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Vascular endothelial growth factor regulates adult hippocampal cell proliferation through MEK/ERK- and PI3K/Akt-dependent signaling

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic protein that exhibits a broad range of neurotrophic and neuroprotective effects in the central nervous system. Given that neurogenesis occurs in close proximity to blood vessels, increasing evidence has suggested that VEGF may constitute an important link between neurogenesis and angiogenesis. Although it is known that VEGF can directly stimulate the proliferation of neuronal progenitors, the underlying signaling pathways responsible in this process are not fully understood. Thus, in the present study, we set out to examine the requirement of two downstream targets of the VEGF/Flk-1 signaling network, the phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) pathways, in producing the mitogenic effects of VEGF. Both *in vivo* and *in vitro* experiments showed that a single treatment of VEGF activated Erk1/2 and Akt signaling pathways in the adult rat hippocampus and in cultured hippocampal neuronal progenitor cells. This effect was blocked with the VEGF/Flk-1 inhibitor SU5416. Importantly, microinfusion of VEGF into the rat brain also induced pCREB expression in the dentate gyrus and increased the number of BrdU-labeled cells in the dentate subgranular zone. Double immunofluorescence labeling revealed that a large proportion of BrdU-labeled cells expressed activated forms of Flk-1, Erk1/2, and Akt. Interestingly, treatment with the SSRI fluoxetine, which is well known to stimulate neurogenesis and VEGF-signaling, also produced a similar expression pattern of Erk1/2 and Akt in proliferating cells. Finally, pharmacological experiments showed that administration of inhibitors of either MAPK/ERK (U0126) or PI3K (LY294002) blocked VEGF-stimulation of hippocampal cell proliferation *in vivo* and *in vitro*. Taken together, our findings demonstrate that the proliferative actions of VEGF require activation of both ERK and Akt signaling cascades and that these intracellular pathways are stimulated almost exclusively in actively proliferating neuronal progenitor cells of the adult hippocampus.

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1. Introduction

Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and key regulator of angiogenesis (Leung et al., 1989; Yancopoulos et al., 2000). In addition to its well-established angiogenic effects, recent evidence has revealed an important role for VEGF in exerting trophic and protective actions on neurons. For example, VEGF stimulates neurite outgrowth and survival of superior cervical, dorsal root ganglion, and cortical neurons in culture (Jin et al., 2006; Khaibullina et al., 2004; Sondell et al., 2000), and protects both HN33 (mouse hippocampal neuron × neuroblastoma) and cortical neurons against cell death induced by hypoxic conditions (Jin et al., 2001, 2000; Li et al., 2005). Conversely, VEGF reduction triggers apoptosis of cultured cortical and hippocampal neurons (Matsuzaki et al., 2001; Ogunshola et al., 2002), and contributes to adult-onset motor neuron degeneration in mice (Oosthuyse et al., 2001). In light of these diverse effects, there has been increasing interest in the development of VEGF for the treatment of various neurodegenerative conditions, such as traumatic brain injury, amyotrophic lateral sclerosis, and stroke (Hermann and Zechariah, 2009; Skold and Kanje, 2008).

In the adult mammalian brain, the dentate subgranular zone (SGZ) and subventricular zone of the lateral ventricle are active sites of neurogenesis (Altman and Das, 1965; Cameron and McKay, 2001; Kempermann et al., 2004). It is well known that signals provided by the local microenvironment regulate the proliferation and differentiation of neural stem/progenitor cells (Suh et al., 2009). Of the proposed regulators, the vasculature represents an important candidate in providing the required molecular signals

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and metabolic demands necessary for maintaining neuronal progenitor pools throughout life. Consistent with this view, neurogenesis has been shown to occur in close proximity to growing blood vessels in the SGZ (Palmer et al., 2000), and accumulating evidence suggests that endothelial cells can influence neural stem/progenitor cell proliferation through the release of various growth factors (Li et al., 2006; Louissaint et al., 2002).

Several studies have found that VEGF can act as a direct stimulator of neurogenesis (Jin et al., 2002; Schanzer et al., 2004). VEGF exerts its biological functions through several receptors, among them VEGFR-2 (Flk-1) is believed to mediate most of the neuron-specific effects of VEGF, including neurogenesis (Ruiz de Almodovar et al., 2009), although there is recent evidence that VEGFR-1 (Flt-1) and VEGFR-3 (Flt-4) also regulate neurogenesis in the subventricular zone and dentate SGZ (Calvo et al., 2011; Wittko et al., 2009). While the exact contribution of VEGF-stimulated neurogenesis in the adult brain is unclear, a large number of studies have shown that VEGF expression is increased, particularly in the hippocampus, after various pro-neurogenic stimuli. For example, hippocampal VEGF expression is upregulated in response to antidepressant treatment, and VEGF signaling is required for the neurogenic as well as the behavioral effects of these drugs (Fournier and Duman, 2011; Greene et al., 2009; Lee et al., 2009; Warner-Schmidt and Duman, 2007). VEGF is also required for the increased cell proliferation and neurogenesis that occurs after adult mice are exposed to environmental enrichment (Cao et al., 2004) or exercise (Fabel et al., 2003), while conditions that reduce hippocampal cell proliferation, such as aging or stress, are associated with reduced levels of VEGF and Flk-1 in the hippocampus (Heine et al., 2005; Shetty et al., 2005).

Although these findings highlight the importance of VEGF/Flk-1 signaling in cell proliferation, the precise downstream intracellular signaling pathways mediating this effect on neuronal progenitor cells remain to be determined. In the present study, we set out to examine the requirement of two downstream targets of the VEGF/Flk-1 signaling network, the phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) pathways, in producing the mitogenic effects of VEGF in the adult hippocampus. Our results reveal that VEGF increases adult hippocampal cell proliferation through activation of ERK and Akt signaling cascades, and that VEGF stimulates proliferation by directly acting on neuronal progenitor cells both *in vivo* and *in vitro*.

2. Materials and methods

Male Sprague–Dawley rats (Charles Rivers) weighing between 175 and 250 g at the time of arrival served as subjects. They were housed in pairs in rectangular polypropylene cages with standard laboratory bedding and kept on an artificial 12:12 h light:dark cycle with lights on at 0700 h local time. Ambient temperature in the housing facility was maintained at 20 °C (±1 °C). Food and water was available *ad libitum* throughout the duration of the experiment. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by the Yale University Animal Care and Use Committees. All efforts were made to minimize the number of animals used in these experiments.

2.1. Drugs

Drugs used included human recombinant $VEGF_{165}$ (Sigma–Aldrich), U0126 (10 mM, 4.3 μg/μl, Cell Signaling), LY294002 (10 mM, 4.6 μg/μl, Cell Signaling), and SU5416 (4 mM, 2.4 μg/μl, Sigma–Aldrich). All drugs were prepared according to the manufacturer's specification in either phosphate buffered saline (pH 7.2) or dimethyl sulfoxide (DMSO), and stored at −20 °C until use. Bromodeoxyuridine (BrdU; 150 mg/kg, 20 mg/ml, Sigma–Aldrich) was dissolved in warm physiological saline (50 °C) and then sterile filtered before administration.

2.1.1. Surgical and microinfusion procedure

After one week of habituation to the animal colony, rats were anesthetized with a ketamine (80 mg/kg, i.m., Fort Dodge Animal Health)-xylazine (6 mg/kg, i.m., Lloyd Laboratories) cocktail and placed into a stereotaxic apparatus. A single guide cannula (22 Ga, Plastic One) was inserted into the lateral ventricle (−0.9 mm anteroposterior, ±1.5 mm mediolateral, and −3.3 mm below dura). The cannula assembly was secured to the skull with four stainless steel screws and dental acrylic, and each animal was fitted with a dummy cannula to prevent the accumulation of debris.

Following a 7–9-day recovery period, compounds were delivered i.c.v. in a 2 μl volume and at a flow rate of 0.25 μl/min. The infusion cannula was left in place for an additional 3 min after delivery before slowly being withdrawn to facilitate diffusion of the compound and to prevent back-filling of the guide. Following the last infusion, animals were sacrificed at various time points according to the purpose of the experiment. For inhibitor experiments, compounds (e.g., DMSO, U0126, LY294002, or SU5416) were delivered 30 min before VEGF or vehicle (PBS) infusion.

2.1.2. Western blot analysis

Dissected hippocampal samples were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 1 mM sodium vanadate, 10 mM NaF, and 1X protease inhibitor cocktail. Protein concentration was determined by BCA assay (Pierce Biotechnology). For Western blotting, equal amounts of protein (10–30 μg) were loaded and separated on a 7.5% or 10% SDS-PAGE gel. To facilitate normalization of band intensities across different gels, the same control samples were loaded on all gels. After electrophoresis, the proteins were electrically transferred to nitrocellulose membranes. Following electro-transfer, membranes were blocked for 1 h in 5% bovine serum albumin in TBS-T (TBS + 0.1% Tween-20) and incubated overnight at 4 °C with primary antibody. The following primary antibodies were used: phospho-Akt (Ser473, 1:1000, Millipore), total Akt, phospho-ERK (Thr202/Tyr204, 1:1000, Millipore), phospho-CREB (Ser133, 1:1000, Millipore), total CREB (1:1000, Millipore), and GAPDH (1:10,000, Millipore). Following incubation, membranes were washed in TBS-T and incubated for 1 h with an appropriate peroxidase-labeled secondary antibody (1:10,000; Vector Laboratories). Bands were visualized with enhanced chemluminescence and exposed to Hyblot CL autoradiography film (Denville Scientific Inc.). Membranes were stripped (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris–HCl, pH 6.8) for 30 min at 50–55 °C and then received several washes with TBS-T. The stripped membranes were placed in blocking solution for 1 h and incubated with a primary antibody direct against the total levels of the respective protein (non-phosphorylated) as a protein loading control.

The intensity of the protein bands was quantified using image analysis software (ImageJ 1.35, National Institute of Mental Health). For each blot, the background signal was determined by tracing an unlabeled area adjacent to each band and subtracting this value from the target band. Resultant values were normalized to the average signal for the total (non-phosphorylated) protein levels (also background adjusted) to simplify gel analysis and reduce inter and intra-gel variability.

2.1.3. Tissue preparation and immunohistochemistry

For *in vivo* analysis of cell proliferation, rats received a single injection of BrdU (150 mg/kg, i.p.) 2 h after VEGF or PBS microinfusion. Approximately, 1 h later, the rats were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and underwent transcardiac perfusion with 0.1 M phosphate buffer saline (PBS, pH = 7.4) followed by 10% buffered formalin. The brains were extracted and postfixed in the same fixative for 48–72 h at 4 °C before undergoing sucrose infiltration (15%, 30% sucrose) and sectioning on a freezing microtome (40 μm). All sections were collected and stored at −20 °C in a cryoprotectant solution consisting of 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol in 0.1 M phosphate buffered saline (PBS; pH = 7.4) until use.

For BrdU immunohistochemistry, free-floating sections were treated with 1 N HCl at 45 °C for 30–45 min to denature the DNA and expose the BrdU antigen. Sections were then incubated for 1 h at room temperature in a blocking solution comprised of 5% normal horse serum, 1% bovine serum albumin (BSA), and 0.3% Triton X-100 dissolved in 0.1 M PBS. After blocking, the sections were treated with a primary anti-mouse BrdU monoclonal antibody (1:500, 48 h, 4 °C, Becton Dickinson) diluted in the previously described blocking solution, followed by incubation with a secondary biotinylated antibody (horse anti-mouse, 1:500, 2 h, room temperature, Vector Laboratories) and then avidin-biotin peroxidase complex (1:200, 1 h, room temperature, Vectastain ABC Elite, Vector Laboratories). Immunolabeled cells were visualized with a solution of 3,3′-diamobenzidine (DAB) according to the manufacturers specification (Vector Laboratories). Before coverslipping, mounted sections were counterstained with NovaRed (Vector Laboratories). For quantification, every 12th section throughout the hippocampus was counted using a modified unbiased stereology protocol (Kuhn et al., 1997). Briefly, BrdU-labeled cells in the dentate SGZ layer ipsilateral and contralateral to site of cannulation were counted at 400× magnification (Olympus BX60). The dentate SGZ was defined here as a two cell-body width zone along the border of the dentate granule cell layer and hilus. To avoid oversampling, cells in the outermost plane of focus were omitted. The number of BrdU-labeled cells counted was then multiplied by 12 to provide an estimate for the total number for BrdU + cells per dentate SGZ layer.

For phospho-ERK, -Akt, -CREB, and Ki-67 immunohistochemistry, sections were treated with anti-mouse phospho-ERK (1:2000, Millipore, 24 h, 4 °C), anti-rabbit phospho-Akt (1:1000, Millipore, 24 h, 4 °C), anti-rabbit phospho-CREB

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