Vascular endothelial growth factor: an attractive target in the treatment of hypoxic/ischemic brain injury

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Abstract
Cerebral hypoxia or ischemia results in cell death and cerebral edema, as well as other cellular reactions such as angiogenesis and the reestablishment of functional microvasculature to promote recovery from brain injury. Vascular endothelial growth factor is expressed in the central nervous system after injury and is involved in the process of brain repair via the regulation of angiogenesis, neurogenesis, neurite outgrowth, and cerebral edema, which all require vascular endothelial growth factor signaling. In this review, we focus on the role of the vascular endothelial growth factor signaling pathway in the response to hypoxic/ischemic brain injury, and discuss potential therapeutic interventions.

Key Words: nerve regeneration; VEGF; VEGFR; HIF1; PI3K/Akt pathway; Akt/eNOS pathway; JAK/STAT; Src-SceCKS pathway; hypoxic/ischemic brain injury; neural regeneration

Introduction
Cerebral hypoxia leads to necrosis and apoptosis, in addition to other cellular reactions, such as angiogenesis, which promote recovery from brain injury (Bhattacharya et al., 2013). The vascular endothelial growth factor (VEGF) family comprises the trophic factors VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor, and stimulates the growth of new blood vessels (Holmes et al., 2007). VEGF is expressed in the central nervous system (CNS) after injury (Dore-Duffy et al., 2007; Chaitanya et al., 2013; Leonard and Gulati, 2013) and is an important regulator of vascular leakage in the brain. Hypoxia induces the expression of VEGF, leading to the formation of cerebral edema (Bauer et al., 2010; Baburamani et al., 2013).

VEGF can reduce the damage caused by hypoxia/ischemia in a number of ways (Shimotake et al., 2010; Zhao et al., 2011; Dzietko et al., 2013). During cerebral ischemia, VEGF promotes neurogenesis (van Rooijen et al., 2010; Moriyama et al., 2013; Rosell et al., 2013), neurite outgrowth (Cesca et al., 2012), and the survival of newborn neuronal precursors. It also has an important role in increasing the size of the subventricular zone after injury (Gotts and Chesselet, 2005). Furthermore, inhibition of VEGF expression after injury may exacerbate neuronal and glial damage (Skold et al., 2006). However, increased endogenous VEGF interacts with its receptors on ischemic vessels, and contributes to the disruption of the blood-brain barrier and subsequent leakage (Zhang et al., 2011). Therefore, understanding the relevant signaling pathway of VEGF in response to hypoxia/ischemia, and devising ways to modulate it, is essential for the successful treatment of hypoxic/ischemic brain injury. In this review, we provide an overview of the VEGF signaling pathway and discuss its role in hypoxic/ischemic brain injury.

VEGF and Its Receptors (VEGFRs)
VEGF is an endothelial cell-specific mitogen and a secreted dimeric protein, and as such can induce angiogenesis in a variety of ways (Dzietko et al., 2013; Morgan et al., 2007; Holzer et al., 2013). The role of VEGF in angiogenesis is crucial for the development and regeneration/restoration of tissue, as well as for tumor formation (Morgan et al., 2007; Dzietko et al., 2013; Holzer et al., 2013). Rodent models of hypoxia/ischemia have demonstrated that angiogenesis
provides the right neurovascular microenvironment for neuro-vascular remodeling (Arai et al., 2009; Xiong et al., 2011). Lin et al. (2003) first found that VEGF promotes the outgrowth of nerve fibers on cultured major pelvic ganglia in vitro. A large number of studies have suggested that activation of the VEGF*VEGFR signaling pathway is beneficial to neurobehavioral recovery and neurovascular remodeling after hypoxic/ischemic brain injury (Shimotake et al., 2010; Zhao et al., 2011; Dzietko et al., 2013).

In most mammalian tissues, VEGF165 is the most common isoform of VEGF, existing as a heparin-binding homodimeric glycoprotein of approximately 45 kDa (Holzer et al., 2013). VEGF regulates physiological and pathological angiogenesis by binding to and activating the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) (Ho et al., 2012). In vascular endothelial cells, VEGF binds to VEGFR-1 and (predominantly) VEGFR-2, and stimulates angiogenesis in the periphery by triggering mitotic and migratory processes (Shibuya and Claesson-Welsh, 2006). VEGFR-2 is considered the major receptor for VEGF-mediated activities (Zachary and Oliki, 2001). VEGFR-1 binds to endogenous VEGF and transmits the proliferation signal for astrocytes and the vasculature (Shih et al., 2003; Krum et al., 2008; Sato et al., 2011). VEGFR-1 is upregulated almost exclusively in reactive astrocytes (Krum and Khaibullina, 2003; Khaibullina et al., 2004), while VEGFR-2 is upregulated in neurons. Furthermore, it has been shown that VEGF stimulates axonal outgrowth by binding to VEGFR-2. In situ hybridization and immunocytochemistry in adult mice revealed that VEGF promotes axonal outgrowth from dorsal root ganglia, and that the VEGFR-2 inhibitor SU5416 prevented this process (Sondel et al., 2000; Olbrich et al., 2012). These findings provide strong evidence that VEGF is necessary for the regeneration of peripheral nerves.

**VEGF and Hypoxia Inducible Factor (HIF)**

HIFs are important regulators of the transcriptional response to oxygen deprivation. In the adult hypoxic brain, the nuclear protein complex HIF-1 is the most ubiquitously expressed member of the HIF family. It is the best-characterized transcription regulator of VEGF, and binds to the consensus sequence in target gene promoters. HIF-1 is a heterodimer composed of an alpha and a beta subunit. The beta subunit has been identified as the aryl hydrocarbon receptor nuclear translocator. Hypoxia induces HIF-1 expression (Josko and Mazurek, 2004; Dery et al., 2005). Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-proteosome system, but remains stable during hypoxia. Conversely, HIF-1α is stable under normoxic conditions. The expression of HIF-1α is increased in different cell types during hypoxia-induced CNS injury (Jin et al., 2000). Furthermore, Marti et al. (2000) revealed that HIF-1 and VEGF mRNA are coexpressed in a mouse model of focal ischemia, and that the number of newly formed vessels is increased at the marginal zone of the cerebral infarction. The same group also analyzed the expression of VEGF and VEGFRs in hypoxic cells, observing a significant increase both in VEGF in the ischemic region and in VEGFRs at the border. They further found that expression of HIF-1 was also increased in the ischemic region. These results strongly suggest that the HIF-1-VEGF-VEGFR signaling pathway may be involved in the growth of new vessels after cerebral ischemic injury.

In another study, Nordin et al. (2004) used immunohistochemistry and in situ hybridization to detect the expression of the HIF-1α subunit and VEGF in the irradiated rat spinal cord. HIF-1α expression was observed in glial cells expressing VEGF (Sondell et al., 2000), and VEGF expression correlated with HIF-1α expression. A number of HIF-1α-mediated regulators of genes such as VEGF and erythropoietin may be relevant in CNS injury responses (Mu et al., 2003). In the ischemic or hypoxic brain, astrocytes are one of the main sources of erythropoietin. The pathway by which HIF-1α mediates the transcriptional activation of erythropoietin expression may promote the survival of neurons during hypoxia via an astrocytic paracrine-dependent mechanism (Fandrey, 2004). By activating the phosphatidylinositol-3-kinase (PI3K)-Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways, erythropoietin increases the secretion of VEGF in neural stem cells (Xiong et al., 2011). Upregulation of VEGF increases vascular permeability and interstitial fluid pressure, and reduces perfusion and edema. Although the precise mechanism by which VEGF increases permeability remains unclear, it may involve action on tight junction proteins or adhesion molecules (Radisavljevic et al., 2000; Fischer et al., 2002). Interrupting this secondary cycle of damage caused by VEGF upregulation may improve neuroprotective strategies against CNS radiation injury. Above all, VEGF may be involved in hypoxic/ischemic brain injury via the HIF-erythropoietin-PI3K-Akt and ERK1/2-VEGF pathways.

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**VEGF and the VEGFR-2-Akt-endothelial nitric oxide synthase (eNOS) pathway**

raumatic brain injury (TBI) remains one of the main causes of serious, long-term disability. One of the most prominent pathophysiological changes after TBI is ischemia and hypoxia in the lesion boundary area, and the volume of ischemic tissue in early focal cerebral ischemia after TBI correlates with neurological outcome (Coles et al., 2004). Following TBI, a substantial increase in angiogenesis occurs, which may provide oxygen and nutrition for cerebral reconstruction (Morgan et al., 2007). TBI-induced angiogenesis and functional recovery in the lesion boundary zone and hippocampus are improved by simvastatin, an effect which may be mediated by activation of the VEGFR-2-Akt-eNOS signaling pathway (Wu et al., 2011). In vitro, simvastatin can stimulate endothelial cell tube formation after oxygen-glucose deprivation. Simvastatin can also augment the expression of VEGF-2 in brain tissue and cultured rat microvascular endothelial cells, and this effect may be related to simvastatin-induced activation of Akt. Furthermore, simvastatin can also induce Akt-dependent eNOS phosphorylation in vivo and in vitro (Wu et al., 2011).

Many of the downstream angiogenic effects of VEGF, such as increased vascular permeability and interstitial fluid pressure, and reduced perfusion and edema, are mediated by the VEGFR-2-Akt-endothelial nitric oxide synthase (eNOS) pathway. This pathway is activated by VEGF binding to VEGFR-2, leading to the activation of the phosphatidylinositol-3-kinase (PI3K)-Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways in endothelial cells. Akt activation results in the phosphorylation and activation of eNOS, leading to the production of nitric oxide (NO), which relaxes smooth muscle cells and increases blood flow. The activation of the VEGFR-2-Akt-endothelial nitric oxide synthase (eNOS) pathway also involves the activation of the mammalian target of rapamycin (mTOR) signaling pathway, which regulates cell growth and metabolism. The combined action of these pathways promotes angiogenesis and neuroprotection after TBI.
as microvascular permeability and endothelial cell proliferation, migration and survival, are mediated by VEGFR-2 (Hicklin and Ellis, 2005). On the surface of endothelial cells, VEGF activates intracellular tyrosine kinases by binding to VEGFR-2, which triggers multiple downstream signals to stimulate angiogenesis. Among these, Akt-dependent eNOS phosphorylation is essential for angiogenesis (Kureishi et al., 2000). Phosphorylation of the protein kinase Akt plays a crucial role in multiple cellular and physiologic effects (Parcellier et al., 2008). The pro-survival effects of Akt include anti-apoptosis, angiogenesis, and neuroprotection after brain injury (Kilic et al., 2006; Shein et al., 2007). In a TBI study (Thau-Zuchman et al., 2010), the effects of VEGF on brain recovery and function were examined by infusing ectogenic VEGF into the lateral ventricles of mice for 7 days after TBI. VEGF had multiple effects, including promotion of neurogenesis and angiogenesis, neuroprotection, and improvement of functional recovery by mediating phospho-Akt signaling (Thau-Zuchman et al., 2010). eNOS is a downstream mediator of VEGFR-2 and is critical for angiogenesis (Fischer et al., 2002). eNOS mediates vasodilation after hypoxic/ischemic episodes by increasing blood flow (Bolanos and Almeida, 1999). Nitric oxide is synthesized by eNOS and is an essential component of the pathological and physiological response to hypoxia/ischemia (Kaur and Ling, 2008). Simvastatin administration can activate Akt-GSK-3 and enhance phosphorylation of eNOS in the TBI model (Thau-Zuchman et al., 2010), and phospho-eNOS in turn induces a series of downstream effects, such as angiogenesis and recruitment of mural cells to immature angiogenic sprouts (Kashiwagi et al., 2005).

VEGF and the VEGFR-2-PI3K-Akt pathway

The pro-angiogenic effects of VEGF are thought to be attributed to VEGFR-2, and the protective effect of VEGF on cerebral cortical neurons may involve VEGFR-2 dimerization to form a receptor complex with neuropilin-1 (Sato et al., 2011). Class IA PI3K and its downstream effector Akt are enabled by the activation of VEGFR-2 (Koch et al., 2011). The PI3K-Akt pathway is crucial for many VEGF-dependent effects, including cell survival and migration, and vasopermeability (Olsson et al., 2006). The VEGF-PI3K-Akt pathway is not only involved in endothelial permeability in vitro (Pedram et al., 2002), but is also attributed to neuroprotection and blood–brain barrier permeability in a mouse model of focal cerebral ischemia. Furthermore, these effects are dependent on VEGFR-2 (Hicklin and Ellis, 2005). This pathway may contribute to the maintenance of mitochondrial function under conditions of tissue oxygen deficit. Akt is activated by phosphorylation of the Bcl-2-associated death promoter, which increases the removal of Bcl-xL from mitochondria, blocks the formation of the mitochondrial permeability transition pore, and maintains the mitochondrial membrane potential. In addition, Bcl-xL depresses the activity of caspases 9 and 3 by blocking the release of cytochrome c from injured mitochondria, thereby restraining DNA cleavage (Cheng et al., 2010; Wu et al., 2011).

The specific mechanism by which VEGF-VEGFR-2 activates PI3K-Akt is still unclear, but a recent report suggested that the receptor tyrosine kinase Axl may be responsible for VEGF-A-dependent activation of PI3K/Akt (Ruan and Kizlauskas, 2012).

VEGF and the JAK-STAT pathway

The Janus kinase (JAK) family comprises four non-receptor tyrosine kinases, JAK1, JAK2, JAK3 and TYK2. The first three are widely expressed in various tissues and cells, but TYK3 exists only in the bone marrow and lymph system. Signal transducer and activator of transcription (STAT) is the substrate for JAK. The STAT family comprises seven latent cytoplasmic transcription factors that are involved in signal transduction mediated by cytokines and growth factors. The JAK-STAT pathway is downstream of the cytokine receptors. Activation of these cytokine receptors initiates JAK phosphorylation and activation, which in turn phosphorylates STAT. Following tyrosine phosphorylation, STAT proteins dimerize through the nuclear membrane into the nucleus, where they combine with genomic regulatory sequences and enhance the transcription of related genes (Lai and Johnson, 2010).

In mammals, the JAK-STAT pathway is considered to be the major signaling mechanism for a number of growth factors and cytokines (Ihle and Kerr, 1995), and mediates a wide variety of biological functions in the CNS including the regeneration of peripheral nerves (Bella et al., 2006; Lin et al., 2006; Xu et al., 2009), and may also be involved in axon regeneration and in the proliferation and migration of Schwann cells after sciatic nerve injury (Xu et al., 2009).

To date, most studies on the interaction between VEGF and the JAK-STAT pathway have focused on tumors (Roorda et al., 2010) and cellular invasiveness. Whether VEGF can directly activate the JAK-STAT pathway to promote neurite outgrowth has not been examined. VEGF may achieve this by promoting angiogenesis or by binding with JAK-STAT directly, similarly to combining with neurotrophic factors. However, activation of STAT3 can increase VEGF expression, which indicates that another signaling pathway may be involved (Wang et al., 2010). VEGF expression can be induced by latent membrane protein 1 via both the JAK-STAT and mitogen-activated protein kinase (MAPK)-ERK pathways (Wang et al., 2010). Furthermore, the increased expression of VEGF induced by elevated phosphorylation of STAT3 after nerve injury may further sensitize the JAK-STAT pathway (Bella, 2007). Therefore, VEGF may interact directly or indirectly with the JAK-STAT pathway. Further understanding of the interaction between VEGF and the JAK-STAT pathway will be beneficial to develop new therapies for neuronal recovery.

VEGF and the Src-SSeCKS pathway

The non-receptor tyrosine kinase Src is another protein considered to be associated with angiogenesis (Theus et al., 2006; Tang et al., 2007). The activity of Src kinase increases significantly during transient global ischemia (Schlessinger,

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Table 1 VEGF pathways involved in hypoxic/ischemic brain injury

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<th>Signaling pathway</th>
<th>Role in hypoxic/ischemic brain injury</th>
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<tr>
<td>VEGF and VEGFR-1/2 pathway</td>
<td>Promotes angiogenesis, increasing vascular permeability and expression of tight junction proteins.</td>
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<tr>
<td>VEGF and HIF-1 pathway</td>
<td>Acts as a neurotrophic factor, transmitting the proliferation signal for astrocytes and the vasculature, and stimulating axonal outgrowth.</td>
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<tr>
<td>VEGF and VEGFR-2-PI3K/Akt pathway</td>
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<td>VEGF and JAK-STAT pathway</td>
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<tr>
<td>VEGF and Src-SScEKS pathway</td>
<td>Promotes neurite outgrowth.</td>
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VEGF: Vascular endothelial growth factor; HIF-1: hypoxia inducible factor-1; VEGFR-1/2: vascular endothelial growth factor receptor-1/2; PI3K: phosphatidylinositol-3-kinase; Akt: protein kinase B; JAK: Janus kinase; STAT: signal transducer and activator of transcription; SScEKS: Src-suppressed C kinase substrate.

2000) and this effect is associated with increased vascular permeability mediated by VEGF (Paul et al., 2001; Zan et al., 2014; He et al., 2015). Mice lacking the Src subtype pp60c-Src are resistant to this increase in VEGF-induced vascular permeability and have smaller infarct volumes after stroke. However, mice lacking pp59c-Fyn, another member of the Src family, do not show these effects (Paul et al., 2001). VEGF induces endothelial activation and vascular leak mainly via VEGFR-2 (Mason et al., 2004) and Src (Eliceiri et al., 1999). Src-suppressed C kinase substrate (SScEKS) is widely expressed in astrocyte-like, neuron-like and endothelial-like cells (Zan et al., 2011). Under ischemic conditions, Src and SScEKS can regulate the expression of VEGF (Lee et al., 2003; Zan et al., 2011). Inhibition of Src decreases VEGF-induced vascular permeability and infarct volume (Bella et al., 2007) and alleviates brain edema and injury (Akiyama et al., 2004; Lennmyr et al., 2004). Accordingly, inhibitors of VEGF or Src kinases can reduce the edema and tissue injury following myocardial ischemia injury (Weis et al., 2004). The inhibition of SScEKS by Src and its regulation of angiogenesis and vascular permeability may be achieved by regulating VEGF and tight junction proteins after ischemic brain injury. Src and SScEKS may have opposing effects on angiogenesis and vascular permeability after focal cerebral ischemia, and angiogenic factors are involved in this process by serving as downstream mediators (Paul et al., 2001). Furthermore, modulation of angiogenesis and vascular leakage by the Src-SScEKS pathway helps improve recovery after focal cerebral ischemia (Bai et al., 2014).

Conclusion

Hypoxic/ischemic brain injury causes severe brain damage, but the specific mechanisms underlying the pathophysiology of such injury, and preventive measures, remain unclear. Cerebral hypoxia/ischemia results in widespread responses at the systemic and cellular levels and regulates many physiological and pathological processes. The upregulation of VEGF is considered to be a crucial stimulus for these processes. As a hypoxia-induced angiogenic protein, VEGF plays a double-edged role in the central nervous system. Based on its trophic influence on neurons and vascular cells, it is a promising candidate for brain injury treatment. Accumulating evidence implicates VEGF in cerebral hypoxia/ischemia via the HIF-1, VEGF-R2-PI3K-Akt, VEGF-R2-Akt-eNOS, JAK-STAT, and Src-SScEKS pathways (Table 1). Thus VEGF becomes an attractive target for the treatment of hypoxic/ischemic brain injury. A variety of therapeutic strategies targeting VEGF are currently in the research pipeline, but most of them are in the experimental stages. Creatinine may be an effective treatment against cerebral hypoxia/ischemia, increasing the expression of VEGF and mediating neovascularization in the ischemic zone (Pedram et al., 2002); however, the downstream intracellular signaling pathways mediating these effects remain unclear. A better understanding of the VEGF signaling pathway will improve therapeutic advances for hypoxic/ischemic brain injury.

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