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Orginal Contribution

Signaling through the vascular endothelial growth factor receptor VEGFR-2 protects hippocampal neurons from mitochondrial dysfunction and oxidative stress

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ABSTRACT

Vascular endothelial growth factor VEGF (VEGF-A or $VEGF_{165}$) is a potent angiogenic factor that also signals neuroprotection through activation of its cognate receptor VEGFR-2. In this capacity, VEGF signaling can rescue neurons from the damage induced by stressful stimuli many of which elicit oxidative stress. However, the regulatory role that VEGFR-2 plays in providing neuroprotection remains elusive. Therefore, we investigated the effects of VEGFR-2 inhibition on primary cultures of mature hippocampal neurons undergoing nutritional stress. We found that neurons cultured under nutritional stress had increased expression of VEGF and its receptors, VEGFR-1, VEGFR-2, and NP-1, as well as enhanced levels of VEGFR-2 phosphorylation. These neurons also showed increased activation of the prosurvival pathways for MEK/ERK1/2 and PI3K/Akt, enhanced phosphorylation (inactivation) of the proapoptotic BAD, and higher levels of the antiapoptotic protein Bcl-xL, all of which were augmented by treatments with exogenous VEGF and blocked by VEGFR-2 inhibition. The blockade of VEGFR-2 function also elicited a cytotoxicity that was accompanied by caspase-3 activation, induction of hemeoxygenase-1 (HO-1), oxidative stress, and a collapse in the mitochondrial membrane potential ($\Delta\Psi_m$). Knockdown of VEGFR-2 by siRNA generated a similar pattern of redox change and mitochondrial impairment. Pretreatments with VEGF, VEGF-B, or the antioxidant *N*-acetylcysteine (NAC) rescued SU1498 or siRNA-treated neurons from the mitochondrial dysfunction and oxidative stress induced by VEGFR-2 inhibition in a timely fashion. These findings suggested that VEGF or VEGF-B can provide neuroprotection by signaling through an alternate VEGF receptor. Together, our findings suggest that VEGF signaling through VEGFR-2 plays a critical regulatory role in protecting stressed hippocampal neurons from the damaging effects of an oxidative insult. These findings also implicate VEGFR-1 or NP-1 as compensatory receptors that mediate neuroprotection when VEGFR-2 function is blocked.

Introduction

Oxidative stress and mitochondrial dysfunction contribute to the pathogenesis of a number of neurodegenerative disorders including Alzheimer's and Parkinson's disease [1]. One approach for protecting neurons from oxidative insults is to administer neurotrophic growth factors. One such factor is VEGF (VEGF-A or $VEGF_{165}$) which is a mitogen that stimulates angiogenesis and neuroprotection through autocrine or paracrine mechanisms [2,3]. In addition, VEGF can stimulate axonal outgrowth, and rescue rat mesencephalic neurons or hippocampal cells from death induced by serum withdrawal, ischemia, hypoxia, and glutamate-induced toxicity [4–6] and elicit neuroprotection via angiogenesis and neurogenesis [7–9]. Delineating this neuroprotective mechanism is complex since VEGF can undergo cell-surface interactions with different cognate tyrosine kinase receptors such as VEGFR-1, VEGFR-2, and the non-tyrosine kinase members of the neuropilin family of class 3 semaphorin receptors Neuropilin-1 and -2 (NP-1, NP-2) receptors [10]. While VEGF mediates most biological effects through VEGFR-2, it can interact with NP-1 as a coreceptor that enhances VEGF signaling. Ligand binding to VEGFR-1 and VEGFR-2 results in receptor dimerization followed by autophosphorylation and activation of downstream signaling cascades [3]. In neurons, NP-1 is a cell-surface receptor for both VEGF and class 3 semaphorins and plays a functional role in axonal pathfinding, retraction, and collapse during development. While the function of VEGFR-1 remains unclear, it is implicated as a decoy receptor that sequesters VEGF from activating VEGFR-2. VEGF-B, which is

Abbreviations: carboxy-H_2DCFCA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; GCM, glial-conditioned medium; MAP, microtubule-associated protein; MEK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NB, Neurobasal; NP-1, NP-2, Neuropilin-1 and -2; PI3K, phosphatidylinositol 3-kinase; TMRE, tetramethylrhodamine methyl; VEGF, vascular endothelial growth factor

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a ligand for VEGFR-1 but not VEGFR-2, is poorly angiogenic in the brain but can protect against mitochondrial membrane permeabilization [11], proapoptotic gene expression [12], and is coexpressed with genes encoding mitochondrial proteins [13]. Thus, VEGF-B may protect against mitochondrial dysfunction independent of angiogenesis.

Oxidative insults are implicated as causative factors of neuronal damage in several different neurological disorders and increasing evidence from *in vitro* and *in vivo* studies shows that VEGF signaling protects neurons from insults which are known to induce oxidative stress [14,15]. In this context, VEGF overexpression has been shown to delay the onset of neuronal death in an animal model of amyotrophic lateral sclerosis (ALS) where oxidative stress is a contributing factor [16]. VEGF has been shown to mediate neuroprotection under stress conditions through the downstream activation of the phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase MEK/ERK1/2 pathways [6,17–19] and by suppressing caspase activation [20]. While several of these studies show that VEGF activates VEGFR-2 to mediate survival, the protective capacity of VEGFR-2 signaling remains unclear. Therefore, the aim of these studies is to determine the impact of VEGFR-2 signaling on the survival of mature hippocampal neurons by blocking its activation using pharmacological and gene silencing methods.

In previous studies we used a neuronal model of serum-deprived SK-N-SH cells to show that VEGF signaling through VEGFR-2 prevents a caspase-dependent cell death [21]. Therefore, we addressed whether VEGF signaling through VEGFR-2 protected rat hippocampal neurons from the harmful effects of nutritional stress. Depriving cultured neurons from vital nutrients provide a viable model to identify the molecular basis of neuronal insults that would occur under pathological conditions *in vivo*. Our findings show that a blockade of VEGFR-2 function in hippocampal neurons leads to a rapid loss in viability that is manifested by induction of markers of oxidative stress, mitochondrial dysfunction, and a loss in the activation of prosurvival pathways. Notably, the inclusion of exogenous VEGF or VEGF-B mediates a time-dependent rescue from this response, suggesting that a molecular switch to an alternate receptor can provide neuroprotection when VEGFR-2 activity is blocked. Our findings establish a link between VEGFR-2 signaling and mitochondrial function in differentiated rat neurons and provide insight on oxidative stress-related pathways that mediate neuronal damage and how exogenous VEGF or VEGF-B may counteract these events.

Materials and methods

Materials

Recombinant human vascular endothelial growth factor 165 ($VEGF_{165}$) and VEGF-B were obtained from PeproTech Inc (Rocky Hill, NJ). The inhibitors of VEGFR-2 (SU1498) and PI3K/Akt (Wortmannin) were obtained from EMD Biosciences Inc. (San Diego, CA). The inhibitor of MEK1/2 (U0126) was obtained from Promega Corporation (Madison, WI). The antioxidant *N*-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO).

Primary cell culture and treatments

All animal studies were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the approval of the Institutional Animal Care Committee at Hunter College of the City University of New York. Rat hippocampal neurons were prepared from Sprague-Dawley (Charles River Laboratories) 1 day postnatal (P1) rat pups using a modification of a previously described procedure [4]. Briefly, hippocampi were isolated and dissected aseptically in ice-cold Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS, Invitrogen/Life Technologies). Following the removal of the meninges, the hippocampal tissue was minced, digested in 0.25% trypsin (Invitrogen/Life Technologies) for 15 min at 37 °C, and then rinsed at 37 °C with Ca^{2+}/Mg^{2+}-free HBSS. The tissue was triturated in DMEM/10% FBS (Invitrogen/Life Technologies) through pasteur pipettes and then passed through a 40-μm mesh strainer. Isolated cells were then resuspended in DMEM/10% FBS, and plated into poly-D-lysine (100 μg/ml)-coated plates as follows: 96-well microtiter plates (2×10^4 cells/well) for viability studies, 6-well plates (5×10^5 cells/well) for Western blot analyses, 8-well chamber slides (1×10^5 cells/well) for immunofluoresence staining, oxidative stress, and TMRE detection and 10 cm plates (5×10^6 cells/well) for RT-PCR. The medium was replaced after 2 h with Neurobasal (Invitrogen/Life Technologies) medium, L-glutamine (GlutaMAX Invitrogen/Life Technologies), and penicillin + streptomycin (antibiotic–antimycotic; Invitrogen/Life Technologies) (NB) supplemented with B27 (NB/B27) with 2 μM cytosine arabinoside (AraC) to inhibit the proliferation of glial cells. At Day 4, one-half of NB/B27 was removed and replaced with a glial-conditioned medium (GCM). Neurons were then cultured in NB/B27/GCM (1/1 ratio) for 13 days *in vitro* (13 DIV), replacing one-half of the medium with NB/B27/GCM (1/1 ratio) every 4 days. Cells were then incubated in NB/B27 or NB and cultured for 48 h (15 DIV). Where indicated, exogenous VEGF or VEGF-B (100 ng/ml) was administered at 0 time and replenished at 24 h of the 48 h-incubation period. Neurons were also pretreated with NAC (5 mM) for the final 24 h as indicated. For inhibitor studies, neurons were treated with predetermined concentrations of each selective agent. Incubations with SU1498 (10 μM) were at different times as indicated within the 48 h time frame while treatments with Wortmannin (100 nM) and U0126 (10 μM) were added for the final 2 h of incubation. DMSO-treated neurons served as the control for cells incubated with SU1498, Wortmannin, and U0126. Culturing the neurons for 15 DIV allowed adequate time for neuronal maturation, network formation, and the development of synapses. At this time, neurons consistently represent >90 to 99% of the cell population as stained with the dendritic marker microtubule-associated protein (MAP-2). The level of glial contamination usually ranges from 1 to 10% which is consistent with that previously reported [4,20,22].

Protein extraction and Western blotting

Total cell lysates from 15 DIV neurons were harvested in a lysis buffer as described previously [23] with the exception that the NP40 concentration was 0.3%. Protein concentrations were determined with a bicinchoninic acid assay (BCA) according to the manufacturer's instructions (Pierce). Equal amounts of protein (30 μg) from each lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were then blocked and incubated overnight at 4 °C with the primary antibodies for the phosphorylated (Y996) and total forms of VEGFR-2 (Santa Cruz), Akt (T308 and S473, Cell Signaling), BAD (S112, Cell signaling), phosphorylated ERK1/2 (T202/Y204, Cell Signaling), and total ERK1/2 (Santa Cruz) as well as Bcl-xL (Cell Signaling) and HO-1 (Santa Cruz). Actin (Sigma-Aldrich) was used as a loading control. Immunoreactive bands were detected with the corresponding anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Endogen). Data were quantified using the ImageJ program from NIH.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's protocol. RNA (2–4 μg) was reverse transcribed at 50 °C for 30 min and amplified for 40