epithelial cells lining the choroid plexus have functional tight junction proteins, forming barrier that regulates the exchange of substances between the blood and the CSF. As the cerebral blood vessels at choroid plexus have

fenestrations and no tight junctions, choroid plexus is a major site for exchange of substances between the CSF and the brain across the BCSFB including ionic solutes, hormones and organic acids (Nilsson et al., 1992).

2.1.3 Blood-arachnoid barrier

The blood-arachnoid barrier (BAB), which is a part of the BCSFB, is the barrier found at the interface between the CSF in the subarachnoid space and the overlaying layer of dura mater (Yasuda et al., 2013). The arachnoid epithelial cells are connected by characteristic tight junction proteins that regulate the movement of substances between the blood and subarachnoid CSF. Despite still poorly studied and understood, some studies have shown that the BAB expressed drug transporters and drug-metabolizing enzymes, thus could play an important role in the transport of drugs across the BAB, then from the CSF into the brain (Yasuda et al., 2013).

2.1.4 Cerebrospinal fluid-brain barrier

The cerebrospinal fluid-brain barrier (CSFBB) is the barrier found at the interface between the CSF and the brain at the ventricles (Saunders et al., 2012). This barrier is shown to be present only in embryo during the developmental stage and not present at adult stage. The neuroependymal cells lining the ventricular wall have characteristics of embryo specific junctional proteins or called “strap junctions” that regulate the movement of substances between the CSF and the brain parenchyma. As with development into the adult stage, these intercellular junctional proteins disappear. The CSFBB plays an important role in the development of the fetus as it allows the entry of small molecules such as sucrose from the CSF into the brain but it excludes the entry of large molecules such as protein (Liddelow, 2011).

2.2 The BBB: a neurovascular unit

The BBB exists at the level of cerebral microvessel endothelium. If the whole network of cerebral microvessels is stretched out in a single file, it will cover a distance of approximately 650 kilometers (Begley and Brightman, 2003). The extensive network of cerebral microvessels is important to ensure that the brain is continuously perfused optimally to meet the high demand of oxygen and nutrient supply to ensure optimum functioning of the brain. The importance of the BBB is also noted in the context that it is estimated that every neuron in the brain has its own capillary and both structures are in close association for efficient cross-talk (Zlokovic, 2005). Therefore, the BBB is not a separate entity, but a complex structure in close association with surrounding neurons and other brain cells forming a dynamic structure which is collectively known as the neurovascular unit (NVU) (Abbott et al., 2006). Much understanding of the structure and components of the BBB was gained in the 1960s through electron microscopy studies. The center to the BBB is the specialized endothelial cells which are tightly sealed by tight junctions and they are closely supported by associating astrocytes and pericytes (Reese and Karnovsky, 1969; Brightman and Reese, 1969). The endothelial cells are separated from the neighboring cells by basement membrane which is made up of extracellular matrix proteins mainly collagen, fibronectin and laminin (Tilling et al., 1998). The NVU plays an important role in maintaining optimal brain functions through efficient active cross-talk involving signaling molecules in various pathways including neurogenesis, angiogenesis, inflammation and blood flow regulation (Zlokovic et al., 2008). **Figure 2.2** shows the components that come together in close association to form the NVU including the endothelial cell, astrocytes, pericyte, basement membrane and neuron.

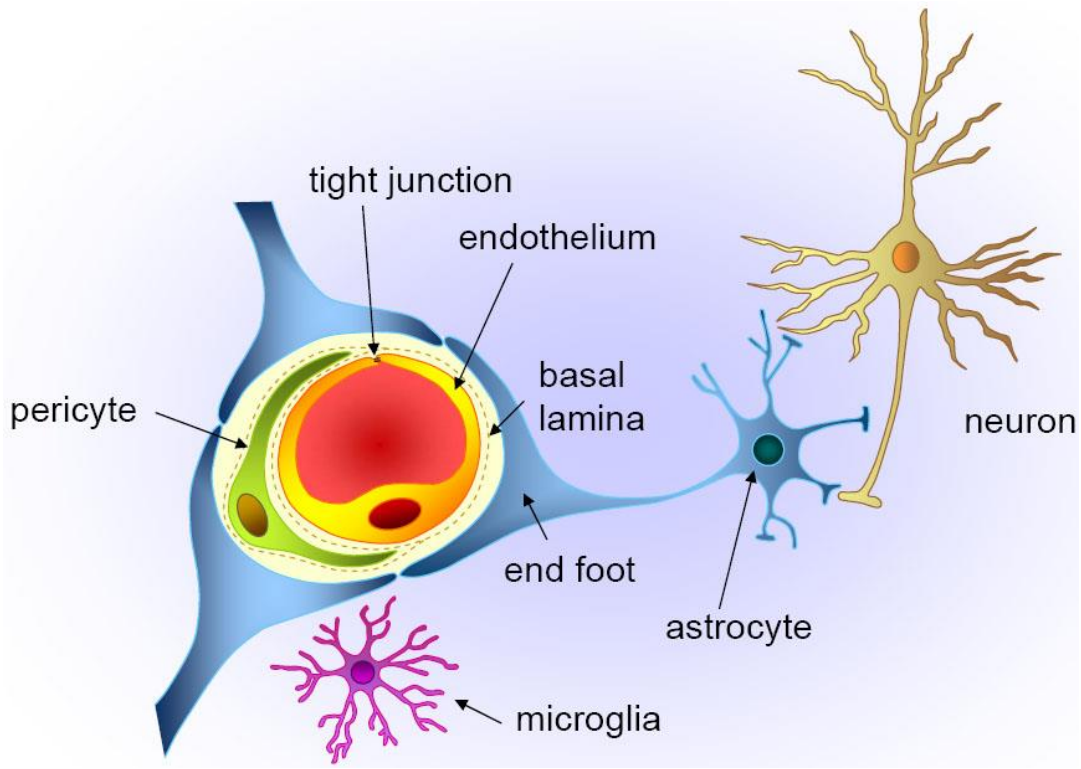

Figure 2.2. Schematic diagram of cross section of the BBB. The BBB is formed by cerebral endothelium which is tightly sealed by tight junction proteins. The endothelial cell is surrounded by basement membrane (basal lamina) that separates the endothelial cell from the neighboring cells. The pericyte enclosed the endothelial cell and the astrocyte end-feet encapsulate the complex. The structure is closely associated with a neuron. Figure is adapted from Abbott and Yusof (2010).

2.2.1 Specialized endothelial cells

Through electron microscopic studies, it is noted that the endothelial cells that formed the BBB are significantly different from the peripheral endothelial cells. The endothelial cells of the BBB are tightly sealed together by tight junction proteins leaving very little to almost no space for movement of substances across the BBB via the intercellular cleft (Laterra et al., 1999). Generally, molecules that are hydrophilic and of size more than 500 Da are excluded by the BBB (Pardridge, 2013). The specialized endothelial cells also have no fenestrations and very little number of vesicles, hence no detectable movement of substances across the BBB by pinocytosis (Laterra et al., 1999). Transcellular movement of hydrophilic substances across the

endothelial cells is only possible through specialized transporter proteins found on the luminal (blood-facing) and abluminal (brain-facing) membranes of the endothelial cells. Transcellular movement of small, lipohilic molecules across the BBB through the membrane lipid bilayer is possible to certain extent, as they will be effluxed by efflux transporters that are highly expressed at the endothelial cells (Banks, 2009).

The endothelial cells are identified to have increased number of mitochondria as compared to periphery endothelial cells to accommodate for the energy required for the strictly regulated active transport of substances across the BBB through the transporter proteins (Carvey et al., 2010).

2.2.2 Basement membrane

Basement membrane, the acellular component of the NVU, is found completely surrounding the endothelial cell separating it from the neighboring brain cells. Despite relatively less studied compared to the other components of the NVU, the basement membrane is found to be important for maintaining the BBB stability, barrier function and it is also involved in vessel formation and inflammatory mechanisms (Sorokin, 2010). The basement membrane is made up of dense extracellular matrix proteins including laminin, collagen type IV, heparan sulfate proteoglycans and nidogens (Baeten and Akassoglou, 2011; Larochelle et al., 2011; Yousif et al., 2013). The structure of the basement membrane is formed by polymerization of laminin and collagen which is linked by nidogens (Yurchenco and Patton, 2009). The basement membrane is an important intermediate component involved in the cross-talk between the endothelial cells and the neighboring cells including astrocytes, pericytes and neurons, as signaling molecules and extracellular matrix receptors are found in the basement membrane (Sixt et al., 2001; Tilling et al., 2002a ; Baeten and Akassoglou,

2011). The basement membrane is also found to be involved in inflammatory mechanism as it is reported that isoform of laminin α5 impedes the entry of leukocytes into the brain parenchyma (Wu et al., 2009). Therefore, it is important to acknowledge the basement membrane as a dynamic non-cellular active component involved in maintaining the integrity of the BBB. Damages to the basement membrane during pathological conditions are often observed concurrently with opening of the BBB (Rosenberg et al., 1993; Rascher et al., 2002; Hawkins and Davis, 2005).

2.2.3 Astrocytes

Astrocyte is an important component of the NVU for maintaining the integrity of the BBB through its active interaction with the specialized endothelial cells through signaling molecules. Astrocyte, one of the major glial cells in the brain, was first visualized in the late 19th century as star-shaped cells using Golgi's staining method under light microscope (Kimelberg and Nedergaard, 2011). Astrocyte's name was derived from the Latin words "astra" and "cyte" which translate to star and cell respectively (Kimelberg and Nedergaard, 2011). It is interesting to note that astrocyte is the most abundant type of cells in the brain as it is estimated that there are five fold more astrocytes in the brain in comparison to neurons (Cherniak, 1990; Nedergaard et al., 2003; Sofroniew and Vinters, 2010). In relation to its abundance in the brain, it is no surprise that astrocytes are involved in many pathways for maintaining the optimal functioning of the brain including synapse development and formation, ion homeostasis maintenance, cerebral blood flow regulation and oxidative mechanism (Clarke and Barres, 2013; Leis et al., 2005; Jou, 2008; Koehler et al., 2009).

Astrocytes formed close contact with endothelial cells of the BBB by projection of many fine processes known as “end-feet” onto the microvessel as seen in **Figure 2.2**. (Abbott et al., 2006). The astrocyte end-feet are seen to completely ensheathed the endothelial cell therefore providing a large surface area for active interaction between the two cells. Astrocytes are known to have direct effect on the integrity of the tight junction proteins of the endothelial cells. *In vitro* studies have shown that the absence of astrocytes caused less tight junctional protein expressions and hence leaky BBB, and these effects were reversed when astrocytes were re-introduced (Janzer and Raff, 1987; Bauer and Bauer, 2000; Thomsen et al., 2015). In *in vivo* studies looking into BBB changes during ischemia, multiple sclerosis, Parkinson’s disease and Alzheimer’s disease, it has been observed that reactive astrogliosis, abnormal increased in number of astrocytes, and glial scarring caused concurrent BBB damages (Bush et al., 1999; Nishie et al., 2004; Tian et al., 2005; Takano et al., 2009; Cabezas et al., 2014). These observations suggest that there is a dynamic cross-talk between the astrocytes and the endothelial cells through signaling molecules and receptors for the maintenance of barrier properties of the BBB.

However to date, there is no clear understanding yet of the signaling mechanism which is involved in for induction of barrier properties of the endothelial cells by astrocytes and only few candidate molecules and pathways have been suggested loosely to be responsible for the interaction between the two cells. Using in *vitro* co-culture of endothelial cells and astrocytes, it was demonstrated that transforming growth factor-beta produced by astrocytes regulates the expression of tissue plasminogen activator and thrombomodulin in endothelial cells thus influencing the integrity of the tight junction proteins (Tran et al., 1999). Other *in vitro* studies suggested basic fibroblast

growth factor and glial cell line derived neurotrophic factor as the most plausible intermediate signaling molecules secreted by astrocytes to be involved in the cross-talk between astrocytes and endothelial cells (Igarashi et al., 1999; Sobue et al., 1999). A study using knockout mouse model have shown bone morphogenetic protein (BMP) acting through the BMP type IA receptor in astrocytes to be the responsible signaling molecule for the induction of BBB tightness (Araya et al., 2008). Although these studies have suggested possible candidates of signaling molecules involved in the cross-talk between endothelial cells and astrocytes, the overall mechanism and pathway involved are yet to be elucidated.

2.2.4 Pericytes

Pericyte, an important component of the NVU, is found embedded in the basement membrane thus placed very closely to the endothelial cells. The role of pericytes in maintaining the integrity of the BBB has been controversial as often its role in maintaining the BBB is downplayed due to the focus given on astrocytes as the key player. However, in recent years, the importance of the pericytes is much acknowledged and the diverse roles played by pericytes have come to light. Pericyte is a prominent nucleated cell with little cytoplasmic content with few processes ensheathing the endothelial cell from the abluminal side (brain-facing) (Hirschi and D'Amore, 1996). In terms of vessel coverage, tissues of the CNS including the brain have the highest percentage of blood vessels covered by pericytes as compared to periphery blood vessels (Zlokovic et al., 2008; Diaz-Flores et al., 2009). The ratio of pericyte to endothelial cell is estimated to be approximately 1:3 (Zlokovic et al., 2008). Therefore, it is highly likely that pericytes support endothelial cell functions and it is

hypothesized that the communication between the two cell types occurs through paracrine signaling (Bergers and Song, 2005).

Pericyte is a multipotent cell as it is capable of differentiating into different types of cells following insults and that explains the diverse role played by pericyte in maintaining the brain homeostasis (Dore-Duffy, 2008). Pericytes are shown to control the BBB-related gene expression in endothelial cells and absence of pericytes is known to cause leaky BBB (Armulik et al., 2010). The most interesting role of pericyte is its involvement in regulating the cerebral blood flow in coordination with endothelial cells through signaling molecules. Similar to cardiac smooth muscle cells, pericytes expressed α-smooth muscle actin and myosin, therefore capable of vasoconstriction and vasodilation, hence regulating vessel diameter, blood volume and flow (Rucker et al., 2000). Vasoconstriction and vasodilation by pericytes are controlled by the binding of vasoactive compound endothelin-1 onto the receptors on pericytes and the production of endothelin-1 is regulated by endothelial cells (Rucker et al., 2000). This is a sophisticated example of mechanism where both the endothelial cell and pericyte work in coordination to maintain the cerebral blood flow. Pericyte is also known as the "cleaning agent" of the BBB as it possesses phagocytotic properties as they have many scavenger receptors capable of displaying scavenging activity (Thomas, 1999). Following downfall of the integrity of the BBB and leakage of substances from the blood into the brain parenchyma, pericytes are observed to scavenge foreign substances by phagocytosis thus removing the unwanted substances from the brain parenchyma (Majno et al., 1961; Kristensson and Olsson, 1973; Balabanov et al., 1996).

As they are in close association within the basement membrane, pericytes and endothelial cells are known to communicate actively to maintain the integrity of the BBB. One of the most highlighted paracrine signaling between the endothelial cells and pericytes is through the platelet derived growth factor beta (PDGF-β) molecule and receptors. Endothelial cells are known to produce PDGF-β which then binds to PDGF-β receptors found on pericytes, regulating recruitment and attachment of pericytes onto microvessels (Enge et al., 2002; Quaegebeur et al., 2010). Another paracrine signaling pathway shown to take place between the pericytes and the endothelial cells is through the angiopoietin molecules and Tie receptor. Tie receptor is expressed by endothelial cells and the signaling molecules Ang-1 and Ang-2 are produced by pericytes (Suri et al., 1996). This signaling pathway controls the attachment of pericytes onto endothelial cells, vessel formation and proliferation (Bergers and Song, 2005). It is important to note that most of these signaling pathways between pericytes and endothelial cells are demonstrated in *in vitro* studies and the complex signaling pathways involved in *in vivo* studies is yet to be explored and understood clearly.

2.2.5 Interaction between neurons and other components of the NVU

It is estimated that every neuron has its own capillary and they lie in extreme close vicinity with a separation distance of approximately 8 μm (Abbott et al., 2006). The interaction between the components of the NVU including endothelial cells, astrocytes, pericytes and neurons is important for the coordination of synaptic activity and maintenance of BBB properties. Neurons and endothelial cells are known to interact through negative feedback mechanism in order to keep the brain microenvironment at optimal condition. One such cross-talk between endothelial cells

and neurons is demonstrated during disruption in cerebral blood flow. Neural signaling to endothelial cells during ischemia insult caused an increase in production of vasoactive compounds by the endothelial cells which then in turn activated the vasodilation and vasoconstriction responses to regulate the cerebral blood flow (Busse and Fleming, 2003; Sokoya et al., 2006).

Besides that, astrocytes are presumed to be a mediator for the cross-talk between the neurons and the endothelial cells. Studies have shown that astrocytes expressed various receptors for neurotransmitters and neuromodulators that are involved in the signaling pathways between neurons and astrocytes (Grosche et al., 1999; Witcher and Ellis, 2012). Astrocytes receive neuronal signals from neurons which then affects the ion homeostasis in astrocytes thus initiating a wave of release of neuroactive compounds (Banerjee and Bhat, 2007). Astrocytes are also involved in signaling to neurons for synaptic strengthening and transmitting mechanisms thus directly influencing neural circuit transmission (Kang et al., 1998; Haydon, 2001; Newman, 2003).

Pericyte, another key player of the NVU, is also involved in neurovascular coupling mechanism. Although there are still unclear data on the signaling pathways between pericytes and neurons, studies have shown that pericytes responded to electrical stimulation, ATP and GABA blockers released by neurons (Peppiatt et al., 2006). Using knockout mouse model, loss and decrease in the number of cerebral pericytes were shown to cause neuronal damages and initiated vascular-mediated neurodegeneration (Bell et al., 2010).

These highlighted studies have clearly shown that neuronal functions are regulated by components of the NVU and vice versa. This co-dependent existence of the members of the dynamic NVU emphasizes the importance of maintaining the integrity of the BBB, thus preserving the well-being of the brain.

2.3 Junctional proteins of the BBB

The BBB tight barrier regulation is due to the presence of tight junction proteins between adjacent endothelial cells. Only the specialized endothelial cells in the CNS have an extensive tight junction structure which is missing in other endothelial cells in the peripheral system. Tight junction proteins are the building blocks for BBB barrier characteristics and the dynamic structure and function of the junctional proteins are modulated by the components of the NVU including pericytes, astrocytes and neurons during physiological condition. Tight junction protein disruption is a common hallmark observed during neurovascular pathological conditions (Luissint et al., 2012).

Tight junction proteins sealed adjacent endothelial cells thus ultimately limiting the paracellular passage of blood-borne substances into the brain parenchyma. This then creates an absolute separation between the brain parenchyma and the circulating blood. The three essential transmembrane proteins that make up the tight junction of the BBB include occludin, claudins and junctional adhesion molecules (JAMs). These membrane spanning junctional proteins are then anchored to the cytoskeleton of the endothelial cells via the zonula occludens (ZO). **Figure 2.3** shows the systematic complex structure of the junctional proteins present at the membrane edge of the specialized endothelial cells.

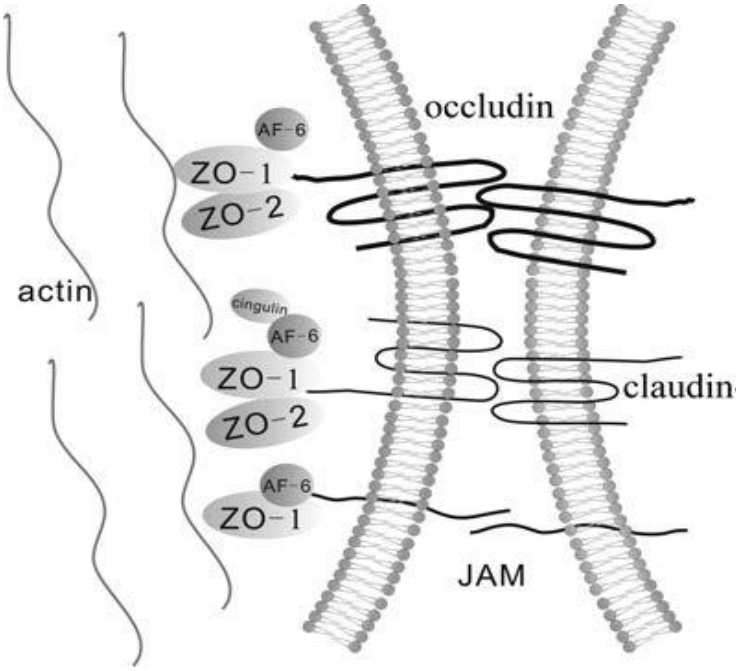

Figure 2.3. Tight junction proteins of the BBB. Occludin, claudins and JAMs are transmembrane proteins that sealed adjacent endothelial cells thus restricting the free passage of blood-borne substances into the brain parenchyma from the blood. These transmembrane proteins are anchored to the cytoskeleton of the endothelial cells via ZO thus maintaining the structure of the tight junction complex. The figure is adapted from Liu et al. (2012)

2.3.1 Occludin

Occludin, a tetra-spanning transmembrane protein of molecular mass of 60 kDa was the first protein of the tight junction complex to be identified (Furuse et al., 1993). Occludin has four membrane spanning domains, three cytoplasmic domains and two extracellular domains with the N- terminus and the C-terminus embedded in the cytoplasm (Feldman et al., 2005). Using occludin knockout mouse model, it was observed that absence of occludin did not alter the tight junction complex suggesting that occludin is not required for the formation of the tight junction (Saitou et al., 1997; Saitou et al., 2000). It was concluded that loss of occludin can be compensated by the presence of the other transmembrane proteins.

However, occludin knockout mouse exhibited gross morphological changes including chronic inflammation and calcifications around microvessels (Saitou et al., 2000). Therefore, occludin function in the tight junction network is rather complex as it is involved in regulatory mechanism for maintaining the integrity of the BBB. Besides regulating the sealing of the tight junctions, occludin is also involved in signaling pathways of the BBB as the C-terminus can bind various regulatory molecules including protein kinases (Nusrat et al., 2000). It has been hypothesized that occludin may be the initial detector of changes in the external environment which then initiates regulatory mechanism to maintain the integrity of the tight junction complex during pathological insults (Sandoval and Witt, 2008).

2.3.2 Claudins

Out of the 24 isoforms of claudins identified, six isoforms of claudins are shown to be expressed in the brain including claudin-1, -2, -3, -5, -11 and -12 although some of the observations are deemed to be debated as some of the antibody detection showed cross reactivity (Huber et al., 2001; Sandoval and Witt, 2008; Romanitan et al., 2010; Liu et al., 2012; Haseloff et al., 2015). However, consistently, it has been observed that claudin-1, -3, -5 and -12 are expressed in the brain microvessels and they have low molecular mass in the range of 20-24 kDa (Liu et al., 2012). Specifically, claudin-5 is expressed abundantly in cerebral microvessels as it is shown that the mRNA level of claudin-5 in the brain is 700 times higher compared to that of claudin-1, -3 and -12 (Daneman et al., 2010). Therefore, claudin-5 is hypothesized to be the key player in sealing the tight junction of the BBB.

Despite sharing similar four transmembrane domains structure, claudins do not share any sequence homology to that of occludin thus distinguishing them into a separate junctional protein group (Wolburg and Lippoldt, 2002). Unlike occludin, claudins have been identified to be important for the formation and maintenance of the tightness of the BBB tight junction as absence of claudins is associated with leaky BBB. Using a mice model deficit of claudin-5 expression, it was observed that the BBB selectively allowed the paracellular movement of substances that are less than 10 kDa but not most of blood-borne proteins across the BBB into the brain parenchyma, indicating its involvement in restricting the paracellular movement of substances across the BBB (Nitta et al., 2003). Loss of claudin-3 has been associated with movement of blood-borne immune cells from the blood into the brain parenchyma, initiating the damaging inflammation process in the brain during pathological conditions (Pfeiffer et al., 2011). With these observations, it is widely accepted that claudins are important for restricting the paracellular movement of substances from the blood into the brain and the loss of claudins results in selective extravasations of certain molecules into the brain.

During neurovascular pathological conditions, loss of claudins is often observed concurrently with tight junction opening of the BBB. In a study examining protein expression of human brain tumor microvessels, it was observed that claudin-1 and claudin-5 expressions were significantly down-regulated indicating loss of the integrity of the BBB (Liebner et al., 2000). In an animal model of multiple sclerosis, selective loss of claudin-3 was observed while the expression of the other isoforms of claudins remained unchanged (Wolburg et al., 2003). It is therefore important to note that the breakdown of the BBB during pathological conditions is not an all loss of tight junction proteins event but rather selective changes in the junctional protein network.

These observations are then rather interesting for further exploration on the mechanism of the changes in the protein network of the BBB during neurovascular diseases which may provide insight into possible target molecules specific for each neurovascular disease that is being scrutinized.

2.3.3 Junctional adhesion molecules

Junctional adhesion molecules (JAMs), which are of approximately 40 kDa, are enriched at the site of tight junctions and they formed the sealing between cell to cell contacts (Liu et al., 2012). JAMs belong to the immunoglobulin superfamily and they are not only expressed at endothelial cells but also at blood-borne cells including leukocytes and platelets (Bazzoni, 2011). Thus, besides forming the tight junction network, JAMs are also involved in various mechanism including inflammation, angiogenesis and vascular injury repair (Bazzoni, 2011). Three isoforms of JAMs including JAM-A, JAM-B and JAM-C or also known as JAM-1, JAM-2 and JAM-3 respectively have been identified to be expressed in the brain especially JAM-A which is predominantly expressed by cerebral endothelial cells (Sandoval and Witt, 2008). JAM-A, -B and -C are observed to be important for maintaining the integrity of the BBB by interacting with the other protein components of the tight junction network.

JAMs are observed to be involved as intermediate receptors in translating BBB pathological changes to external responses through various mechanisms including inflammation and angiogenesis, for initiating vascular damage or repair. JAM-A is shown to be involved in formation of the tight junction as expression of JAM-A causes less paracellular movement of substances across the BBB (Dejana et al., 2000). Another interesting role of JAM-A is its involvement in transmigration of leukocytes

as absence of the expression of JAM-A causes less movement of leukocytes from the blood into the brain parenchyma across the BBB (Del Maschio et al., 1999). Similar to JAM-A, JAM-B is needed for the formation of the tight junction network as JAM-B restricts paracellular movement of substances across the BBB (Wolburg and Lippoldt, 2002). JAM-C is involved in regulating monocyte trafficking across the BBB as expression of JAM-C inhibits the movement of monocytes into brain parenchyma (Palmeri et al., 2000). Overall, the JAMs are important for maintaining the structure of the BBB by excluding paracellular movement of substances across the BBB and they are involved as intermediates in regulating cerebrovascular inflammation mechanism.

2.3.4 Zonula occludens

Zonula occludens (ZOs) belong to the family of membrane-associated guanylate kinases (MAGUK). They are scaffold proteins that anchor the transmembrane proteins including occludin, claudins and JAMs to actin cytoskeleton thus maintaining the tight junction proteins in place at the tight junction network. In brain microvessels, three isoforms of ZOs have been identified including ZO-1 (220 kDa), -2 (160 kDa) and -3 (130 kDa) (Bauer et al., 2010), although detection of ZO-3 is yet to be definitely confirmed.

ZO-1 is known to bind to occludin and claudins at the N-terminus and to actin cytoskeleton at the C-terminus. Therefore, ZO-1 is important for the assembly of both occludin and claudins at the tight junction network. Loss of ZO-1 is associated with increased permeability of the BBB with dissolution of claudins (Hawkins and Davis, 2005). ZO-2, sharing homologues sequences to that of ZO-1, has similar role in

anchoring the transmembrane junctional proteins to the cytoskeleton. The role of ZO-3 as a nuclear scaffolding protein is yet to be observed. However, ZO-3 has been shown to be involved in signaling mechanism similar to ZO-1 and ZO-2 thus suggesting dual-role of ZOs as anchoring structure and signaling molecule at the BBB tight junction network (Bauer et al., 2010). The ZOs has three domains namely SH3 domain, guanylate cyclase domain and PDZ domain which are involved in signal transduction (Wolburg and Lippoldt, 2002). Signaling and regulatory molecules including protein kinases and catenins are associated with these domains, initiating a cascade of BBB signaling mechanism in response to pathological changes in the brain (Bauer et al., 2010).

2.4 Movement of substances across the BBB

The entry and exit of substances into and out of the brain is tightly regulated by the BBB. BBB acts as a checkpoint that allows only the crossing of certain substances that passes strict criteria while excluding almost most of the blood content from crossing the BBB. As rules of thumb, molecules that are lipophilic, have molecular weight of less than 400 Da and have less than eight hydrogen bonds are predicted to be able to trespass the BBB (Muehlbacher et al., 2011; Pardridge, 2012). However, this rule of thumb is not always true as studies have shown that almost 98% of small drug molecules of molecular size less than 400 Da are not able to gain entry into the brain across the BBB (Pardridge, 2005). Such highly sophisticated regulation is of uttermost importance for maintaining the homeostasis of the brain microenvironment as any distortion in the balance of the brain fluid may result in rapid deteriorating effects on the brain cells. The movement of substances across the BBB occurs via

either the paracellular or the transcellular pathways which are both highly regulated by the BBB structure (**Figure 2.4**).

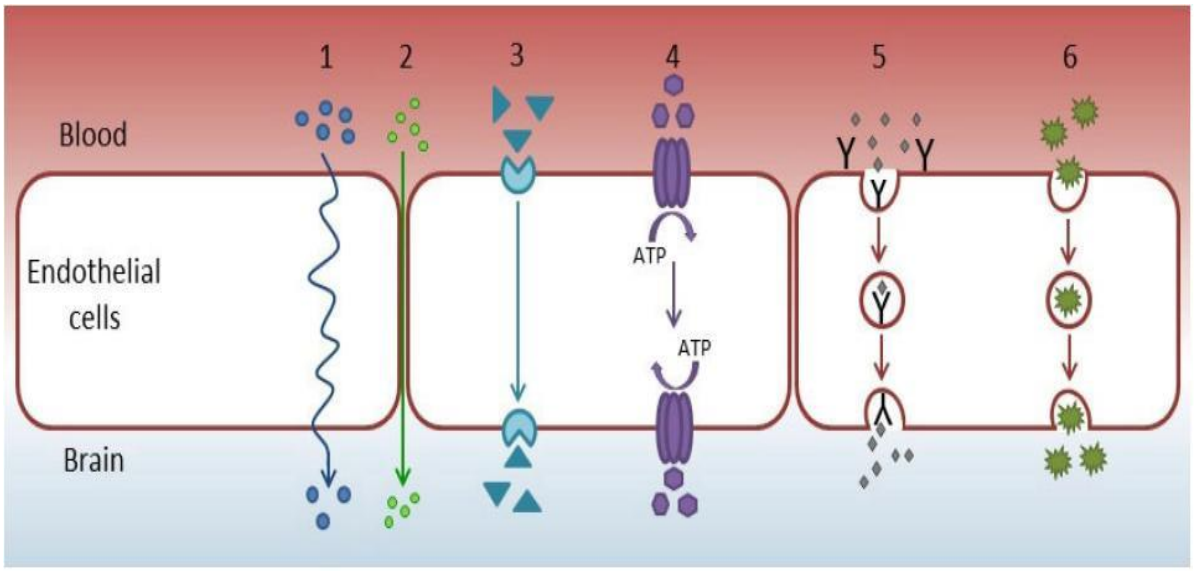

Figure 2.4. Movement of substances across the BBB. Molecules can cross the BBB either by the paracellular pathway which involves the movement through the junction between two endothelial cells (2) or the transcellular pathway which involves the movement through the endothelial cells (1). While paracellular movement involves only passive diffusion (2), transcellular movement involves passive diffusion (1), influx carrier-mediated transport (3), ATP-mediated efflux transport (4), receptor-mediated transcytosis (5) and adsorptive-mediated transcytosis (6). Figure is adapted from Cerna et al. (2016).

2.4.1 Paracellular pathway

Paracellular movement of substances across the BBB involves the movement through the junction between two adjacent endothelial cells. In physiological condition, there is almost no paracellular movement of substances across the BBB (Van Meer et al., 1986; Pardridge, 2005; Hawkins et al., 2006; Pardridge, 2012). This is due to the presence of highly intricate tight junction network including occludin, claudins and JAMs that sealed adjacent endothelial cells. As a result, it is almost impossible for molecules to transverse across the paracellular pathway. During pathological conditions, however, certain factors such as oxidative and inflammatory factors cause