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Role of Vasodilator stimulated phosphoprotein in VEGF induced blood-brain barrier permeability in endothelial cell monolayers

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Abstract

The blood-brain barrier (BBB) plays an important role in the pathophysiology of central nervous system (CNS) disorders such as stroke and hypoxic-ischemic brain injury. Vascular endothelial growth factor (VEGF) is involved in angiogenesis and vasogenic edema during stroke and hypoxia. However, the role of VEGF in BBB permeability after hypoxia has not been fully elucidated. We therefore investigated VEGF effects in an in vitro BBB model using rbce4 endothelial cell line with the stimulation of VEGF or hypoxia. In this study, BBB permeability was studied using ¹⁴C-sucrose detection. The expression of BBB tight junction protein ZO-1, and the expression and phosphorylation of vasodilator stimulated phosphoprotein (VASP), VEGF and VEGF receptor 2 (VEGFR2) were determined using fluorescent immunocytochemistry and western blot analyses. We found that hypoxia upregulated VEGF expression, and VEGF increased BBB permeability. Hypoxia also increased VASP phosphorylation, which is mediated, in part, through VEGFR2. We also found that VASP at tight junctions was co-localized with ZO-1 in cell-cell contacts. Our findings show that VASP phosphorylation is affected by hypoxia and VEGFR2 inhibition suggesting a role for VASP in BBB permeability.

Keywords

Blood-brain barrier; VEGF; Hypoxia; VEGFR2; Permeability; Vasodilator stimulated phosphoprotein

The blood-brain barrier (BBB) plays an important role in the pathophysiology of central nervous system (CNS) disorders such as hypoxia, hypoxia-ischemia, and stroke. Increased BBB permeability after CNS disorders can lead to cerebral edema resulting in an

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exacerbation of injury (Stamatovic et al., 2008). BBB permeability can be changed in response to a variety of stimulators or stressors. These molecular stimulators, including vascular endothelial growth factor (VEGF), can exert beneficial or deleterious effects on the brain, depending on the context, timing, and functional cellular outcomes of signaling (Roux and Couraud, 2005). Early after stroke, VEGF signaling increases paracellular permeability of endothelial cells, which causes vasogenic edema and contributes to cerebral swelling (Abumiya et al., 2005). At later points, VEGF signaling initiates the formation of new cerebral blood vessels (Wang et al., 2007). Studies that examine whether VEGF has beneficial or deleterious roles following cerebral stroke provide mixed results.

The controversial effects of exogenous VEGF on brain ischemia or stroke may be due to the timing and route of VEGF administration. One study which looked specifically at the timing of VEGF administration by intravenous infusion following stroke illustrated that early VEGF administration at 1 hr after stroke increased BBB leakage. Conversely, delayed VEGF administration at 48 hr after stroke enhanced angiogenesis and improved neurological outcome (Zhang et al., 2000). Another study suggested beneficial effects of intracerebroventricular administration of VEGF following stroke was associated with activation of the PI3K-Akt pathway without affecting BBB permeability and angiogenesis (Kaya et al., 2005). Early intravenous VEGF administration in the same study was deleterious and increased infarct volume (Kaya et al., 2005).

In vitro studies further reveal consequences of the route of VEGF intervention. Studies utilizing in vitro BBB models containing endothelial monolayers which are in contact with media from separate apical and basolateral compartments demonstrate an ability of VEGF to increase BBB monolayer permeability in a directional way, with the majority of the effect being exerted from the basolateral side of the monolayer (Nitz et al., 2003). Attempts to inhibit the permeability effects of VEGF have demonstrated limited efficacy in hypoxic-ischemic brain due to a variety of factors in vivo (Chi et al., 2005). Therefore, studying the BBB in vitro is scientifically and biologically important.

Using neutralizing antibody to VEGF, monolayers of endothelial cells have been shown to respond to hypoxia with increases in tight junction permeability in a VEGF-dependent manner (Fischer et al., 2002). VEGF-induced permeability changes are dependent on downstream signal transduction mediated via activation of the VEGF receptors (VEGFR). The precise mechanisms by which VEGF signaling increases vascular permeability are not well understood and may depend on the context of the insult, but changes in ZO-1 at the tight junctions and actin reorganization appear to be major contributing factors.

Vasodilator stimulated phosphoprotein (VASP) can induce permeability changes and dynamic changes in localization of the cytoskeletal protein actin. VASP is phosphorylated by kinases partially dependent on VEGFR2 signaling, including PKA, cGMP-dependent protein kinase (PKG), and AMP-activated protein kinase (AMPK) at residues S157, S239, and T278, respectively. VASP has also been shown to interact with the tight junction protein ZO-1 in human microvascular endothelial cells (Comerford et al., 2002). It can also interact with a structural protein alpha II-spectrin (SPCN) in HUVEC cells in a ZO-1 independent manner (Benz et al., 2008). However, the roles of VASP in changes of BBB permeability in response to stressors are largely unknown. We therefore studied VASP phosphorylation following VEGFR2 inhibition or hypoxia stimulation using the immortalized rbcc4 cell monolayer.

1. Materials and methods

1.1. Immortalized Endothelial Cell cultures

The immortalized brain endothelial cell line, rbcec4 cells, retain an endothelial-like morphology and express the endothelial cell markers such as von Willebrand factor, alkaline phosphatase, and γ -glutamyl transpeptidase (Blasig et al., 2001) and therefore can be utilized as an in vitro BBB model. This cell line was derived from isolated rat brain endothelial cells transformed with the polyoma virus large T antigen. Rbcec4 cells were kindly donated by Dr. Ingolf E. Blasig (Forschungsinstitute für Molekulare Pharmakologie, Berlin, Germany). Rbcec4 cells were plated on collagen I (6 μ g/ml)-coated dishes and maintained in DME/Ham's F12 (2:1) medium supplemented with 10% heat inactivated fetal calf serum. Cultures were kept in a humidified incubator with 5% CO₂/95% air at 37°C for 5–7 days until confluence, with media changes every two days. Cells were then passaged once and grown on transwell inserts on day in vitro (DIV) 5 till monolayer formation. Monolayer integrity and tight junction formation were tested when the cells became confluent.

1.2 Hypoxia and VEGF treatments

Hypoxia was induced by placing endothelial cells in a modular incubator chamber (Anaerobic Chamber, Plas Labs, Lansing, MI) gassed with a mixture consisting of 5% CO₂ and 95% N₂. Prior to the hypoxia experiments, the growth medium was replaced by the medium that had been equilibrated with a gas mixture of 5% CO₂ and 95% N₂ for 30 min before bathing the cells. Hypoxia was maintained for different periods of time, depending on protocols.

The cells were incubated with recombinant VEGF (5–50 ng/ml, Sigma) for 20, 40 or 60 min for acute measurements of tight junction permeability.

1.3 Fluorescence immunolabeling

The presence of tight junctions in rbcec4 monolayer was determined by protein expression of ZO-1 using fluorescence immunolabeling as previously described (Mu et al. 2005). Briefly, cultured rbcec4 cells on DIV 5 were fixed with 4% paraformaldehyde for 10 min. Endogenous peroxidase was inhibited with 0.3% hydrogen peroxide in methanol at room temperature (RT) for 20 min. Cells were incubated with Clean Vision™ blocking solution (Immuno Vision Technologies Co, CA, USA) at RT for 1–2 h. After three washes with PBS, cells were incubated with monoclonal ZO-1 antibody (1:50, Zymed) at 4°C overnight. After washing with PBS 3 times, cells were incubated with TRITC-conjugated secondary antibody (1:50, Sigma) at RT for 1 h. To delineate the cellular localization and expression of VEGFR2 and phosphorylated VEGFR2 on rbcec4 cells after stimulation with VEGF, fluorescence immunolabeling was performed using Texas red-conjugated anti-phospho Y1054+Y1059 VEGFR2 antibody (1:100 Abcam) and FITC-conjugated anti-VEGFR2 c-terminus (1:100 Santa Cruz) as described above. After incubation with secondary antibodies and washing with PBS, cells were coverslipped with ProLong Gold antifade reagent (Invitrogen) and images were captured with Openlab software (Improvision, England) in both the FITC and TRITC channels. As negative controls, cells were processed in parallel without primary antibody.

1.4 VASP-eGFP transfection for rbcec4 cells

For analysis of expression and localization of VASP and ZO-1 proteins, cells were seeded overnight onto collagen-coated dishes and transfected with calcium phosphate precipitates of the eukaryotic vectors coding for EGFP-VASP constructs (pEGFP-C2, Clontech, with corresponding inserts) according to standard protocols. Forty-eight hrs after transfection, cells were washed 3 times with PBS containing 2 mM MgCl₂ and 10% glycerol and fixed

with the bifunctional membrane permeable crosslinker DSP (Pierce, Rockford, IL, USA) for 30 min at 37°C. After additional washing, cells were permeabilized with 0.2% Triton X-100 and excess crosslinker was quenched with 0.2 M glycine. Counterstaining for ZO-1 and fluorescent microscopy was performed to verify their co-localization.

1.5 Western Blot analysis

Western blot was performed as previously described (Jiang et al., 2008). Briefly, whole cell lysates were used to quantify expression of phosphorylated VEGFR2 (p-VEGFR2) and phosphorylated VASP (pVASP) using the following antibodies and conditions: VEGFR2, pVEGFR2 antibodies (1:100, overnight, 4°C; Santa Cruz), VASP, pVASP Ser-239 antibodies (1:100, overnight, 4°C; Upstate) or VEGF antibody (1:200, overnight, 4°C; Santa Cruz). The phosphospecific antibody detects VEGFR2 molecules that are autophosphorylated at Y1054 and Y1059 in the catalytic domain of VEGFR2. The signal was normalized to μ -actin expression (anti- β -actin antibody, 1:1500; Santa Cruz).

1.6 Inducible permeability of rbcec4 monolayers

Permeability studies with ^{14}C -sucrose (672 mCi/mmol, New England Nuclear Research, Boston, MA) were used to determine paracellular flux across confluent rbcec4 monolayers. Apical-to basolateral flux was determined by dividing the p moles of radioactive marker appearing in the receiver chamber by the time in minutes. The apparent permeability coefficient (PC) was calculated using the equation: $\text{PC (cm/min)} = \text{Flux}/(\text{A} \times \text{CDo})$, where flux is the slope of the line, A is the area of the membrane and CDo is the initial donor concentration of radioactive marker (Franke et al., 2000). Functional integrity of tight junctions in the monolayers was confirmed via measurement of trans-endothelial electrical resistance (TEER). TEER measurement can be easily performed with the ENDOHM-24TM chamber and the EVOHMTM voltmeter (World Precision Instruments, Berlin, Germany) as previously described (Franke et al., 2000).

1.7 Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). One-way ANOVA with Bonferroni/Dunnnett post-hoc tests was performed for multiple comparisons. $p < 0.05$ was considered statistically significant.

2. Results

2.1 Immortalized brain endothelial monolayers form tight junctions of BBB

In this study, immortal rat brain cell line rbcec4 cells were employed to set up the in vitro BBB model. Rbcec4 cells have been immortalized with the polyoma large T antigen and express the endothelial cell marker factor VIII and the mature BBB markers, gamma-glutamyl transpeptidase and alkaline phosphatase (Blasig et al., 2001). Rbcec4 cells grown to monolayers had an endothelial morphology and formed tight junctions on DIV5 as determined by expression of the tight junction protein, ZO-1, using fluorescent immunocytochemistry. ZO-1 was observed at cell-cell contacts between rbcec4 cells (Fig 1A), suggesting that these cultures could be used as an in vitro model for study of BBB permeability and function.

Functional integrity of tight junctions in the monolayers was confirmed via measurement of trans-endothelial electrical resistance (TEER) as previously described (Franke et al., 2000). Intact monolayers exhibiting TEER above 100 ohms were included in data analysis. These characteristics of rbcec4 cell are in agreement with previous reported BBB models (Deli et al., 2005).

2.2 VEGFR2 activation after stimulation with VEGF and hypoxia

To study the effect of VEGF on BBB permeability, we first determined whether VEGF could activate its receptor VEGFR2, the predominant VEGF receptor in the brain. Fig. 1B shows that both VEGF and hypoxia significantly increased VEGFR2 and phosphorylation of VEGFR2 in whole cell lysates. Compared to untreated controls, VEGFR2 increased approximately 1.4 fold and 1.3 fold, whereas phosphorylation of VEGFR2 increased approximately 1.7 fold and 1.4 fold by hypoxia and VEGF respectively (Fig. 1B, C, * $p < 0.05$, ** $p < 0.01$). We also found that hypoxia stimulates VEGF expression at approximately 1.4 fold in this system (Fig. 1. D, E, * $p < 0.05$).

We found that VEGFR2 expression was much stronger in VEGF-stimulated cells (Fig. 1G, in green) than that in DMEM control cells (Fig. 1H, in green). There is no positive staining of cells in negative controls (Fig. 1F). Phosphorylation of VEGFR2, which is necessary for the downstream kinase activity of activated VEGFR2 (Dougher and Terman, 1999), was increased in VEGF treated cells (Fig. 1E, red) compared with that seen in DMEM control cells (Fig. 1F, red). These results suggest that both VEGF and hypoxia stimulate VEGFR2 expression and phosphorylation in cultured rbcec4 cells.

2.3 BBB permeability is increased with VEGF stimulation

To determine whether VEGF increases permeability of a monolayer of rbcec4 cells to sucrose, we measured accumulation of ^{14}C -sucrose in non-stimulated cells at 20 min, 40 min and 60 min following VEGF exposure (Fig. 2). Compared to controls, there was a transient and significant increase in ^{14}C -sucrose permeability at 20 min and 40 min after exposure to 20 or 50 ng/ml VEGF (Fig. 2). At lower VEGF concentration (5ng/ml), there was no difference in ^{14}C -sucrose permeability at any time points studied (Fig. 2). Our findings suggest that VEGF could increase BBB permeability at doses higher than 20 ng/ml, and as early as 20 min following administration in this in vitro model.

2.4 VASP phosphorylation is increased by hypoxia

VEGF could increase BBB permeability but the underlying mechanism is not clear. VASP has been shown to interact with the tight junction protein, ZO-1, in human vascular endothelial cells. Increased VASP phosphorylation has been shown to correlate with increased barrier function (Comerford et al., 2002). To investigate whether VASP is involved in VEGF signaling following hypoxia, we performed Western blot analysis using pVASP S239 phosphospecific antibody. We found that VASP phosphorylation was increased when measured immediately following either 24 h and 48 h duration of hypoxia by an approximate 1.3 fold ($p < 0.05$) and 1.6 fold ($p < 0.01$), respectively (Fig. 3A). However, total VASP expression was not changed following hypoxia compared with the controls (Fig. 3A).

To test whether VEGF/VEGFR2 signaling is involved in VASP phosphorylation, Western blot analysis of rbcec4 monolayers treated with the VEGFR2 tyrosine kinase inhibitor SU5416 were performed. The induction of pVASP immediately following 24 h and 48 h of hypoxia was abolished after SU5416 administration (Fig. 3B), suggesting that VEGFR2 signaling pathway is involved in VASP phosphorylation following hypoxia and that VASP is downstream of VEGF signaling following hypoxia. Total VASP levels did not change with SU5416 treatment under hypoxia or control (Fig. 3B).

2.5 Colocalization of VASP with the tight junction protein ZO-1

Since VASP and VEGF may be involved in BBB permeability, we next investigated the relationship between VASP and ZO-1 expression using fluorescent immunolabeling for ZO-1 in VASP-eGFP transfected rbcec4 cells (Fig. 4; green-VASP and red-ZO1). VASP

expression was observed as the characteristic “chicken-wire” staining pattern, while staining for ZO-1 resulted in a nearly identical staining pattern at the regions of cell-cell contacts (Fig. 4A, 4B). Colocalization of VASP with ZO-1 to the tight junction complex (Fig. 4C; yellow) may suggest its possible contribution to modulation of BBB permeability.

3. Discussion

Here we show for the first time that VASP phosphorylation initiated by hypoxia is modulated by inhibition of VEGFR2 signaling in the in vitro rbcec4 cell BBB model. We also show that VASP and the tight junction protein ZO-1 co-localize at cellular contacts, likely contributing to the increased BBB permeability after exposure to VEGF.

In this rbcec4 BBB model, we found that VEGF can induce BBB permeability, which is consistent with previous findings that VEGF is involved in vasodilation and endothelial cell permeability (Gavard and Gutkind, 2006). Since VEGF functions through its receptors especially VEGFR2, we showed that both VEGF and hypoxia significantly increased VEGFR2 expression and phosphorylation. VEGFR2 dimerization and tyrosine kinase activity leads to the activation of several intracellular signaling cascades. Notably, the release of intracellular calcium stores occurs in parallel with PI3K/Akt activation. Subsequently, an increase in NO and cGMP activates PKG. PKG has many substrates, including VASP (Comerford et al., 2002; Fischer S et al. 2004).

We decided to focus on the downstream signaling of VASP. VASP is composed of three major domains, an EVH1 domain, an EVH2 domain, and a central proline-rich region, and contains both actin binding and actin monomer-nucleating capabilities. As a major actin binding protein, VASP has been shown to colocalize with ZO-1 at tight junctions and play a key role in establishing and maintaining barrier function (Comerford et al., 2002). Loss of VASP function results in loose associations between epithelia and failure to reseal barriers after an insult (Vasioukhin et al., 2000). Inactivation of VASP prior to hypoxia in an in vitro epithelial barrier model by siRNA increased barrier permeability whereas VASP overexpression protected from hypoxia-induced changes in permeability (Rosenberger et al., 2007). In endothelial cells, VASP can function in membrane ruffling, aggregation, and tethering of actin filaments during the formation of endothelial cell-substrate and cell-cell contacts. Data that VASP depletion is associated with the more severe loss of ZO-1 peripheral staining in response to LPS also suggest that VASP can regulate ZO-1 expression and that PKA-dependent VASP phosphorylation contributes to the protective effect against LPS-induced BBB disruption in vitro (Bogatcheva et al., 2009).

A recent study performed in HUVEC cells also revealed that patterns of subcellular VASP distribution vary, depending on cell confluence and VASP phosphorylation (Benz et al., 2008). Expression of total VASP which has been shown to interact with a spectrin isoform via the SH3 domain at sites of inter-endothelial adhesion was observed in cell-cell contacts, with almost complete spectrin isoform and ZO-1 localization (Benz et al., 2008). pS157-VASP, in turn, was mainly found at focal adhesions, with minor colocalization with spectrin isoform, suggesting multiple functions of this protein in endothelial cells (Benz et al., 2008). Data in human placenta also suggest that VEGF mediated angiogenic and vasculogenic effects could be via stimulation of VASP expression (Kayisli et al., 2002).

Our results support the hypothesis that VASP phosphorylation after hypoxia is VEGFR2 dependent since the VEGFR2 inhibitor, SU5416, showed a concurrent inhibition of VASP phosphorylation on s239 following hypoxia. Together these results suggest that VASP is a downstream effector of VEGF signaling following hypoxia, specifically via its substrate serine 239 in rbcec4 BBB model.

VASP has also been shown to interact with ZO-1, a main BBB protein in human vascular endothelial cells. In this study, VASP was phosphorylated immediately after 24 h to 48 h of hypoxia. This timeframe is in agreement with previous reports of tight junction breakdown and loss of ZO-1 tight junction localization following hypoxia in a different BBB model (Mark and Davis, 2002).

We also demonstrated that VASP participates in the BBB tight junction complex with ZO-1. VASP co-localized with the tight junction protein ZO-1 at regions of cell-cell contacts and, to a lesser extent, to focal adhesions. Since VASP interacts with the cytoskeletal protein actin and participates in actin polymerization, VASP has the potential of being actively involved in transmitting structural changes within cells (Collard et al., 2002).

It still needs to be determined whether VASP phosphorylation following hypoxia is directly involved in modulation of BBB permeability in our model. However, from our results, VASP acts downstream of VEGF/VEGFR2 signaling and suggests its possible role in VEGF-mediated vascular permeability. Our previous in vivo data from neonatal stroke model demonstrate that VEGFR2 inhibition with SU516 when given 48 hours after the stroke worsens injury and reduces endothelial cell proliferation in injured areas (Shimotake et al., 2009). Again, whether VEGFR2 inhibition has beneficial or deleterious effects on the brain depends on the stimulus, timing, cell type and pathways involved. Our data support the role of VEGF signaling in early disruption of the structural integrity of the BBB, and it is possible that VASP phosphorylation is a critical step in disrupting the structural integrity needed for cell to cell contact by increasing a migratory phenotype of the cells.

In conclusion, activation of VASP following hypoxia is mediated, at least in part, by the VEGF/VEGFR2 signaling pathway. VASP might play a role in VEGF-induced BBB permeability. The ability to modulate this active regulation might be useful for efficient CNS drug delivery and the treatment of cerebral vasogenic edema and inflammation following stroke.

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Abbreviations

BBB	blood-brain barrier
CNS	central nervous system
VEGF	vascular endothelial growth factor
VEGFR2	VEGF receptor 2
VASP	vasodilator stimulated phosphoprotein
ZO-1	zonula occludens 1
cGMP	cyclic guanosine monophosphate
PKA	protein kinase A
PKG	cGMP-dependent protein kinase
AMPK	AMP-activated protein kinase
PI3K	phosphatidylinositol-3-kinase

PBS	phosphate buffered saline
TEER	trans-endothelial electrical resistance
DIV	day in vitro
DMEM	dulbecco's modified eagle's medium

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Fig. 1. VEGF and hypoxia stimulate VEGFR2 phosphorylation in rbcec4 cells

A. Rbcec4 cells form tight junctions, as demarcated by ZO-1 immunolabeling (red). The nuclei are stained with DAPI (blue). **B.** VEGFR2 and VEGFR2 phosphorylation is increased following VEGF incubation or 24 h of hypoxia treatment, as demonstrated with Western blot. **C.** Quantification of results from 1B (n=5, 5 independent experiments). **D.** VEGF expression is increased following hypoxia, as demonstrated with Western blot. **E.** Quantification of results from 1D (n=4, 4 independent experiments). **F–H.** Both VEGFR2 (G, green, arrows) and phosphorylated VEGFR2 (G, H, red, arrowheads) expression are increased in the VEGF treated cells (G) than that seen in control cells (H). There is no positive staining in the negative controls (F).

*p<0.05, **p<0.01; Scale bar = 50 μ M

Fig. 2. VEGF increases permeability of rbce4 cells in a dose-dependent manner

Vehicle (0, medium), 5, 20 or 50 ng/ml of VEGF and C¹⁴-sucrose were administered to rbce4 cell monolayer. Media was collected from basolateral chamber at 20, 40, and 60 min after administration. Permeability is expressed in proportion to controls. 20 or 50 ng/ml of VEGF significantly and transiently increase C¹⁴-sucrose permeability at 20 min or 40 min after VEGF administration. *p<0.05, **p<0.01, ***p<0.001 vs medium controls (n=5, 5 independent experiments).

Fig. 3. Hypoxia increases VASP phosphorylation

A. Rbce4 monolayers were either subjected to hypoxia for 12–60 h or left normoxic (C, control). Western blot analysis with VASP antibody and S239 VASP phosphospecific antibody was used to detect total VASP and VASP phosphorylation (pVASP). pVASP but not total VASP is significantly induced immediately following a 24 h or 48 h exposure of the cells to hypoxia. **B.** Increase in VASP phosphorylation induced by hypoxia was abolished by pre-treatment with VEGFR2 inhibitor SU5416. Total VASP expression did not change with or without SU5416 treatment. Graph shows densitometry in proportion to control. * $p < 0.05$, ** $p < 0.01$; $n = 5$.

Fig. 4. VASP and ZO-1 expression is co-localized in rbcec4 cells

A. Rbcec4 cells with VASP-eGFP expression show tight junctions in green (arrows). **B.** ZO-1 expression in rbcec4 cells transfected with VASP-eGFP is seen in cell-cell contacts in red (arrows). **C.** Overlay of immunofluorescent micrographs shown in A and B. Colocalized expression of ZO-1 and VASP is shown in yellow (arrows). Nuclei were stained blue with DAPI. Scale bar = 50 μ M