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VEGF SIGNALING MECHANISMS IN INCREASED BLOOD BRAIN BARRIER  
PERMEABILITY FOLLOWING HYPOXIA

by

Brandon James Davis

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the



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**UNIVERSITY OF CALIFORNIA,  
SAN FRANCISCO**

**ABSTRACT**

**VEGF SIGNALING MECHANISMS IN  
INCREASED BBB PERMEABILITY  
FOLLOWING HYPOXIA**

by Brandon J. Davis

Signal transduction mechanisms initiated by VEGF signaling leading to tight junction loss and redistribution of ZO-1 have not been fully elucidated. One potential transducer is vasodilator stimulated phosphoprotein (VASP). Site specific phosphorylation of VASP via PKG at s239 may be critical for tight junction loss in this pathway. Here we demonstrate the localization of VASP to tight junctions and changes in VASP phosphorylation following hypoxia and VEGF signaling. We also demonstrate that these changes are dependent on VEGFR2 kinase activity. Further studies may give additional insight into molecular targets for modulating BBB integrity.

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## **Hypothesis**

Several hypotheses were evaluated in this thesis:

- VEGF signaling leads to increased blood brain barrier permeability in rbcec4 cell monolayers and primary brain endothelial cell monolayers.
- This VEGF induced permeability is mediated by the activation of VEGFR2 through autophosphorylation of the receptor dimers.
- VEGF signals VASP via the kinase activity of VEGFR2 and subsequent downstream phosphorylation of VASP.
- VASP is a component of tight junction protein complexes in these cell monolayers and can modulate barrier function.

## **Objectives**

- To isolate and maintain primary brain endothelial cells from rodent brains with negligible cellular contaminations.
- To establish an in vitro blood brain barrier model using primary brain endothelial cells and immortalized brain endothelial, rbcec4, cells.
- To validate the blood brain barrier model by measuring transendothelial electrical resistance, radioactive sucrose permeability, and endothelial marker expression.
- To study the effect of hypoxia and VEGF administration on barrier function.
- To evaluate the use of fluorescent fusion protein transfection and mutation in rbcec4 monolayer permeability models.

## **Background**

Stroke in the newborn is as frequent as stroke in the elderly (1/4000 live births), but the lifelong burden is much greater in children due to longer survival periods of children who have multiple disabilities from the event. Increased blood brain barrier (BBB) permeability after stroke can lead to cerebral edema, which can exacerbate injury. Preliminary data shows that the newborn brain has an attenuated increase in BBB permeability in the first 24 hrs after stroke when compared to the adult. This difference in BBB integrity may affect the pharmacodynamics of drugs crossing the blood brain barrier. These findings suggest that therapies aimed at tissue preservation must consider the possibility that the BBB may be impermeable early on. This leads us to further study neonatal BBB permeability after stroke and seek to understand its mechanisms and attenuation.

Vascular endothelial growth factor (VEGF) is upregulated after hypoxia and stroke, and can induce BBB endothelial cells to become more permeable. VEGF is expressed more in the normal newborn brain than in the adult brain, and may be related to developmental cerebral angiogenesis. This higher baseline level of VEGF expression may activate feedback inhibition mechanisms which attenuate VEGF signaling and paradoxically attenuate VEGF-mediated increases in BBB permeability. Consequently, when relatively large amounts of parenchymal VEGF are expressed during stroke, the responses to this excess expression are more pronounced in the adult leading to increased BBB permeability, while the response is attenuated in the newborn.

Acute cerebral ischemia during the perinatal period is a major cause of neurological morbidity in neonates. It can be manifested as global hypoxia-ischemia from diffuse brain injury like asphyxia, or focal brain injury from arterial or venous thrombosis from stroke. The

initial neuronal response to acute ischemia involves a complex integration of cell dysfunction and necrosis, followed by a second phase of delayed death after tissue reperfusion. This delayed injury compromises a significant portion of the total brain injury, and includes mechanisms involving cerebral edema, excitotoxicity, oxygen toxicity via free radical formation, and cell death signaling cascades [15].

### **Vascular Endothelial Cells**

Microvascular endothelial cells are important in the compartmentalization of body tissues.

These specialized barrier cells regulate blood and fluid extravasations into tissue parenchyma. Under appropriate inflammatory conditions these endothelial cells mediate the entrance of immunity blood cells into localized areas of body tissue. Vascular endothelial cells are also very dynamic. They must both locate and migrate to hypoxic and developing tissues through angiogenesis, and then form a structurally and hemodynamically stable conduction system for blood cells, nutrients, and metabolites throughout the body.

#### *Barrier function*

The blood brain barrier is very efficient at excluding molecules from the neural compartment. Nearly 100% of large molecules greater than 400 Da, and 98% of smaller molecules are unable to cross the BBB unassisted (Kim, Park et al. 2006).

Adjacent endothelial cells are connected by several types of junction proteins. Two types of junction complexes, adherens junctions and tight junctions, contribute to the character of the endothelial barrier. Adherens junctions directly connect adjacent endothelial cells and provide structural strength to intercellular connections.

Focal adhesions anchor the endothelium to the vessel substratum. These protein anchors contain integrins and interact with focal adhesion kinase, and also can affect barrier permeability(Wu 2005).

### *Inflammation*

Emerging evidence suggest that chemokine not only localize leukocytes to areas of inflammation , but also directly activate endothelial cells leading to BBB disruption (Stamatovic, Dimitrijevic et al. 2006).

## **Vascular Endothelial Growth Factor**

### *Discovery*

Vascular endothelial growth factor (VEGF) was earlier identified as the purified protein vascular permeability factor (VPF) (Senger, Galli et al. 1983). Even earlier experiments sought to define soluble angiogenic factors secreted from tumors through porous filter graphs (Ehrmann and Knoth 1968) .

### *Cloning*

A growth factor was identified in the media conditioned by bovine pituitary follicular and described to have mitogenic effects on endothelial cells, terming it vascular endothelial growth factor (Ferrara and Henzel 1989). VEGF is currently known to signal a large range of cellular functions.

### *Structure*

involved in undergoes post translational modifications as well as pos transcriptional gene splicing event. The larger two isoforms VEGF<sub>206</sub> and VEGF<sub>189</sub> contain heparin binding domains. These heparin binding domains can bind to the extracellular matrix proteoglycan

components heparin sulfate and heparan sulfate. VEGF<sub>165</sub> , the most dominant form, undergoes proteolytic cleavage to an activated non-heparin binding form which is freely diffusible and equipotent to VEGF<sub>121</sub> , which contains no heparin binding domain at all. VEGF dimerizes in an antiparallel, fashion as the VEGF monomers are covalently linked by two symmetrical disulfide bonds between Cys51 and Cys60 (Muller, Christinger et al. 1997) .

Ligand binding, and dimerization of the VEGFR2 results in intrinsic tyrosine kinase activity on the intracellular side of the receptor allowing communication of signal across the plasma membrane. The extracellular domain of VEGFR2 is composed of seven immunoglobulin-like domains and domains 2 and 3 have been shown to mediate ligand binding (Ruch, Skiniotis et al. 2007).

### *VEGF and Angiogenesis*

Angiogenesis is accompanied by an increase in endothelial cell permeability. This permeability allows the extravasation of fibrin and other plasma proteins into the extracellular space. This promotes the formation of a provisional matrix that can be used as a substrate for new vessel formation. VEGF the major angiogenic protein which is responsible for the induction of this permeability response.

Angiogenesis illustrates the proliferative and migratory capacities of the blood vessel endothelial cells. Endothelial cells receive many protein and molecular signals from surrounding or distant tissues. During angiogenesis developmental and regenerative signals from growing or regenerating tissues.

## Stroke and Edema

<b>Cytotoxic Edema</b>	<b>Vasogenic Edema</b>
Early Hypoxia	Late Hypoxia
Early Ischemia	Late Ischemia

### *Cytotoxic edema*

Following stroke, cytotoxic edema is caused by the intracellular accumulation of water due to energy failure with ATP depletion. ATP-dependent ion channels, particularly the Na<sup>+</sup>/K<sup>++</sup> ATPase, regulates osmotic gradients across the cellular plasma membrane, and maintain cellular volume in the normal physiological state. During hypoxia and ischemia the net movement of ions, particularly sodium and chlorine into the cell, which is then followed by net water movement into the cell, causes cellular swelling.

Cytotoxic edema is seen in stroke patients shortly after onset as a reduction in the average apparent diffusion coefficient (ADC) on a diffusion weighted imaging (DWI) magnetic resonance image (MRI) (Albers 1998).

### *Vasogenic edema*

Vasogenic edema occurs 24 hours to several days following ischemia, and is seen as an increase in the average apparent diffusion coefficient (ADC) on a diffusion weighted imaging (DWI) magnetic resonance image (MRI) (Schaefer, Buonanno et al. 1997). Vasogenic edema involves increased blood brain barrier (BBB) permeability, and the subsequent extravasation of plasma proteins and water from the intravascular compartment into the extracellular compartment of the brain. This increase in BBB permeability is mediated by endothelial cells,

and includes both transcellular and paracellular processes. Cerebral edema also correlates with midline shift in experimental stroke (Walberer, Blaes et al. 2007).

#### *Role of Aquaporin-4 in brain edema*

Aquaporin 4 (AQP4) is the predominant water channel expressed in the brain. Aquaporins primarily transport water and also other small polar molecules. AQP4 is expressed in astrocyte foot processes which surround endothelial capillaries that comprise the BBB. An important phenomenon in brain edema is the elimination of excess water. This can occur via the arachnoid villi into the CSF and venous sinus circulation, the ependymal layer into the CSF, and also into the peripheral blood circulation through the capillary BBB. AQP4 null mice have been shown to have reduced mortality from cytotoxic brain swelling following ischemic stroke and injection induced hyponatremia. These findings are thought to be due to a reduction in increased BBB water permeability caused by AQP4 activity. In contrast, AQP4 has also been shown to increase brain swelling in models of vasogenic edema, suggesting an important role of AQP4 in brain water elimination. (Papadopoulos and Verkman 2007). These data suggest AQP4 inhibition may protect the brain during cytotoxic edema formation immediately following stroke. However, AQP4 activity may be beneficial during vasogenic edema formation during the later phases of stroke.

#### *Predictors of fatal brain edema following stroke*

Nausea, vomiting and major hypodensity on CT are predictive of herniation. In patients with large middle cerebral artery (MCA) infarctions, increased fatality is associated with a history of hypertension, heart failure, increased baseline WBC count, involvement of additional vascular

territories, and early hypodensity on CT involving over half of the MCA territory. Of 201 patients with large MCA strokes 47% died of brain swelling. (Kasner, Demchuk et al. 2001).

### *Brain edema management*

In patients brain swelling from stroke typically occurs with large MCA infarcts from occlusion of the MCA stem. Brain swelling can occur late at around 4 days following stroke, or early at 24 hrs in "malignant" edema as a result of reperfusion effects. Large hypoattenuation on enhanced CT and large hypoperfusion on CT perfusion maps predicted the development of malignant edema with sensitivity and specificity greater than 90%.

Management of initial cerebral swelling involves avoiding factors that could exacerbate swelling including: free water consumption, hypoxemia, hypercarbia, hyperthermia, and anti-hypertensive agents that cause cerebral vasodilation. Also, the head of the bed can be elevated to 20-30 degrees to increase venous drainage from the brain.

Increases in intracranial pressure can become rapidly life threatening. Decompressive hemicraniectomy remains an important and life saving intervention albeit with risks of possibly poor neurological outcomes. Additional less aggressive interventions aimed at decreasing intracranial pressure include: mannitol, furosemide, and glycerol. (Adams, Del Zoppo et al. 2007).

Osmotherapy has traditionally been at the foundation for management of cerebral edema (Bhardwaj and Ulatowski 2004). Osmotic agents work through establishing osmotic pressure gradients that favor the egress of water from the extracellular compartment of the brain into the intravascular compartment. Experimental stroke models have shown significant efficacy of mannitol and hypertonic saline at attenuating water content increases in the brain following middle cerebral artery occlusion (MCAO). These same studies have also shown that



these hyperosmotic agents do not significantly alter the permeability of the blood brain barrier to albumin bound Evans blue. (Chen, Toung et al. 2006). There are studies, however, that report hyperosmotic agents, while unable to increase BBB permeability to larger Evans blue bound proteins, can indeed affect the permeability of the smaller molecules such as C14 sucrose (Brown, Egleton et al. 2004).

Hypothermia is also recently being considered as an option in controlling brain swelling, and involves a reduction in the cranial temperature to 33 degrees Celsius. (Adams, Del Zoppo et al. 2007).

### **Hypoxia and angiogenesis**

VEGF is an angiogenic peptide that is released in response to tissue hypoxia during normal development. During tissue development, underperfused and hypoxic areas of new tissue growth signals angiogenesis while increasing tissue perfusion through increased VEGF expression. This developmentally normal mild hypoxic and VEGF signaling may recapitulate some of the molecular signaling involved in preconditioning, and this can protect the neonatal brain in specific ways from stroke, specifically a resistance to loss of BBB integrity after stroke.

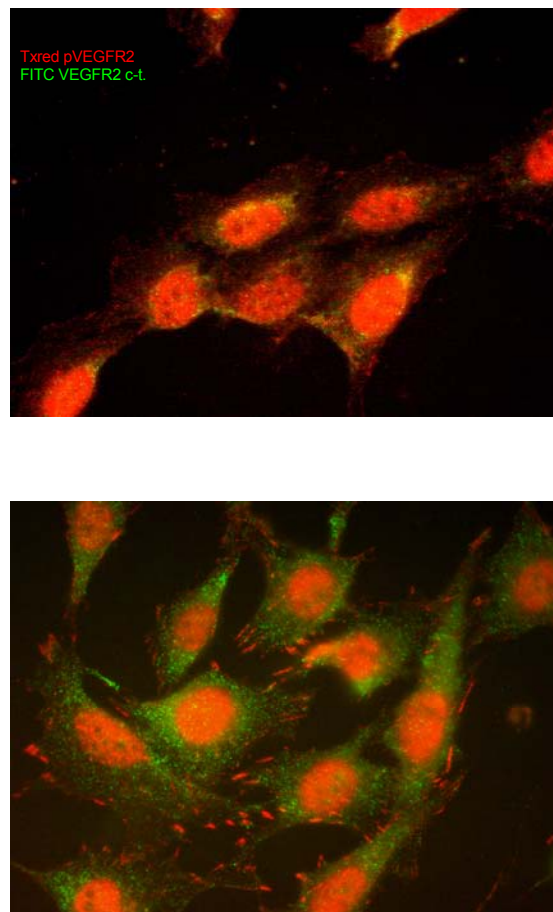
### **VEGF Receptor 1 and VEGF Receptor 2**

VEGFR2 is thought to be responsible for most biological signaling by VEGF. Autophosphorylation and tyrosine kinase activity of dimerized VEGFR2 leads to binding and activation of SH2 containing cellular proteins. VEGFR1 has poor kinase activity, but has high affinity binding of VEGF with a Kd of 10pM, versus VEGFR2's Kd of 75-125pM. VEGFR1 null mice have excessive proliferation of endothelial cells. VEGFR1 is implicated in negative regulation of VEGF signaling. Also, VEGFR1 can be secreted as sVEGFR1 to further inhibit

VEGF signaling through VEGFR2. VEGFR1 is a good candidate for modulating feedback inhibition of VEGF signaling in the neonatal BBB, as it is increased by hypoxia and VEGF stimulation.

## Figures

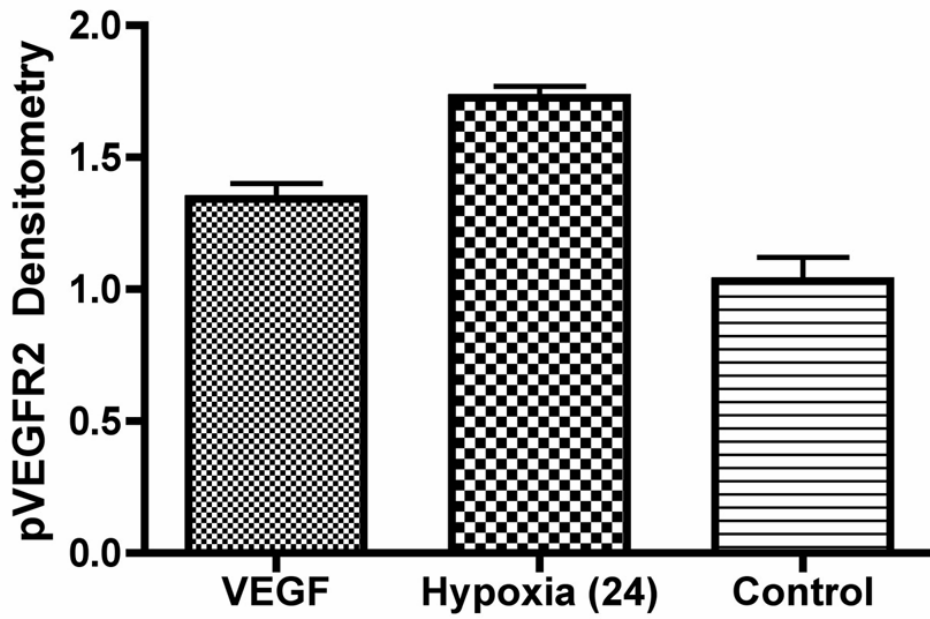
**Figure 1.**



**Fig 1.** VEGF induced rbceec4 monolayer permeability assay. Vehicle, 5, 20 and 50 nanograms of VEGF and C14 sucrose were administered to rbceec4 monolayers and media was collected

from basolateral chamber at 20, 40, and 60 min. Permeability is expressed in proportion to control.

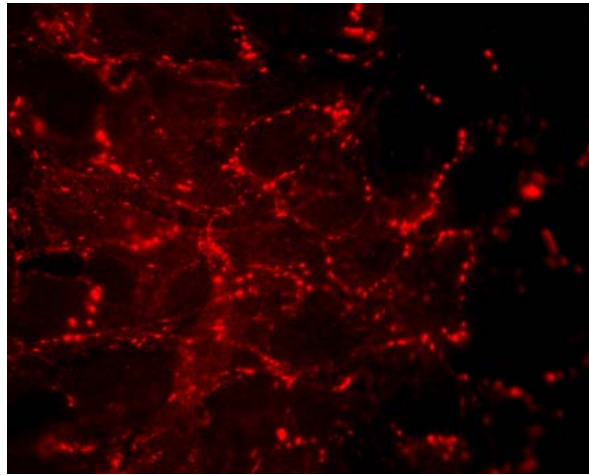
Figure 2.



B.

**Fig 2.** A. Western blot analysis using the Y1054 + Y1059 phosphospecific VEGFR2 antibody.  
Graph, densitometric plot of band intensity in proportion to control.

**Figure 3.**



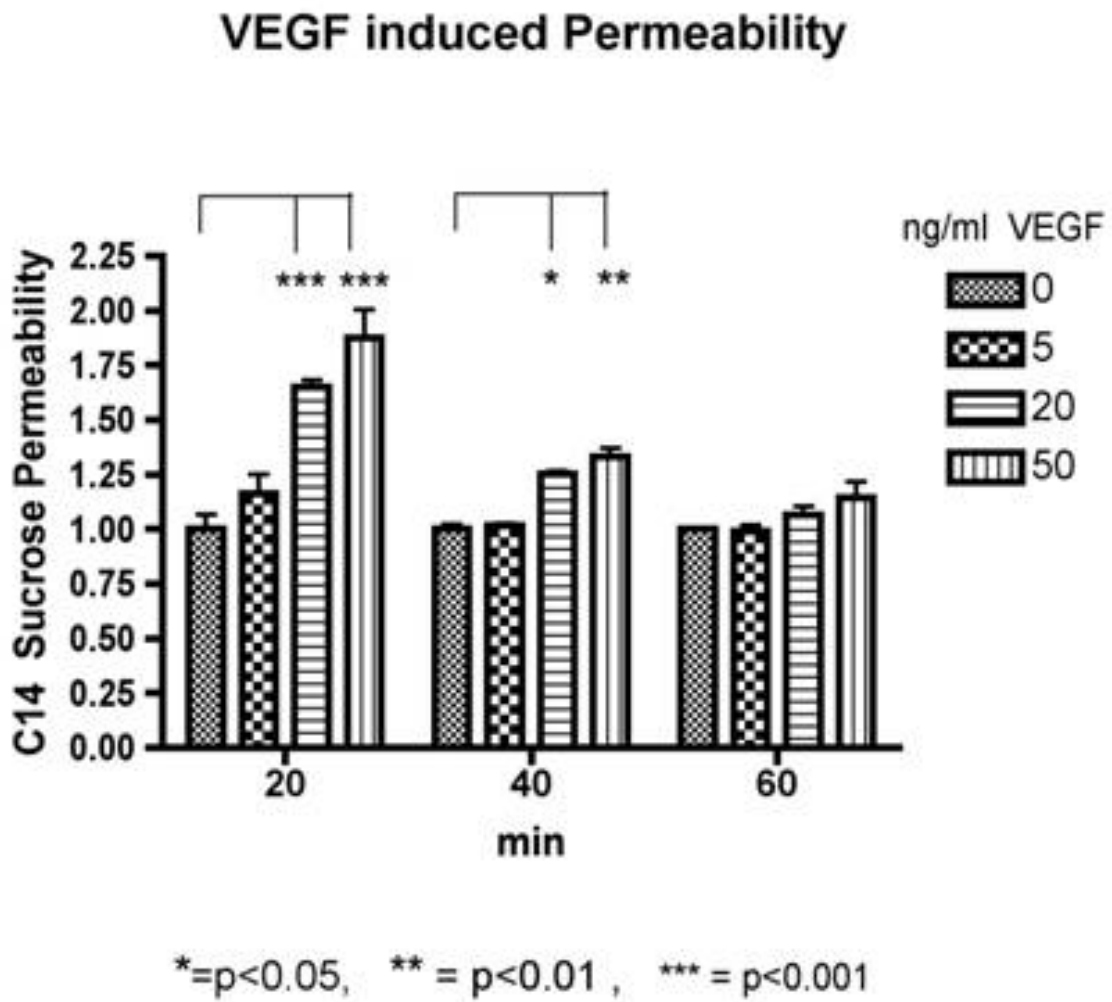
**Fig 3.** Immunofluorescent micrograph of rbcec4 cells expressing ZO-1 at tight junctions.  
Monoclonal ZO-1 (Zymed) staining shown in red.

**Table 1.**

<u>TEER</u>	<u><sup>14</sup>C DPM</u>
189	197
215	245
178	283
259	224
38	14034

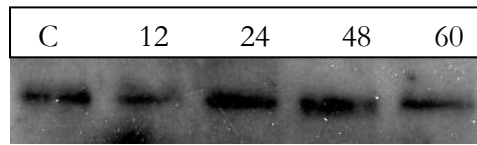
**Table 1.** Transendothelial Electrical Resistance (TEER) in ohms is shown to indicate an intact monolayer. Disintegrations per minute (DPM) measurements in subsequent 20 min C14 permeability assays confirm TEER indications.

Figure 4.

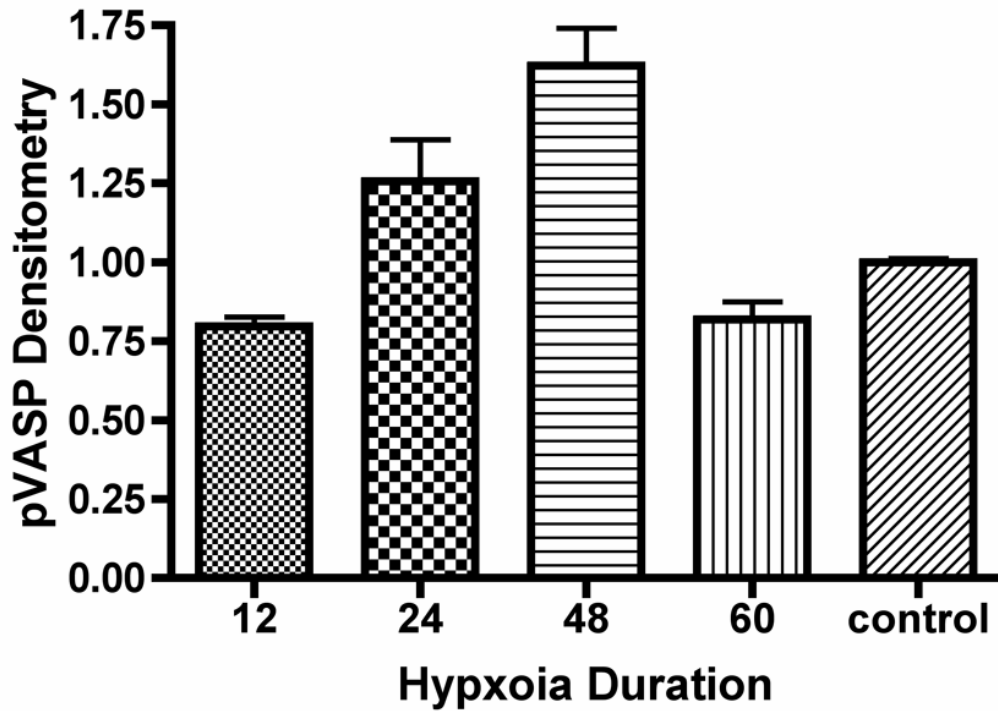


**Fig4 .** VEGF induced rbcec4 monolayer permeability assay. Vehicle, 5, 20 and 50 nanograms of VEGF and C14 sucrose were administered to rbcec4 monolayers and media was collected from basolateral chamber at 20, 40, and 60 min. Permeability is expressed in proportion to control.

**Figure 5.**



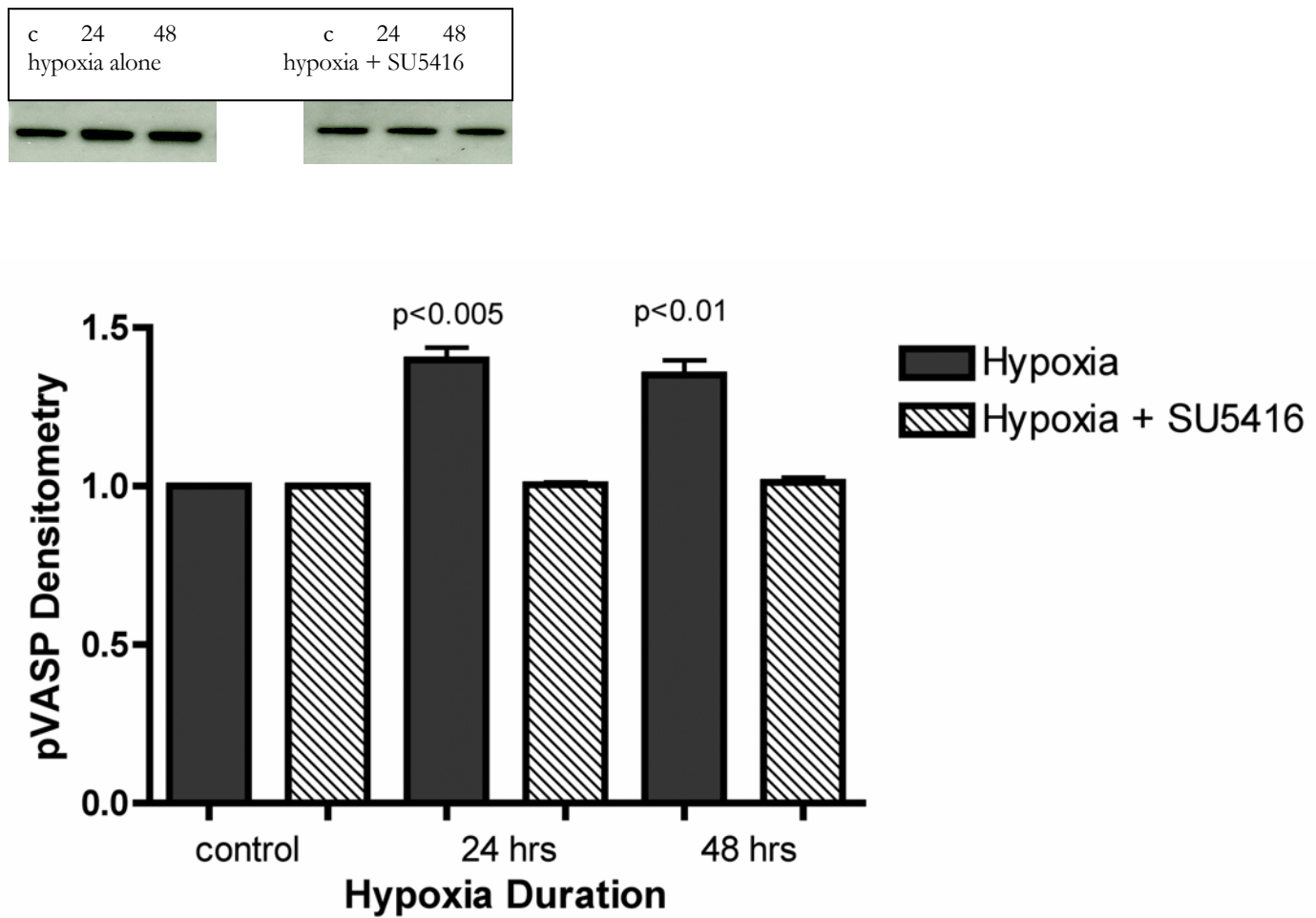
### VASP phosphorylation hypoxia time course





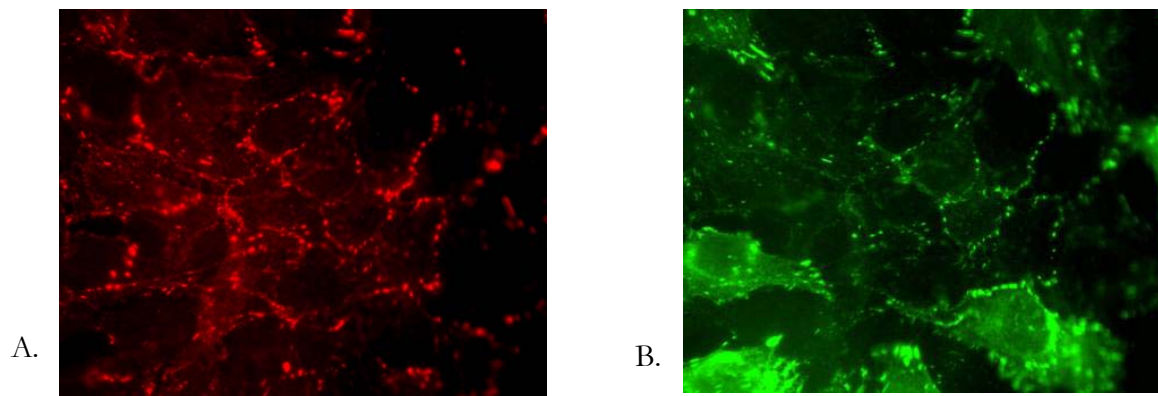
**Fig 5.** Western blot of VASP phosphorylation during hypoxia. Rbcec4 monolayers are subjected to hypoxia for 12-60 hrs. Lane C represents 60 minutes of normoxia. S239 VASP phosphospecific antibody Western blot in upper panel. Graph shows densitometry in proportion to control.

**Figure 6.**



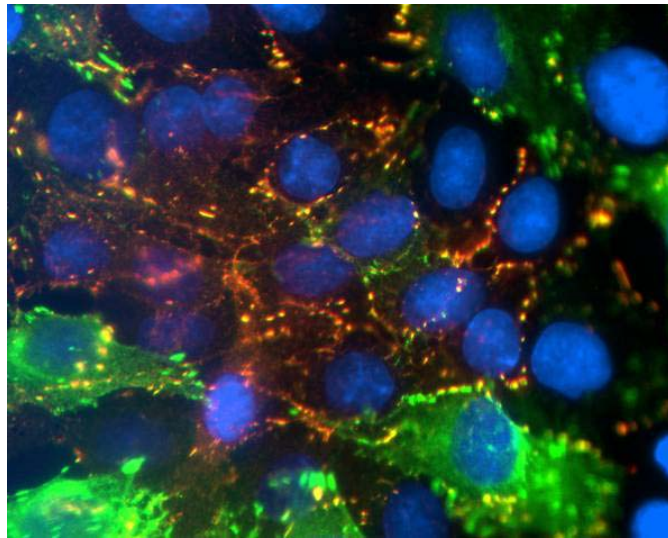
**Fig 6** Western blot of VASP using phosphospecific s239 VASP antibody. Lysate from monolayer subjected to hypoxia alone (left) or with SU5416 (right). Graph, densitometric plot as proportion of control.

**Figure 7.**



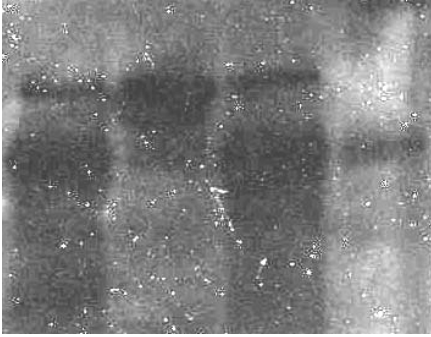
**Fig 7.** Immunofluorescent micrograph of rbcec4 cells expressing ZO-1 and VASP-eGFP. A). Monoclonal ZO-1 staining shown in red. B). Cell line containing stable transfection with VASP-eGFP shows tight junctions in green.

**Figure 7c.**



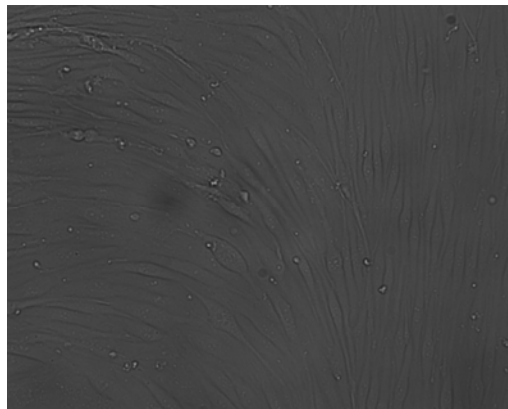
**Fig 7c.** Overlay of immunofluorescent micrographs showing co-localization. Monoclonal ZO-1 (Zymed) staining shown in red, VASP-eGFP in green, and yellow-orange show co-localization. Nuclei are stained blue with DAPI.

**Figure 8.**



**Fig 8.** co-immunoprecipitation of ZO-1 at 200 kd (arrow) using VASP polyclonal antibody.

**Figure 9a.**



**Fig 9 a.** Phase contrast of isolated rat brain endothelial cell monolayer

**Figure 9b.**

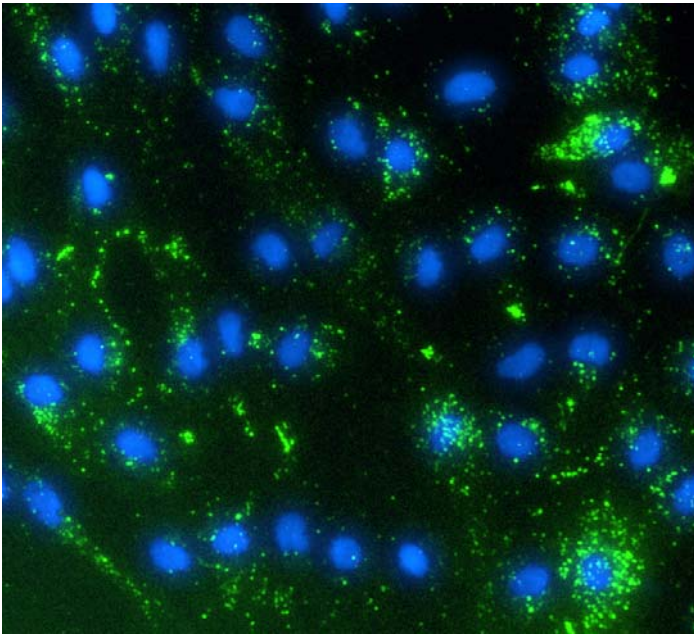
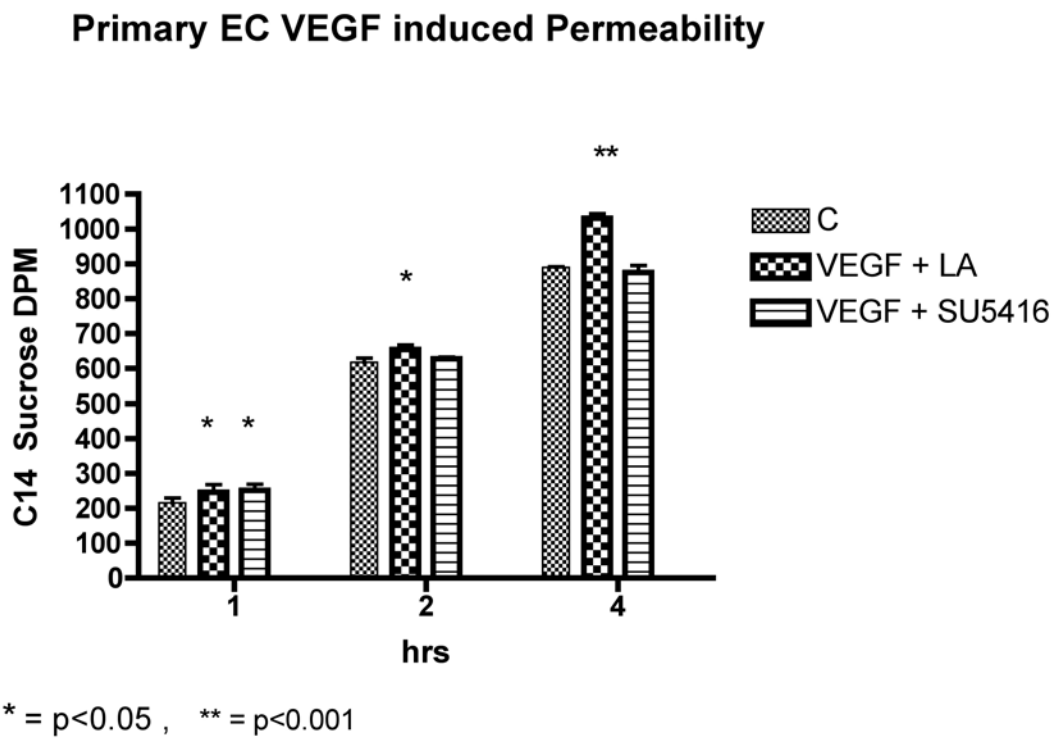


Fig 9b. Primary Rat Brain Endothelial Cells. Immunofluorescence micrograph of VWF in green and nuclei stained with DAPI in blue.

Figure 10.



**Fig 10.** Graph of diffusion of C14 sucrose across primary endothelial cell monolayer at various time points after addition of VEGF and the VEGFR2 kinase inhibitor SU5416

### **III. VEGF Signaling & Barrier Function in RBCEC4 Monolayers**

#### **Abstract**

VEGF signaling in endothelial cells induces increases in paracellular permeability in the blood brain barrier (BBB). A better understanding of the mechanisms involved in this signaling process may lead to additional targets for the consequences of increased BBB permeability following stroke. These consequences include the formation of brain edema, hemorrhagic transformation, and cerebral inflammation. We investigate the role of vasodilator stimulated phosphoprotein (VASP) in VEGF signaling and tight junction integrity using an in vitro BBB model of hypoxia. We demonstrate VASP at tight junctions with the tight junction protein ZO-1. VASP is also phosphorylated following hypoxia, and this phosphorylation is blocked by VEGFR2 inhibition using SU5416. VASP overexpression was shown to interfere with the formation of the BBB phenotype.

#### **Introduction**

The blood brain barrier responds to a variety of stimulators from other tissues and from endothelial cells themselves through autocrine stimulation. These molecular stimulators can have beneficial or deleterious consequences on the brain depending on the context, timing, and functional cellular outcomes of signaling. In the case of angiogenesis, VEGF signaling increases paracellular endothelial permeability and initiates the formation of



new vessels. In the case of cerebral stroke, early VEGF signaling increases paracellular endothelial permeability causing vasogenic edema, which contributes to damaging cerebral swelling. Studies which examine whether VEGF is beneficial or deleterious following cerebral stroke provide mixed results (see table below). This is likely due to the timing and route of VEGF administration or inhibition.

VEGF	Reagent	Route	Outcome	Model	Citation
Overexpression	VEGF containing Plasmids	Intraventricular 2hrs post	Infarct volume reduction, angiogenesis, neurogenesis.	tMCAO, peak VEGF at 3 days detectable 4hrs	(Wang, Guo et al. 2007)
Overexpression	HIF-1 responsive VEGF Plasmids	Intraventricular and intracerebral 5 days pre	Infarct volume reduction, reduced TUNEL.	tMCAO, peak VEGF at 1wk detectable 1 day	(Shen, Su et al. 2006)
Inhibition	VEGF neutralizing antibody	Topical on exposed cerebral cortex 1 hr pre	Reduced ipsilateral brain edema	MCA coagulation	(Chi, Hunter et al. 2007)
Inhibition	Adenoviral sFlt-1 vector	Intraventricular 90 min post sFlt-1 detectable at 6 hrs	Reduced infarct volume, edema, permeability, and inflammation.	Photochemical MCAO sFlt-1 exp. peak 1 day	(Kumai, Ooboshi et al. 2006)
Inhibition	VEGF neutralizing antibody	Intraperitoneal immediately post	Infarct volume reduction, vasogenic edema reduction	2 vein photochemical occlusion	(Kimura, Nakase et al. 2005)
Inhibition	VEGF neutralizing mFlt(1-3)-IgG	Intraperitoneal 12-16 hrs pre	Infarct volume reduction, edematous tissue volume reduction	tMCAO and tCCAO	(van Bruggen, Thibodeaux et al. 1999)
Increased	Recombinant VEGF	Intra-arterial, internal carotid 2hrs post	Increased hemorrhagic transformation and vessel density	tMCAO	(Abumiya, Yokota et al. 2005)
Increased	Recombinant VEGF	Intracerebroventricular infusion 1-3 days post	Infarct volume reduction, increased neurogenesis, neurological outcome	tMCAO	(Sun, Jin et al. 2003)
Inhibition	VEGF neutralizing antibody	Intraventricular infusion over 1-7 days post	Infarct volume increase	tMCAO	(Bao, Lu et al. 1999)
Increased	Recombinant VEGF	Intraventricular infusion	Infarct volume reduction, edema reduction,	tMCAO	(Harrigan, Ennis et al. 2003)

Indeed, one study which looked specifically at the timing of VEGF administration by gradual intravenous infusion following focal cerebral embolic ischemia, illustrated that early administration of VEGF, 1 hr following stroke, increased BBB leakage, ischemic lesions, and hemorrhagic transformation. Conversely, delayed VEGF administration at 48 hrs following stroke enhanced angiogenesis in injured brain and improved neurological outcome. (Zhang, Zhang et al. 2000). Additional studies suggest that intracerebroventricular

administration of VEGF may be beneficial without increased BBB leakage at early and late time points following stroke. The benefits of early administration were associated with phospho-Akt levels and not angiogenesis. In the same study, early intravenous administration once again was deleterious and increased infarct volume (Kaya, Gursoy-Ozdemir et al. 2005). In vitro studies further reveal consequences of the route of VEGF intervention. In vitro BBB models contain endothelial monolayers which are in contact with media from separate apical and basolateral compartments. These studies demonstrate a polarized ability of VEGF to increase porcine BBB monolayer permeability, with the majority of the effect being exerted from the basolateral side of the monolayer (Nitz, Eisenblatter et al. 2003). Polarized responses to VEGF seem to exist in vivo as shown by administration route, however, how these relate to in vitro results is still unclear. There are many factors to consider including effective concentrations and interactions from additional cell types.

Attempts to inhibit the permeability effects of VEGF with NOS inhibition have demonstrated limited efficacy (Chi, Hunter et al. 2005). However, inhibition of NOS could potentially have deleterious effects on hemodynamics and tissue perfusion in the injured brain, and must be carefully studied and administered.

Studying the blood brain barrier outside of the body is a scientifically and biological challenge. The relatively tight barrier characteristics unique to the BBB can be recapitulated in vitro to a useful degree. In our model of the blood brain barrier we utilize the immortalized brain endothelial cell line, rbceec4. This line is derived from isolated rat brain endothelial cells transformed with the polyoma virus large T antigen. Rbceec4 cells also retain an endothelial-like morphology and express the endothelial cell markers von Willebrand factor, alkaline phosphatase, and  $\gamma$ -glutamyl transpeptidase (Blasig, Giese et al. 2001).

Blood brain barrier monolayers isolated from bovine brains have been shown to respond to hypoxia with increases in barrier permeability and redistribution of the tight junction protein ZO-1 in BBB endothelial cells. These specific consequences of hypoxia were blocked using a polyclonal antibody to vascular endothelial growth factor (VEGF), demonstrating that VEGF is an active inducer of these effects (Fischer, Wobben et al. 2002). VEGF- induced permeability changes are dependent on several events downstream of the activation of the VEGF tyrosine kinase receptors. Particularly, permeability changes are dependent upon distinct activation cascades: the activation of phospholipase C gamma (PLC $\gamma$ ) which leads to the release of intracellular calcium, and the activation of phosphatidylinositol 3-kinase and Akt (PI3-K/Akt). These signaling events converge on the activation of iNOS leading to increased NO, activation of soluble guanylyl cylcase, increased cGMP, and activation of cGMP- dependent protein kinase also called protein kinase G (PKG). PKG has many substrates, and how its activation leads to tight junction alterations is not know (fig A). What is known about the tight junction alterations is that ZO-1 is redistributed from the cell membrane to the cellular cytoplasm following hypoxia and VEGF stimulation (Silvia Fischer 2004). We seek to further elucidate how these signaling events lead to increased barrier permeability, and specifically how the activation of PKG leads to changes in the tight junction protein localization of ZO-1.

Vasodilator stimulated phosphoprotein (VASP) is a PKG substrate that has dynamic interactions with the cytoskeletal protein actin. VASP has also been shown to interact with ZO-1 in human microvascular endothelial monolayers (HMVEC). In addition, increases in VASP phosphorylation at Ser 157 following cAMP dependent protein kinase (PKA) activation have been shown to correlate with increased barrier function (COMERFORD, LAWRENCE et al. 2002). We are interested in the role of VASP in mediating PKG signaling

following VEGF stimulation and hypoxia. Here we study VASP activity in our blood brain barrier model using the immortalized rbcec4 cell monolayers.

## Results

### *Immortalized brain endothelial monolayers form tight junctions and respond to VEGF stimulation with increased permeability.*

The blood brain barrier has several characteristics which we sought to recapitulate in vitro for our studies. Rbcec4 cells have been immortalized with the polyoma large T antigen, express the endothelial cell markers factor VIII, angiotensin-converting enzyme, and the mature blood brain barrier markers gamma-glutamyl transpeptidase and alkaline phosphatase (blasig et al. 2001). As we are studying the molecular events involved in VEGF induced BBB permeability, we sought to demonstrate: VEGF receptor expression, tight junction formation, barrier formation, and increased permeability following VEGF administration. VEGF increases BBB permeability in vivo and in primary brain endothelial cell in vitro models. This increased permeability is mediated primarily through the activation of the tyrosine kinase receptor VEGFR2 or Flk-1 (Murohara, Horowitz et al. 1998). Here we characterize the responsiveness to VEGF stimulation in the rbcec4 immortalized brain endothelial model. We demonstrate expression and activation of VEGFR2 in rbcec4 cells using antibodies to VEGFR2 and phosphorylated VEGFR2. This phosphospecific antibody detects VEGFR2 molecules which have autophosphorylated at Y1054 + Y1059 in the catalytic domain of VEGFR2. This autophosphorylation is necessary for the downstream kinase activity of activated VEGFR2 (Dougher and Terman 1999). In the rbcec4 cells upon

stimulation with VEGF there is an increase in phosphorylated VEGFR2 intensity by immunohistochemistry (fig 1), and also by western blot (fig2). Hypoxia alone also stimulated the phosphorylation of VEGFR2 (fig2). Tight junctions are the structural component of the endothelial barrier. After 5 days in vitro we see a change in cellular morphology as cells become confluent. The extension of cellular processes become less frequent as cells cease to migrate. At this time the rbcec4 have formed tight junctions with neighboring endothelial cells.

We verified the existence of these tight junctions by immunostaining for the tight junction protein ZO-1 (fig3). To assess whether our cultures have formed intact monolayers we measure transendothelial electrical resistance (TEER) only intact monolayers exhibiting TEER above 100 ohms were included in data analysis in subsequent permeability assays (table 1). To quantify the barrier function of the endothelial monolayers we measure specifically the changes in paracellular permeability by paracellular diffusion of C14 sucrose following VEGF exposure. Endothelial monolayers show increased paracellular permeability following 20 minutes of VEGF administration (fig 4).

***VASP is phosphorylated after hypoxia and this phosphorylation is blocked with a VEGFR2 kinase inhibitor in brain endothelial monolayers.***

To investigate whether VASP is involved in VEGF signaling following hypoxia, we performed western blot analysis using pVASP 239 phosphospecific antibodies. We show that VASP phosphorylation increases 12-24 hours after hypoxia (fig 5). This timeframe correlates with previous reports of tight junction breakdown, loss of ZO-1 tight junction localization, and increased BBB permeability following hypoxia. We also show that VASP phosphorylation following hypoxia is mediated by activation of VEGFR2. Western blot

analysis of rbcec4 monolayers treated with the VEGFR2 tyrosine kinase inhibitor SU5416 show a concurrent inhibition of VASP phosphorylation on s239 following hypoxia (fig 6). Together these results suggest VASP is downstream of VEGF signaling following hypoxia, specifically via its PKG substrate serine 239.

***VASP participates in the tight junction complex with ZO-1, and modulates the barrier morphology of brain endothelial cells.***

The composition of the tight junction complex formed in immortalized brain endothelial cell monolayers was investigated by immunocytochemistry. Fluorescent microscopy shows that VASP co-localizes with the tight junction protein ZO-1 at regions of cell-cell contact (fig 7). To determine a physical interaction between ZO-1 and VASP co-immunoprecipitation assays were performed. We found significant protein-protein interactions between ZO-1 and VASP demonstrating the potential for signaling interactions (fig 7). In cells highly over expressing VASP, a migratory morphology is retained, and VASP is localized to points of substrate adhesion with no ZO-1 co-localization (fig 7b,c).

These results provide further evidence that VASP can directly modulate the barrier function of brain endothelial cells, and that in cells exhibiting barrier morphology VASP localization supports a tight junction modulatory function.

**Discussion**

A better understanding of how VEGF increases vascular permeability can lead to the development of useful therapeutics that has neuroprotective effects while avoiding the risks of exacerbating cerebral edema. Regulating the permeability effects of VEGF would increase the therapeutic window for VEGF administration and would allow immediate, delayed and even prophylactic treatment options. In addition, regulating the permeability effects of VEGF could possibly allow for intra-arterial and intravenous routes of administration, which would be more expeditious options during clinical management of stroke.

In vitro blood brain barrier experiments can be useful, but its limitations must be considered. Here we verify the VEGF signaling pathway under study by demonstrating a functional response of increased permeability to VEGF administration in our blood brain barrier model using rbceec4 cell monolayers. We also verify the formation of tight junction complexes by demonstrating the localization of the tight junction protein ZO-1 at cellular contacts.

Little is know about how active signaling processes involved in endothelial barrier function modulation translate second messenger signals into physical changes in the tight junction protein complex. Here we further elucidate the connection between the intracellular consequences of VEGF signaling and tight junction alterations associated with increased BBB permeability. The intracellular signaling cascade following VEGFR2 dimerization and tyrosine kinase activity leads to the activation of several second messengers. Notably, activation of PLC and the release of intracellular calcium stores occur in parallel with PI3K/Akt activation. Subsequently, an increase in NO and cGMP ends in PKG activation. PKG has many substrates, which includes vasodilator stimulated phosphoprotein (VASP).

Here we show that VASP is located at tight junctions in BBB monolayers. This physical location supports a role for VASP in modulating the function of tight junction proteins. Particularly, because VASP interacts with the cytoskeletal protein actin, and in some cases is involved in actin polymerization, VASP has the potential of being actively involved in transmitting structural changes within the cell. We also show that VASP is phosphorylated following hypoxia and that this phosphorylation is blocked with inhibition of VEGFR2. This is additional evidence that VASP is activated following hypoxia, and that this activation is mediated by VEGF signaling.

It is also interesting that we see an effect of VASP overexpression on the behavior and morphology of transfected endothelial cells. Those cells which have marked overexpression of VASP by immunohistochemistry show a migratory phenotype against the stationary BBB background. These cells also show VASP containing adhesions to the culture substrate that do not co-localize with ZO-1. These findings suggest multiple functions for the VASP protein under varying conditions. In both conditions VASP is intimately involved in the structures necessary for specialized cellular adhesion.

#### **IV. Evaluation of Primary Brain Endothelial Cell Monolayers**

##### **Abstract**

Establishing in vitro models of the blood brain barrier (BBB) can be achieved through the isolation and purification of cerebral microvessels. Successful culture and formation of useful BBB monolayers depends largely on the purity of endothelial cultures. We demonstrate the use of pure primary rat brain endothelial cell cultures, which respond to VEGF stimulation with increased barrier permeability.



## **Introduction**

In vitro models of the BBB have been developed from cocultures between bovine, porcine, rodent, or human BMECs with rodent or human astrocytes. However, there are a number of drawbacks with these primary culture methods. BMECs rapidly de-differentiate in vitro, losing the characteristics of BBB endothelial cells after a few passages in culture, which limits their use for biochemical or permeability studies. In a primary culture, it is difficult to eliminate all nonendothelial cell contaminants (pericytes, leptomenigeal cells, smooth muscle cells). However, when contaminating cells occupy space within the monolayer, they frequently cause a hole in the layer, because the endothelial cells appear to be unable to grow over them. This becomes a limitation for studies of transendothelial permeability and transport. Also, BMEC lifespan is quite limited, which is the case with rat BMECs (Roux and Couraud 2005) . We utilize a method of isolating rat brain endothelial cells which utilizes separation by percoll density gradients, in combination with the use of specialized serum. Plasma derived serum (PDS) lacks growth factors derived from platelet activation. This prevents the further growth of pericyte and smooth muscle cell contaminants.

## **Results**

***Isolated primary rat brain endothelial cells form confluent homogenous monolayers in vitro.***

Following the extensive isolation of the brain endothelial capillaries the plating of the 4-8 cell vessel fragments results in the proliferation and migration of the endothelial cells across the substrate. Coating the plates with collagen is an important step which fosters adhesion of the capillary fragments and acts as a mitogen for the newly proliferating endothelial cells. Successful and uncontaminated cultures show uniform growth of elongated endothelial cells running in parallel to each other (fig 8).

***Primary brain endothelial cell monolayers express the endothelial marker von Willebrand Factor (VWF) and demonstrate high purity.***

Contamination of primary endothelial cell cultures with menigeal cells and pericytes is a particular problem. Contamination can cause defects in the monolayer, and confounded permeability measurements. Primary brain endothelial cell capillaries are also very slow growing. Large vessel endothelial cells and other cell types which contaminate the preparation will soon outcompete the BBB cells during culture growth. Immunostaining for von Willebrand Factor identifies the purity of the endothelial cells (fig 9). In addition, the use of puromycin prevents the growth of contaminant cells.

Passaging of these cells is very difficult and can alter the morphology of the surviving cells. We perform assays directly on cultures derived from the initial plating. Twelve days following isolation the monolayers have become confluent and have formed an effective barrier. We verify the response to VEGF by measuring monolayer permeability to c14 sucrose. This

VEGF induced permeability is also blocked with SU5416, demonstrating the importance of VEGFR2 activity for VEGF signaling (fig10).

## **Discussion**

We were able to isolate and maintain pure cultures of primary endothelial cells. These cells are isolated as capillary fragments, from which the endothelial cells proliferate and migrate into the culture dish substrate. We employed several newer culture methods to promote the growth of pure endothelial cultures. Plasma derived serum (PDS) instead of fetal bovine serum, lacks platelet derived growth factors that promote the growth of smooth muscle cells and pericytes, potential contaminants of the primary cultures. In addition, puromycin is added during plating of the capillary fragments. Blood brain barrier endothelial cells contain the drug resistance transporter p-glycoprotein, which selectively protect BBB cells against puromycin toxicity, resulting in extremely pure endothelial cultures. (Perriere, Demeuse et al. 2005). After a significant culture time of 12 days these cells form a confluent monolayer. Under confluent conditions these cells form tight junctions and effective monolayer barriers. These cells also responded with increased permeability to VEGF stimulation. This model requires substantial time and maintenance, but yields native protein expressions from genetically intact brain endothelial cells. This offers an invaluable tool for comparisons to results from immortalized endothelial cells. Also, primary brain endothelial cells display more developed barrier properties than immortalized cell lines. Demonstration of the viability and experimental proof of concept for these primary brain endothelial cells, allow us to design future experiments using this model. Used in conjunction with the faster growing and hardier

immortalized endothelial cell lines, this model will enhance our understanding of blood brain barrier signaling and function.

#### **IV. Concluding Discussion**

In this thesis we identify VASP as a mediator of tight junction integrity and barrier function in the blood brain barrier. It is important to elucidate mechanisms that involve active signaling of increased BBB permeability. The ability to modulate this active regulation can be useful for the treatment of cerebral vasogenic edema, inflammation, and efficient CNS drug delivery. Understanding the proteins and signaling events involved will yield new therapeutic targets for treatment. Also, for prophylactic treatment of cerebral edema the consequences of sealing the blood brain barrier needs investigation. This approach may predisposes to infection or have some other harmful side effect which should be considered. Before we arrive at the ability to control the BBB, further study must be undertaken on the tight junction and cytoskeletal biology that characterizes an effective barrier. Gene therapy represents a promising avenue for delivering a steady supply of native or altered proteins that could allow control over the permeability state of the blood brain barrier. VASP is situated at the intersection of actin dynamics, second messenger signaling through cAMP and cGMP, and physical tight junction protein association. Further study of this particular protein may directly or indirectly yield important events that determine the character of the endothelial barrier.

## **Methods**

Isolation and Culture of rat brain capillaries

### Preparations:

#### *Collagen coating*

24 well plates are coated with .3mg/ml Rat Tail Collagen (Roche) in sterile Hank's BSS, then incubated for 2hrs at 37°C (or 4°C overnight). Excess collagen is removed and plates are placed in a sealed plastic box containing gauze soaked in 8.5% ammonia solution, for ammonia vapor fixation. Plates are then rinsed in HBBS 3 times until HBBS remains red. Plates are left in incubator with HBBS until plating.

#### *Percoll gradients*

1.68ml 10X HBBS (w/Ca/Mg no phenol red), 13.32ml 1X HBBS(w/Ca/Mg no phenol red) and 15ml Percoll solution are mixed and poured into two 12 ml clear ultracentrifuge tubes (previously sterilized with ethanol and washed and left shaking for 20 min in dissection buffer until use). 50%Percoll mixture is centrifuged for 1hr at 25,000 X g at 4°C, using a SA-600 Sorvall fixed angle rotor. Gradients can be stored overnight at 4°C.

#### *Dissection Buffer*

150 ml HBSS (Ca/Mg free)

1.5 ml 1M HEPES

1.5 ml Antibiotics/Antimycotics

1.7 ml of 25% sterile BSA solution (add last)

add sterile NaOH dropwise until the solution returns to HBSS original color

Oxygenated by bubbling for 5min with 95%O<sub>2</sub> 5%CO<sub>2</sub>

*1 mg/ml Collagenase Dispase solution*

dissolve 100 mg Collagenase/Dipase (Roche-CCF) in 100 ml HBSS (Ca/Mg Free)

1 ml HEPES

1 ml Pen/Strep (100X)

-Sterile filter into 15 ml aliquots and store -20°C.

Just before use: Thaw and add 300 U Dnase I [20 units/ml] (Roche-CCF) (150 µl of 2000 U/ml Dnase I stock soln); and add 2.205 µg TLCK [.147 µg/ml] (sigma) (15 µl of 0.147 mg/ml TLCK stock soln)

Oxygenate for 2 min and adjust to pH 7.3

*Growth Media 100 ml*

80 ml Hams F10

16 ml Calf Plasma Derived Serum (PDS)

4 ml 2 mM Glutamine soln w/

0.3 mg ECGS (Sigma E2759)

0.32 mg Heparin grade I (Sigma)

1 ml Pen/Strep

## **Dissection**

1. 4 three month old rats are deeply anesthetized with halothane, heads sprayed with 70% ethanol and decapitated onto ice. The brains are then removed and placed into dissection buffer.
2. One at a time brains are placed in a sterile Petri dish filled with dissection buffer. The Cerebellum, hippocampus, and white matter are removed.
3. Under a dissecting microscope blood vessels and meninges are removed with fine straight forceps. Cortices are rolled on across a Kim wipe to remove surface cells.
4. This is repeated for all brains, and then cortices are minced in dissection buffer using a sterile scalpel.

## **Isolation**

1. Minced tissue is centrifuged at 600 x g for 5 min.
2. 15 ml of 1mg/ml collagen/dispase solution was made, and 10 ml was added to the pellet after the supernatant was removed.
3. The tube was incubated at 37°C for 1 hr.
4. After incubation a Pasteur pipette is used to titrate the solution to break up the larger pieces. After ~2min a Pasteur pipette is narrowed at the tip with a Bunsen burner, and the solution titrated. This is repeated with an even narrower tip until the suspension is creamy.

5. The suspension is centrifuged at 600 x g for 5 min.
6. The supernatant is pipetted off with a Pasteur and the pellet mixed with 2 ml of isotonic 25% BSA (in PBS) solution. After resuspension, 10 ml more BSA soln is added and mixed.
7. The mixture is centrifuged at 1000 x g for 20min.
8. After centrifugation the tube is rolled gently to remove the myelin from the sides (myelin will be on top). The tube is quickly tipped to remove the myelin. The tube is left upside down with the top on to prevent any myelin from sliding back down to contaminate. All traces of myelin are removed from the tube.
9. The pellet is resuspended in dissection buffer and centrifuged at 600 x g for 5 min.
10. The supernatant is removed and the pellet is resuspended in 5 ml of the remaining collagenase/dispase solution and incubated for 2 hours at 37°C.

\*\*\*\*\* 2hr \*\*\*\*\*

11. After incubation for 2-3hrs the fragments are spun at 600 x g for 5 min.
12. The pellet is resuspended in 1ml dissection buffer and carefully dribbled on top of the percoll gradient, while holding the percoll tube at an angle.
13. The gradient is then spun at 1000 x g for 10 min on low brake.
14. After spinning, a Pasteur pipette tip is lowered (while applying positive pressure to prevent aspiration of the upper layer) to the capillary layer, about 4/5<sup>th</sup> of the way down the gradient, above a band containing red blood cells, and below the single cell contaminants. Aspirate about 2ml into a clean tube.



15. 10 ml of dissection buffer is added to wash the fragments and the mixture is spun at 600 x g for 5 min.

16. The pellet is resuspended in Growth Media and cells are seeded onto collagen coated 24 well plates at 1 ml per well 50,000 cells/cm<sup>2</sup> (95,000 cells per well on 24 well plate). Plates are incubated for 7-10 days until confluent. With media changes every two days.

At the initiation of the hypoxia experiments the cell culture medium is removed and replaced with fresh medium equilibrated with nitrogen. The cells are subsequently exposed to an atmosphere of 0% O<sub>2</sub>/5% CO<sub>2</sub> and 95% N<sub>2</sub> at 37 °C for 20 hrs, using humidified Modular Incubator Chamber (Billups-Rothenberg Inc, Del Mar, CA)

Hypoxia is induced by placing endothelial cell in a modular incubator chamber (Anaerobic Chamber, Plas Labs, Lansing, MI) gassed with a mixture consisting of 4.5% CO<sub>2</sub> and 95.5% N<sub>2</sub>. The growth medium is replaced by the medium that has been equilibrated with a gas mixture consisting of 4.5% CO<sub>2</sub> and 95.5% N<sub>2</sub> for 30 min before bathing the cells. The hypoxia is maintained for 3 h for preconditioning, the duration known to induce tolerance *in vivo*.

Permeability studies with [14C]-sucrose will be used to determine paracellular flux across confluent BBB monolayers. Apical-to basolateral flux is determined by dividing the pmoles of radioactive marker appearing in the receiver chamber by the time in minutes.

## **Materials & Methods**

## IP Materials

*Reagents should be maintained at 4° C on ice.*

1X Cell Lysis Buffer (cell signaling). Add 1mM PMSF

50% (v/v) protein A–Sepharose bead (Sigma, Amersham Pharmacia Biotech) slurry in PBS containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide (NaN<sub>3</sub>).

Antibody and Control isotype.

10% (w/v) BSA

### ***Wash buffer***

0.1% (w/v) Triton X-100 (store at room temperature in dark)

50 mM Tris□ Cl, pH 7.4

300 mM NaCl

5 mM EDTA

0.02% (w/v) sodium azide

Store up to 6 months at 4°C

### **Procedure**

1. Rinse cells attached to a tissue culture plate twice with ice-cold PBS. Remove the PBS by aspiration with a Pasteur pipet attached to a vacuum trap.
2. Place the tissue culture plate on ice.
3. Add 1 ml ice-cold nondenaturing lysis buffer to the 35 mm tissue culture plate.

4. Scrape the cells off the plate with a rubber policeman, and transfer the suspension to a 1.5-ml conical microcentrifuge tube using an adjustable pipettor fitted with a disposable tip. Vortex gently for 3 sec and keep tubes on ice for 15 to 30 min.

5. Clear the lysate by microcentrifuging 15 min at  $16,000 \times g$  (maximum speed),  $4^{\circ}\text{C}$ .

6. Transfer the supernatant to a fresh microcentrifuge tube using an adjustable pipet fitted with a disposable tip. Do not disturb the pellet, and leave the last 20 to 40  $\mu\text{l}$  of supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing or addition of antibody beads.

7. In a 1.5-ml conical microcentrifuge tube, combine 30  $\mu\text{l}$  of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and 1 to 5  $\mu\text{l}$  polyclonal antiserum, or 1  $\mu\text{g}$  purified monoclonal antibody.

8. Set up a nonspecific immunoprecipitation control in a 1.5-ml conical microcentrifuge tube by incubating 30  $\mu\text{l}$  of 50% protein A–Sepharose bead slurry, 0.5 ml ice-cold PBS, and the appropriate control antibody.

9. Mix suspensions thoroughly. Tumble incubation mixtures end over end  $\square$  1 hr at  $4^{\circ}\text{C}$  in a tube rotator.

*Addition of 0.01% (w/v) Triton X-100 may facilitate mixing of the suspension during tumbling. Incubations can be carried out for as long as 24 hr. This allows preparation of the antibody-conjugated beads prior to immunoprecipitation.*

10. Microcentrifuge 2 sec at  $16,000 \times g$  (maximum speed),  $4^{\circ}\text{C}$ .

11. Aspirate the supernatant (containing unbound antibodies) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

12. Add 1 ml nondenaturing lysis buffer and resuspend the beads by inverting the tube three or four times.

13. Wash by repeating steps 10 to 12, and then steps 10 and 11 once more.

### ***Preclear lysate***

14. In a microcentrifuge tube, combine 1 ml cell lysate (from step 6) and 30  $\mu$ l of 50% protein A–Sepharose bead slurry.

15. Tumble end over end 30 min at 4°C in a tube rotator.

16. Microcentrifuge 5 min at 16,000  $\times g$  (maximum speed), 4°C.

### ***Immunoprecipitate***

17. Add 10  $\mu$ l of 10% BSA to the tube containing specific antibody bound to protein A–Sepharose beads (step 13), and transfer to this tube the entire volume of cleared lysate (from step 6 or 16). If a nonspecific immunoprecipitation control is performed, divide lysate in two  $\sim$ 0.4-ml aliquots, one for the specific antibody and the other for the nonspecific control.

18. Incubate 1 to 2 hr at 4°C while mixing end over end in a tube rotator.

19. Microcentrifuge 5 sec at 16,000  $\times g$  (maximum speed), 4°C

20. Aspirate the supernatant (containing unbound proteins) using a fine-tipped Pasteur pipet connected to a vacuum aspirator.

21. Add 1 ml ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

22. Microcentrifuge 2 sec at  $16,000 \times g$  (maximum speed),  $4^{\circ}\text{C}$ .

23. Aspirate the supernatant, leaving  $\sim 20 \mu\text{l}$  supernatant on top of the beads.

24. Wash beads three more times.

25. Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a drawn-out Pasteur pipet or an adjustable pipet fitted with a disposable tip.

*The final product should be  $15 \mu\text{l}$  of settled beads containing bound antigen.*

26. Analyze immunoprecipitates.

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