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Multiple neurotrophic effects of VEGF on cultured neurons

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Abstract

A large literature demonstrates the multifunctional nature of vascular endothelial growth factor (VEGF). Though initially characterized as an endothelial cell-specific factor, recent studies reveal that VEGF has numerous effects on diverse cell types in the brain including neurons. The objective of this study is to examine the effects of VEGF in cultured cortical neurons on survival, p38 mitogen-activated protein kinase (p38 MAP kinase) activity, pro- and anti-apoptotic protein expression and on release of neurotrophic and neurotoxic factors. The results show that VEGF dose-dependently enhances the survival of neurons in culture. VEGF decreases active caspase 3 levels and increases expression of the anti-apoptotic protein Bcl-2. VEGF decreases phosphorylated p38 MAP kinase level and activity in cortical neurons. In addition to modulating survival/death pathways in cortical neurons, VEGF also regulates release of proteins that affect neuronal viability. VEGF causes a dose-dependent release of the neurotrophic protein pigment epithelial-derived factor (PEDF), while significantly decreasing release of the neurotoxic protein amyloid beta. The VEGF-mediated decrease in amyloid beta is dependent on a functional Flt-1 receptor and is inhibited by dicoumarol, a multifunctional inhibitor of stress activated protein kinase (SAPK)/JNK and NFkappaB pathways. Taken together, these data demonstrate that the neurotrophic effects of VEGF are likely mediated directly by increasing survival and decreasing apoptotic proteins and signals as well as indirectly by modulating release of proteins that affect neuronal viability.

Keywords

PEDF; amyloid beta; apoptosis; survival; p38 MAP kinase; Bcl-2; caspase 3

Introduction

A large literature demonstrates the multifunctional nature of vascular endothelial growth factor (VEGF) (Ng *et al.*, 2006; Nieves *et al.*, 2009). Initially identified as a vascular permeability factor, VEGF is also a potent mitogen for endothelial cells and a key regulator of angiogenesis (Leung *et al.*, 1989; Dvorak *et al.* 1995; Yancopoulos *et al.*, 2000). VEGF promotes vasculogenesis during development and physiological angiogenesis that occurs in wound repair (Swift *et al.* 1999, Grazul-Bilska *et al.* 2003) and inflammation (Dvorak *et al.* 1995; Detmar *et al.* 1998) in the adult. This multifaceted protein also plays a role in pathologic angiogenesis associated with tumor growth (Kim *et al.* 1993; Dvorak *et al.* 1995; Ferrara and Davis-Smyth 1997) and age-related macular degeneration (Bhisitkul and Rutar, 2006).

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Though initial studies over a decade ago indicate that VEGF is an endothelial cell-specific factor, more recent findings reveal that VEGF has direct effects on the nervous system on neuronal growth, axonal outgrowth and neuroprotection. Application of VEGF causes axonal outgrowth (Sondell *et al.* 2000; Khaibullina *et al.* 2004) and protects neurons against ischemic, hypoxic, and excitotoxic injury (Jin *et al.* 2000a, 2001; Matsuzaki *et al.* 2001). Genetic studies show that mice with reduced VEGF develop adult-onset motor neuron degeneration, reminiscent of the human neurodegenerative disease amyotrophic lateral sclerosis (ALS), and that VEGF overexpression delays neurodegeneration and prolongs survival in ALS mice (Oosthuysen *et al.*, 2001; Wang *et al.*, 2007). VEGF is up-regulated in the brain after stroke (Issa *et al.*, 1999) and has been implicated in other neurological disorders such as diabetic and ischemic neuropathy, nerve degeneration, Parkinson's disease, Alzheimer disease, and multiple sclerosis (Storkebaum *et al.*, 2004; Ruiz de Almodovar *et al.*, 2009).

In endothelial cells the signaling cascades that mediate VEGF-induced changes in proliferation, migration, permeability, and cell survival have been extensively documented (Kumar *et al.*, 2004; Youn *et al.*, 2009). In contrast, the underlying mechanisms that contribute to VEGF's neuroprotective functions remain to be defined. In neuronal cells, the neuroprotective effects of VEGF have been attributed to its binding primarily to VEGF Flk-1 receptor (Sondell *et al.* 2000) and activation downstream of phosphatidylinositol 3'-kinase/protein kinase B (PI3/Akt), the mitogen activated protein kinase kinase/extracellular signal-regulated protein kinases (MAPK/ERK 1/2) or both (Jin *et al.* 2000b; Matsuzaki *et al.* 2001; Wick *et al.* 2002; Kilic *et al.* 2006a; 2006b). In the retina, overexpression of VEGF reduces phosphorylation of p38 MAP kinase, a stress-activated enzyme that can initiate apoptosis in neurons (Hou *et al.*, 2002; Jeohn *et al.*, 2002), and protects against neurodegeneration (Kilic *et al.* 2006b). The role of p38 MAP kinase, in VEGF's neuronal signaling remains unclear.

Many signaling pathways that affect neuronal survival/death decisions converge downstream on common targets. Among the most well studied of these are the anti-apoptotic protein Bcl-2 and the pro-apoptotic protease caspase 3 (Yuan and Yankner, 2000; Antonsson and Martinou, 2000; Sadowski-Debbing *et al.*, 2002). We have previously shown in cultured neurons that acetaminophen-mediated protection against oxidant injury is associated with an increase in Bcl-2 and a decrease in caspase 3 (Tripathy and Grammas, 2009). Whether these pathways are regulated by VEGF in neurons is unknown.

VEGF could also promote neuronal survival indirectly by interacting with other proteins with neurotrophic or neurotoxic properties. In this regard, expression of pigment epithelium-derived factor (PEDF), a neurotrophic protein widely distributed in the CNS (Barnstable and Tombran-Tink, 2004), has been shown to be correlated with VEGF in the striatum of patients with Parkinson's disease (Yasuda *et al.*, 2007). However, whether VEGF affects expression of PEDF in neurons is unknown. Similarly, a recent study suggests that VEGF can affect processing of the amyloid precursor protein in brain slice cultures derived from transgenic Alzheimer's disease mice (Bürger *et al.*, 2009). The ability of VEGF to affect amyloid beta (A β) levels may contribute to the multi-factor nature of VEGF-mediated neuroprotection.

The objective of this study is to examine in primary cultured neurons the effects of VEGF on neuronal survival, p38 MAP kinase, pro- and anti-apoptotic protein expression and on release of neurotrophic and neurotoxic factors.

Materials and methods

Primary neuronal cultures and culture treatments

Cell culture reagents and media were purchased from Invitrogen (Carlsbad, CA). Rat cerebral cortical cultures were prepared from cortices of 18-day gestation fetuses, as previously

described (Grammas *et al.*, 1999; Reimann-Philipp *et al.*, 2001). The cells were seeded on 6-well poly-L-lysine coated plates at a density of $3-5 \times 10^5$ cells per ml and incubated in Neurobasal medium containing B-27 supplement, antibiotic/antimycotic, glutamine (0.5 mM) and 5-fluoro-2'-deoxyuridine (20 μ g/ml) to inhibit proliferation of glial cells. On day 5, fresh medium without 5-fluoro-2'-deoxyuridine was added. Neuronal cultures were used for experiments after 8–9 days in culture. The primary cerebral cortical cultures utilized were enriched for neurons (>95%). The neuronal identity of the cultures was confirmed using immunofluorescent labeling for beta-3 tubulin, a neuron-specific protein (Promega, Madison, WI) and glial fibrillary acidic protein (Promega).

Recombinant rat VEGF (CRV017A) was obtained from Cell Sciences (Canton, MA). VEGF was reconstituted in sterile, deionized water frozen until use and diluted in treatment media to attain the experimental doses (0–100 ng/ml). The p38 MAP kinase inhibitor SB203580 was obtained from EMD Biosciences (La Jolla, CA) and used at a final concentration of 100 μ M in treatment media (neurobasal media containing N-2 supplement, 0.5 mM glutamine, and antibiotic/antimycotic). The p38 MAP kinase activator anisomycin was also purchased from EMD Biosciences and used at a final concentration of 7.5 μ M. VEGF receptor Flt-1 and Flk-1 neutralizing antibodies were from R&D Systems (Minneapolis, MN). VEGF receptor antibodies were used together with VEGF (100 ng/ml) at a final concentration of 4 μ g/ml. The MAP kinase kinase (MEK) 1 and MEK2 inhibitor (U0126), phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) and the c-Jun N-terminal kinase (JNK) inhibitor (JNK Inhibitor II) were purchased from Calbiochem (La Jolla, CA) and used at final concentrations of 5 μ M, 5 μ M, and 10 μ M, respectively. Tyrosine kinase (Genistein) and protein kinase C (PKC) (bisindolylmaleimide) inhibitors were purchased from Sigma (St. Louis, MA) and used at 100 μ M and 10 μ M final concentrations, respectively. Dicoumarol was obtained from Calbiochem and used at a final concentration of 50 μ M.

Assessment of cell viability

Cell viability was assayed using the MTT assay (Promega) as follows. Treatment medium was replaced with fresh treatment medium containing 25 μ l/ml of the Cell Titer 96 Aqueous One Solution and incubated for 10 min at 37°C after which optical density was measured at 490 nm using a microplate reader. The quantity of soluble formazan product, as measured by the amount of absorbance, was directly proportional to the number of viable cells. The optical density of untreated controls was set at 100%. The number of viable cells after treatment was determined by measuring optical density and expressing viability as percent of untreated controls.

Quantitation of proteins in culture media by ELISA

An indirect ELISA procedure was used to measure the amyloid beta1–42 (A β 1–42) and pigment epithelium-derived factor (PEDF) proteins. After treatments, cell media were collected and used for ELISA. ELISA plates (96-well flat bottom) were coated overnight at 4°C with 100 μ l cell media mixed with 100 μ l of coating buffer (0.1 M sodium bicarbonate buffer, pH 9.5), blocked with 1% bovine serum albumin solution at 37°C for 45 min and then incubated with the primary antibody (for A β 1–42, AB5758P, Chemicon, Temecula, CA; for PEDF 07–280, Upstate Cell Signaling Solutions, Lake Placid, NY) diluted in sodium bicarbonate buffer (1:1000 dilution). The plate was incubated with the peroxidase-conjugated secondary antibody (1:1000) (BioRad, Hercules, CA) for 1 h and washed three times. Color reaction was developed by addition of 200 μ l/well of o-phenylene diamine H₂O₂ (Thermoscientific, Rockford, IL). Optical density was measured at 450 nm using a microplate reader. The A β 1–42 (03–111) and PEDF (01-211) peptides used for standard calculation were from Biosource (Camarillo, CA USA), and Upstate Cell Signaling Solutions, respectively.

Western blot analysis

Total protein was extracted from neurons using lysis buffer containing 0.1% SDS, 1% Triton X-100 and 0.5% phenylmethyl sulfonylfluoride. Protein was determined by the Bradford method using Bio-Rad protein reagents. Equal amounts of protein were run on a 12% polyacrylamide gel, transferred on to a PVDF membrane, blocked with 5 % milk solution (non-fat dry milk in Tris-buffered saline Tween-20) and immuno-blotted with primary antibodies. Primary antibodies from Cell Signaling Technology (Danvers, MA) include cleaved form of caspase 3 (#9664, 1:200), total p38 MAPK (#9212, 1:200), and phospho-p38 MAP kinase (#9211, 1:200). The Bcl-2 (ab7973, 1:300) antibody was from AbCam (Cambridge, MA). Membranes were washed with Tris-buffered saline Tween-20 and incubated with peroxidase-conjugated secondary antibodies. After extensive washing to remove unbound antibodies, membranes were developed with chemiluminescence reagents. Band intensities were quantified using Quantity One software (Bio-Rad) and graphically expressed as intensity units which reflects the average intensity over the area of the band. Densitometric measurements of bands were normalized to corresponding GAPDH levels with control samples set to 100 and treatment values expressed as percent of control.

p38 MAP kinase assay

Activity of p38 MAP kinase was determined using the nonradioactive p38 MAP kinase assay kit from Cell Signaling (#9820). After treatments, cell lysates were collected in cell lysis buffer and used for kinase assay according to manufacturer's protocol. p38 MAP kinase was immunoprecipitated overnight from samples (100 µg) using phospho p38 (Thr180/Try184) MAP kinase monoclonal antibody immobilized to agarose hydrozide beads. The immunoprecipitated phospho p38 MAP kinase pellet was resuspended in kinase buffer containing 2 µg ATF-2 fusion protein and 200 µM cold ATP. The reaction mix was incubated at 30°C for 30 min and the reaction was terminated by addition of sample buffer. Activity of phospho p38 MAP kinase was determined by detecting ATF-2 phosphorylation using phospho-ATF-2 (Thr71) antibody through western blotting and chemiluminescent detection.

Statistical Analysis

Data from each experiment were expressed as mean $\pm$ standard error (SEM). Comparisons between control and treatment groups were conducted using the one-way ANOVA followed by Bonferroni's multiple comparison test for multiple samples. Statistical significance was determined at $p < 0.05$.

Results

VEGF improves neuronal survival in vitro

Exposure of eight-day old neuronal cultures to various concentrations (10 -100 ng/ml) of VEGF for 24 h increased neuronal survival (Fig. 1A). This increase was significant ($p < 0.05$) at 25 ng/ml and highly significant at 50 ng/ml ($p < 0.001$). At 100 ng/ml there was a 45% increase in neuronal cell survival compared to untreated cell cultures.

To determine whether the neuroprotective effect of VEGF on primary cortical neurons was mediated by changes in cell death machinery/mediators we determined if VEGF blocked the activation of caspase 3. Neuron cultures were exposed to increasing doses of VEGF (10–100 ng/ml) for 24 h and western blots were performed using an antibody specific for the 17 kDa active (cleaved) form of caspase 3. Figure 1B shows that exposure to VEGF significantly ($p < 0.01$ – 0.001) reduced immunoreactivity for the active form of caspase 3.

Experiments to determine other possible targets in the apoptotic cascade that might be affected by VEGF showed that the level of Bcl-2, an anti-apoptotic protein upstream of caspase 3, was

also altered. In contrast to the suppression of active caspase 3 by VEGF, the expression of the anti-apoptotic protein Bcl-2 was significantly ($p<0.05$ – 0.01) increased in a dose-dependent manner by VEGF (Fig. 1C).

VEGF decreases phosphorylated p38 MAP kinase level and activity in cortical neurons

Since the p38 MAP kinase pathway is linked to caspase activation (Junn and Mouradian 2001; Choi *et al.* 2004) and apoptosis induced by A β (Zhu *et al.* 2005), we examined the role of p38 MAP kinase in VEGF signaling. Neuronal cultures were exposed to increasing doses of VEGF (10–100 ng/ml) for 24 h and western blots were performed using an antibody specific for the phosphorylated form of p38 MAP kinase. Figure 2A shows that VEGF treatment had no significant effect on total p38 MAP kinase but increasing concentrations of VEGF reduced immunoreactivity for the phosphorylated form of p38 MAP kinase (Fig. 2A).

Experiments to determine the activity of phosphorylated p38 MAP kinase were performed using the ATF-2 fusion protein as a substrate and its level detected by western blot analysis. The data showed that increasing doses of VEGF (10–100 ng/ml) caused a decrease in phosphorylation of ATF (Fig. 2B).

Dissociation of VEGF effects on p38 MAP kinase activity and on neuronal survival

We examined the ability of VEGF to affect p38 MAP kinase activity, as indicated by ATF-2 phosphorylation (Fig. 3A), and neuronal survival (Fig. 3B) in the presence of a p38 MAP kinase inhibitor (SB203580) or the p38 MAP kinase activator anisomycin. Addition of VEGF (100 ng/ml) caused a decrease in p38 MAP kinase activity as did addition of the p38 MAP kinase inhibitor SB203580 (100 μ M). The combination of VEGF and SB203580 completely inhibited p38 MAP kinase activity to levels below detection (Fig. 3A). Addition of the p38 MAP kinase activator anisomycin (7.5 μ M) increased p38 MAP kinase activity. Co-incubation of VEGF with anisomycin significantly ($p<0.001$) reduced p38 MAP kinase activity compared to anisomycin alone (Fig. 3A).

In contrast the effects of VEGF in combination with SB203580 or anisomycin on neuronal cell survival were complex. Incubation of neuronal cultures with either VEGF or SB203580 alone caused a significant ($p<0.001$) increase in cell survival. However, survival of cultures exposed to VEGF plus SB203580, although higher than untreated cultures ($p<0.05$), was significantly ($p<0.05$) less than either agent alone (Fig. 3B). Addition of anisomycin caused a pronounced (72%) and highly significant ($p<0.001$) decrease in neuronal survival. Neuronal survival was similar in cultures exposed to anisomycin alone and in cultures treated with anisomycin plus VEGF and significantly ($p<0.001$) less than VEGF alone (Fig. 3B).

VEGF affects release of neurotoxic and neurotrophic factors from cultured neurons

To determine other mediators/pathways that may be involved in VEGF's neurotrophic effects, we examined the ability of VEGF to alter the release of the neurotoxic peptide A β 1-42 and the neurotrophic protein PEDF from cultured neurons. Analysis of media by indirect ELISA from cultured neurons at 24 h showed release of A β 1-42 (Fig. 4A). Neuronal cultures exposed to increasing concentrations of VEGF (5–100 ng/ml) showed a strong, dose-dependent decrease in the level of A β 1-42 detectable in culture supernatants. The decrease was significant ($p<0.05$) at 50 ng/ml VEGF and 100 ng/ml VEGF caused a 51% decrease in released A β 1-42 (Fig. 4A).

In contrast, exposure of neuronal cultures to similar concentrations of VEGF for 24 h resulted in a dose-dependent increase in the level of PEDF released into the supernatant. VEGF was less potent in its ability to evoke PEDF release compared to the inhibition of A β 1-42 release. The increase in PEDF was significant ($p<0.05$) at 50 ng/ml VEGF (Fig. 4B).

VEGF-mediated decrease in A β 1-42 is mediated by Flt-1 receptor and is blocked by dicoumarol

In an effort to understand the mechanism behind the VEGF-mediated decrease in release of A β 1-42, cultured neurons were exposed to VEGF in combination with neutralizing antibodies for the VEGF receptors Flk-1 and Flt-1. Incubation of neurons with an antibody to the Flk-1 receptor during VEGF treatment did not affect the decrease in A β 1-42 release induced by VEGF. In contrast, in neuronal cultures exposed to a Flt-1 receptor antibody the ability of VEGF to inhibit A β 1-42 release was significantly ($p < 0.001$) diminished (Figure 5).

Co-incubation of neurons with VEGF and single-target kinase inhibitors of MEK 1/2, PI3K, JNK, PKC or tyrosine kinase did not alter the effect of VEGF on A β 1-42 release (Table 1). In contrast, the use of VEGF in combination with dicoumarol, a multifunctional inhibitor of several signaling pathways including stress activated protein kinase (SAPK)/JNK and NF κ B (Cross *et al.*, 1999), abolished the VEGF-induced decrease in A β 1-42 (Table 1).

Discussion

Considerable literature now supports the idea that VEGF, a key angiogenic factor, also plays a crucial role in the nervous system. This protein can regulate neuronal migration in the CNS (Schwarz *et al.*, 2004) and stimulate axonal outgrowth of cortical neurons and retinal ganglion cells (Sondell *et al.*, 2000; Böcker-Meffert *et al.*, 2002; Khaibullina *et al.* 2004). VEGF has been implicated in adult neurogenesis (Cao *et al.*, 2004). The notion that VEGF may be a central mediator of vascular-neuronal interactions is supported by data showing that neuropilins are shared co-receptors between axon guidance molecules (semaphorins) and VEGF (Salikhova *et al.*, 2008; Sköld and Kanje, 2008). Also, VEGF shares common receptor signaling with the neuronal guidance molecule SEMA3A and thus could link the coordinated patterning of the developing vascular and nervous systems (Rosenstein and Krum, 2004).

In the normal adult brain VEGF is expressed at relatively low levels; primarily in neurons (Merrill and Oldfield, 2005). The physiologic functions of this basal expression are unknown. In the current study we document VEGF dose-dependently enhances the survival of uninjured neurons in culture. These data suggest that VEGF has neurotrophic effects as well as the widely documented ability to protect neurons from exogenous injury. VEGF can inhibit or reduce neuronal cell death evoked *in vitro* by hypoxia, glutamate, or serum-deprivation (Wick *et al.*, 2002; Tolosa *et al.*, 2008; 2009). *In vivo*, VEGF induces neuritic growth and provides neuroprotection, particularly after ischemia or spinal cord injuries (Widenfalk *et al.*, 2003; Kilic *et al.*, 2006a). Also, VEGF is a modifier of motor neuron degeneration in humans and in a mouse model of ALS (Storkebaum *et al.*, 2004). Despite these protective effects, VEGF overexpression contributes to the phenotype associated with many CNS disorders. In this regard, marked upregulation of VEGF is observed in traumatic injury, stroke, aneurysms, and AD (Skirgaudas *et al.*, 1996; Kalaria *et al.*, 1998; Shore *et al.*, 2004; Storkebaum *et al.*, 2004; Merrill and Oldfield, 2005). This increase could be a compensatory response to injury or could directly relate to disease pathogenesis. Because VEGF has a potentiating effect on CNS inflammation and increases vascular permeability (Merrill and Oldfield, 2005), continuous upregulation of VEGF, at sites of brain injury or inflammation, may contribute to the pathogenesis of chronic neuroinflammatory disease. Defining the pathways and secondary mediators regulated by this multifaceted protein is important for developing targeted therapeutics that best harness the neurotrophic/protective effects of VEGF and minimize untoward effects.

VEGF has been shown to affect expression/activity of numerous mediators in neurons that are part of the apoptotic cascade. VEGF protects motor neurons from serum deprivation-induced down regulation of the anti-apoptotic protein Bcl-2 (Tolosa *et al.*, 2009). Chronic glutamate

excitotoxicity in spinal cord neurons induces down regulation of Bcl-2 that is prevented by VEGF (Tolosa *et al.*, 2008). We have previously shown the induction of Bcl-2 in cultured cortical neurons by the drug acetaminophen enhances neuronal survival when cells are exposed to oxidant stress (Tripathy and Grammas, 2009). In the current study, we document a dose-dependent increase in Bcl-2 expression in cultured neurons exposed to VEGF. Increased expression of Bcl-2 may be a common and important target of neuroprotective/trophic proteins in neurons. In mouse cortical neuronal cultures subjected to hypoxia, the neuroprotective effects of VEGF also involve suppression of cell death pathways mediated by caspase 3. In those cultures VEGF triggers the PI3K/Akt signaling cascade leading to inhibition of caspase 3 (Jin *et al.*, 2001). VEGF reduces ischemic brain infarct and decreases hypoxic neuronal death via inhibition of caspase 3 (Shen *et al.*, 2006). In the current study we document that VEGF dose-dependently inhibits expression of the cleaved (active) form of caspase 3 in uninjured neurons. Thus, in neurons VEGF improves cell survival both by enhancing pro-survival signals (Bcl-2) as well as inhibiting pro-apoptotic proteins (caspase 3).

VEGF has been shown to activate several signaling cascades in neurons. The ability of VEGF to protect motor neurons from chronic glutamate excitotoxicity is mediated by activation of PI3K/Akt (Tolosa *et al.*, 2008). VEGF protects motor neurons from serum deprivation-induced cell death through PI3K mediated p38 MAP kinase inhibition (Tolosa *et al.*, 2009). In cerebral ischemia VEGF increases phosphorylation of Akt and ERK-1/2 and decreases phosphorylation of c-Jun NH2-terminal kinase (JNK)-1/2 and p38 MAP kinase (Kilic *et al.*, 2006a). After axotomy, VEGF transgenic animals demonstrate, compared to control mice, increased phosphorylated form of ERK-1/2 and Akt and reduced phosphorylated p38 MAP kinase and protection from neuronal degeneration (Kilic *et al.*, 2006b). Here we show VEGF-mediated effects on phosphorylation of p38 MAP kinase in uninjured neurons. However, our experiments with a specific inhibitor (SB203580) and stimulator (anisomycin) of p38 MAP kinase reveal a partial dissociation between VEGF's effects on p38 MAP kinase activity and on neuronal survival. Incubation of neuronal cultures with either VEGF or SB203580 alone causes a significant increase in cell survival. However, survival of cultures exposed to VEGF plus SB203580, is significantly less than either agent alone. The mechanism underlying this observation is unclear. However, it is possible that the combination of both SB203580 and VEGF may result in a high, possibly noxious level of VEGF. In this regard, SB203580 has been shown to increase VEGF secretion in fibroblast-like cells, and furthermore, in tumors p38 MAP kinase itself can stimulate VEGF secretion (Yoshino *et al.*, 2006; Lee *et al.*, 2009). Alternatively, the extreme suppression of p38 MAP kinase evoked by the combination of SB203580 and VEGF treatment may abrogate functions of p38 MAP kinase recently reported to play a role in initiation/maintenance of cell cycle checkpoints and survival (Thornton and Rincon, 2009). Addition of the p38 MAP kinase stimulator anisomycin causes a pronounced decrease in neuronal survival. Neuronal survival is similar in cultures exposed to anisomycin alone and in cultures treated with anisomycin plus VEGF. Thus, VEGF is unable to mitigate the toxic effect of anisomycin on neuronal cultures. This suggests that other signals/pathways evoked by anisomycin have a greater influence over neuronal survival than p38 MAP kinase. Taken together these data suggest that the trophic effect of VEGF on neurons is complex and likely involves multiple mechanisms and/or mediators.

In addition to the direct effects of VEGF on neurons, VEGF could affect neuronal survival indirectly by inhibiting release of neurotoxic factors, promoting release of neurotrophic proteins, or both. In this regard, there could be a heretofore underexplored relationship between VEGF and the neurotoxic protein amyloid beta (A β). Data in the literature suggests that there is an interaction between VEGF and A β protein. In the neurodegenerative disorder Alzheimer's disease, VEGF is co-localized in senile plaques with A β (Yang *et al.*, 2004). Structural interactions between these two proteins have been reported *in vitro* (Yang *et al.*, 2005). VEGF receptor (Flt-1) is increased in Alzheimer's disease and in rat hippocampi injected with A β 1-42

(Ryu *et al.*, 2009). In the current study we show, for the first time, that treatment of cultured neurons with VEGF results in a dose-dependent decrease in the release of A β 1-42 into the media. This effect, unlike other neurotrophic effects documented for VEGF that utilize the Flk-1 receptor, is dependent on a functional Flt-1 receptor.

The intracellular mechanisms that mediate the VEGF response are numerous and complex and thus likely to be unaffected by single-target kinase inhibitors. In this regard, we find that inhibitors that block either MEK 1/2, PI3K, JNK, PKC, or tyrosine kinase are unable to affect the action of VEGF on A β secretion. In contrast, use of dicoumarol, a pharmacologic inhibitor of several signaling systems including stress activated protein kinase (SAPK)/JNK and NF κ B (Cross *et al.*, 1999) completely blocks the VEGF-mediated decrease of A β release. Furthermore, dicoumarol, a long-established anticoagulant, has multiple modes of action that involve redox perturbations including inhibition of heat shock proteins and effects on protein degradation (Seanor *et al.*, 2003; Hernandez *et al.*, 2008; Alard *et al.*, 2009).

Finally, another mechanism that may underlie VEGF's effect on A β 1-42 is endocytosis. VEGF affects endocytosis (Salikhova *et al.*, 2008), a process required for amyloidogenic processing of amyloid precursor protein and subsequent release of A β (Schneider *et al.*, 2008). This mechanism may be especially relevant for our observation that VEGF mediates a decrease in A β 1-42 levels in light of a recent report using brain slices from Alzheimer's disease transgenic mice that shows that VEGF exposure reduces the formation of soluble, SDS-extractable A β 1-42. The VEGF-mediated decrease in A β 1-42 in that study is accompanied by a transient decrease in β -secretase activity indicating that VEGF affects the processing of amyloid precursor protein (Bürger *et al.*, 2009).

Another protein that may play a role in VEGF's trophic effect is PEDF. Interestingly, although both PEDF and VEGF are neurotrophic, PEDF has opposite functions from VEGF in terms of angiogenesis and permeability. For example, it has been shown that intradermal injections of PEDF in nude mice block VEGF-induced vascular hyperpermeability (Yamagishi *et al.*, 2007). The dissociation between VEGF's neurotrophic and permeability effects is important. Indeed, the use of VEGF as a neuroprotectant therapeutic has been limited by concerns that neuroprotective and permeability effects are both mediated by activation of PI3K/Akt. Thus, the demonstration that VEGF can release the neurotrophic protein PEDF from neurons and that this protein antagonizes the permeability effects of VEGF suggests that further dissecting the mechanisms/mediators regulated by the versatile protein VEGF in the brain will lead to more targeted approaches that take advantage of the beneficial actions while minimizing potentially toxic side effects.

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Figure 1.

Eight-day old cultured neurons were treated with increasing doses of VEGF for 24 h. **A.** Cell survival was determined by MTT assay. Data are mean $\pm$ SEM expressed as percent of control (untreated cells) from 4 separate experiments.

* $p < 0.05$ vs. control; *** $p < 0.001$ vs. control.

B. Total protein was extracted and western blot analysis performed using specific antibodies for cleaved caspase 3. Data are normalized to GAPDH and expressed as percent of control (untreated). Relative expression determined from densitometric scans from 2 separate experiments is shown below representative western blot.

** $p < 0.01$ vs. control; *** $p < 0.001$ vs. control.

C. Western blot analysis was performed using specific antibodies for Bcl-2 and relative expression determined from densitometric scans from 2 separate experiments is shown below representative western blot.

* $p < 0.05$ vs. control; ** $p < 0.01$ vs. control.

Figure 2.

Eight day old neurons were treated with increasing dose of VEGF for 24 h. **A.** Total protein was extracted and western blot analysis performed using specific antibodies for phosphorylated p38 MAP kinase, total p38 MAP kinase and GAPDH. Data are normalized to total p38 MAP kinase and expressed as percent of control (untreated). Relative expression determined from densitometric scans from 4 separate experiments is shown below representative western blot. * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control.

B. Cell lysates were used to measure p38 MAP kinase activity by measuring ATF-2 phosphorylation *in vitro*. Activity of phospho p38 MAP kinase was determined by western blot using antibody to phosphorylated ATF-2. The figure is representative of 2 experiments.

Figure 3.

Eight-day old neurons were treated for 24 h as follows: Control, VEGF (100 ng/ml), SB203580 (100 μ M), SB203580+VEGF, anisomycin (7.5 μ M) and anisomycin+VEGF. **A.** Cell lysates were used to determine p38 MAP kinase activity by measuring ATF-2 phosphorylation *in vitro*. Activity of phospho p38 MAP kinase was determined by western blot using antibody to phosphorylated ATF-2. Relative expression determined from densitometric scans from 3 separate experiments is shown below representative western blot.

*** $p < 0.001$ vs control; ^a $p < 0.05$ vs SB203580+VEGF; ^b $p < 0.001$ vs anisomycin.

B. Cell survival was determined by MTT assay. Data are mean $\pm$ SEM expressed as percent of control (untreated cells) from 2 separate experiments.

*** $p < 0.001$ vs control; * $p < 0.05$ vs control; ^a $p < 0.05$ vs SB20358+VEGF; ^c $p < 0.001$ vs VEGF.

Figure 4.

Primary neuronal cultures were treated with increasing doses of VEGF for 24 h, media collected and analyzed by indirect ELISA for levels of (A) Aβ1-42 or (B) PEDF. Data are mean + SEM from 5 separate experiments. *p<0.05 vs. control; ***p<0.001 vs. control.

Figure 5.

Neuron cultures were exposed to media (control), VEGF (100 ng/ml), VEGF + Flt-1 or Flk-1 neutralizing antibodies (4 μ g/ml) for 24 h and A β 1-42 released into media assessed by ELISA. Data are mean $\pm$ SEM from 3 separate experiments. *** p<0.001 vs control.

Table 1Effects of signaling inhibitors on VEGF-mediated A β 1-42 decrease.

Treatments			
	DMSO (0.25%)	VEGF (100 ng/ml)	A β 1-42 (ng/ml)
Control	-	-	175.8 $\pm$ 6.0
DMSO	+	-	178.5 $\pm$ 5.2
VEGF (100 ng/ml)	-	+	143.6 $\pm$ 3.2
Genistein (100 μ M)	-	+	153.1 $\pm$ 2.0
PKC Inhibitor (10 μ M)	-	+	157.5 $\pm$ 3.6
MEK 1/2 Inhibitor (5 μ M)	-	+	137.6 $\pm$ 6.0
P13K Inhibitor (5 μ M)	-	+	136.4 $\pm$ 6.3
JNK Inhibitor II (10 μ M)	-	+	148.3 $\pm$ 3.1
Dicoumarol (50 μ M)	-	+	186.5 $\pm$ 4.0***

Neuronal cultures were treated as indicated for 24 h and A β 1-42 levels in the media determined by ELISA. Data are mean $\pm$ SEM from 4 separate experiments.

***p<.001 vs VEGF