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Research paper

VEGF-mediated NF-κB activation protects PC12 cells from damage induced by hypoxia

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HIGHLIGHTS

- Endogenous VEGF is required for protection against PC12 cells apoptosis under hypoxia condition mimicked by $CoCl_2$.
- Treatment of PC12 cells with VEGF significantly decreased hypoxic damage induced by $CoCl_2$.
- VEGF restored NF-κB activity inhibited by $CoCl_2$-induced hypoxic damage in PC12 cells.
- VEGF mediated cytoprotection against $CoCl_2$-induced hypoxic damage via activating NF-κB.

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ABSTRACT

Neuronal apoptosis is a contributing cause of disability and death in cerebral ischemia. Nuclear factor-κB (NF-κB) may become a potential therapeutic target for hypoxic/ischemic neuron damage because NF-κB is inactivated after hypoxia exposure. Vascular endothelial growth factor (VEGF) has been found to improve neurological function recovery in cerebral ischemic injury although the exact molecular mechanisms that underlie the neuroprotective function of VEGF remain largely unknown. Here we defined the mechanism by which VEGF antagonized neuron-like PC12 cells apoptosis induced by hypoxia mimetic agent cobalt chloride ($CoCl_2$) is through restoration of NF-κB activity. Depletion of VEGF with small interfering RNA (siRNA) in PC12 cells conferred $CoCl_2$-induced cytotoxicity which was mitigated by VEGF administration. Treatment of PC12 cells with VEGF attenuated the $CoCl_2$-induced cytotoxicity in both dose- and time-dependent manner. Mechanistically, VEGF increased IκBα phosphorylation and ubiquitination, promoted P65 nuclear translocation as well as upregulated *XIAP* and *CCND1* expression. Meanwhile, VEGF administration reversed the dysregulation of IκBα phosphorylation and ubiquitination, P65 nuclear translocation as well as *XIAP* and *CCND1* expression induced by $CoCl_2$. Notably, the VEGF-dependent cytoprotection was abolished by pretreatment with BAY 11-7085, a specific inhibitor of NF-κB. Our data suggest that VEGF/NF-κB signalling pathway represents an adaptive mechanism that protects neural cells against hypoxic damage.

1. Introduction

Cerebral ischemia is a common cardiovascular disease, occurring as the second most prevalent noncancer-related disease in China, exhibiting considerable geographic variation and ranking as the third main inducer of deaths. The main pathogenesis of cerebral ischemia includes inflammatory response, edema, hypoxia and neuronal apoptosis, which result in several motor dysfunctions, including hemiplegia and paraplegia [5,18]. Accumulating evidences suggest that the prognosis of cerebral ischemia is dependent on the neuron survival and improved neurological outcome [19]. Accordingly, the development of innovative therapies preventing neural damage caused by hypoxic–ischemic insult has high priority.

Vascular endothelial growth factor (VEGF), a member of downstream targets of hypoxia-inducible factor-1 (HIF-1), exert its

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biological activities via multiple signaling transducers, such as the SRC, MAPK, PI3K/Akt, and Smad [1,2,16,20]. Functioning as a prototypical cytokine to mediate the phosphorylation of vascular endothelial growth factor receptor (VEGFR), VEGF plays a important role in regulating angiogenesis, cell differentiation, cell invasion, and metabolism. Autocrine secretion of VEGF-A in human pancreatic cancer cells is essential for the maintenance of the tumorigenic phenotypes, including cell proliferation and malignant vascularization [3]. It has been reported that VEGF/STAT3 signaling is required for the neuronal differentiation of neural stem/progenitor cells [13]. In addition, promotion of VEGF-A mRNA splicing by RNA splicing protein YT521 in endometrial carcinoma (EC) results in an increase of EC cells invasion [21]. Disruption of constitutively activated ERK/VEGF signaling has also been found to inhibit high glucos-induced human adult retinal pigmented epithelium (ARPE) cell growth [22]. However, the biological roles and underlying mechanisms of VEGF in hypoxia/ischemia-induced neural injury is poorly understood.

In this report, we validated the protective action of VEGF in hypoxic PC12 cells induced by $CoCl_2$, and identified the possible mechanism: activation of nuclear factor-κB (NF-κB) signalling pathway. Our study may open up the possibility for further investigation of VEGF in clinical trials of patients with brain ischemia.

2. Materials and methods

2.1. Cell culture and transfection

PC12 cells, the rat cell line derived from phaeochromocytoma cells, which can be induced into neuronal-like cells by nerve growth factor (NGF), were cultured as previously described [15,23]. Briefly, neural PC12 cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, USA) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum (FBS), 50 ng/mL purified recombinant mouse beta-NGF (R&D Systems, USA), 100 U/mL penicillin and 100 mg/mL streptomycin, at 37 °C and 5% CO_2 under a humidified atmosphere.

Transient transfection of siRNA was performed according to manufacturer's guidelines as previously described [9,15]. For detailed methods, please see Supplemental information.

2.2. Cell viability assay

Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (MTT) assay as previously described [9,15]. For detailed methods, please see Supplemental information.

2.3. Western-blotting

Western blotting analyses were performed with precast gradient gels (Bio-Rad) using standard methods as described previously [9,15]. For detailed methods, please see Supplemental information.

2.4. Quantitative real-time PCR (qRT-PCR)

For quantitative real-time PCR (qRT-PCR) experiments, total RNA was extracted from the indicated PC12 cells using Trizol (Invitrogen) and was then reverse-transcribed to complementary DNA with PrimeScript® RT reagent Kit (Takana, Dalian, China) using Super Array PCR master mix (SuperArray Bioscience, Frederick, Maryland, USA). Real-time PCR was then performed on each sample with the double-stranded DNA dye SYBR Green PCR Mastermix in Takana SYBR® Primix Ex Taq™ Kit (Takana, Dalian, China) according to the manufacturer's instructions. Primers included were the following: XIAP forward, 5′-GACCCTCCCCTTGGAC-3′; XIAP reverse, 5′-CTGTTAAAAGTCATCTTCTCTTGAA-3′; CCND1 forward, 5′-GGCGGATTGGAAATGAACT-3′; CCND1 reverse, 5′-TCCTCTCCAAAATGCCAGA-3′; GAPDH forward, 5′-AATCCCATCACCATCTTCC-3′; GAPDH reverse, 5′-TGGACTCCACGACGTACTC-3′. All reactions were run on an Applied Biosystems 7900HT Fast Real-Time PCR instrument with a 15 min hot start at 95 °C followed by 40 cycles of a three step thermocycling program: denaturation: 30 s at 95 °C, annealing: 5 s at 95 °C, and extension: 30 s at 60 °C. Melting curve analysis was performed at the end of every run to ensure that a single PCR product of the expected melting temperature was produced in a given well.

2.5. Annexin-V/PI staining

Annexin-V/Propidium iodide (PI) double staining was performed on PC12 cells by flow cytometric analysis as described previously [9,15]. Briefly, cells were seeded in 24-well plates, exposed to 0.6 mmol/L $CoCl_2$ with or without 100 ng/mL VEGF for 24 h, and then harvested, washed and incubated in binding buffer containing 25 μg/mL Annexin V-FITC and 25 μg/mL PI for at 37 °C. After 20 min, the cells suspensions were underwent FACS analysis. The percentage of apoptosis was corrected for background levels found in the corresponding untreated controls.

2.6. Caspase-3 activity assay

Caspase-3 activity was carried out using caspase-3 activity detection kit (Bestbio, Shanghai, China). The detailed procedures for Caspase-3 activity were described previously [15]. Briefly, after treatment with the indicated conditions, cellular proteins were extracted and the protein concentrations were determined. Then, the protein liquids were mixed with the reaction buffer and 10 μL Ac-DEVD-*p*NA substrate, followed by a 1 h incubation at 37 °C. Caspase-3 activity was measured at 405 nm with a plate reader. Three independent experiments were performed separately.

2.7. Hoechst-PI staining

To quantify the apoptotic and necrotic cells by Hoechst-PI staining, cells were grown in six-well plates, underwent various treatments as indicated and washed twice with 1 × PBS. Subsequently, Hoechst 33342, a blue-fluorescent dye that stains all cells and propidiumiodide (PI), a red-fluorescent dye that stains dead cells, were added to culture media at a final concentration of 5 μg/mL. After incubation for 15 min, 3 fields were randomly selected, quantification of apoptotic cells were observed and quantified using fluorescent microscope (IX71; Olympus, Japan). The percentage of apoptotic cells was expressed as the mean ± SD of three experiments.

2.8. Statistical analysis

SigmaStat Statistical Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses and data were expressed as mean ± standard deviation of at least three independent experiments. Differences between two groups were assessed by an unpaired, two-tailed Student's *t* test (SPSS software 17.0). One-way ANOVA was performed to make statistical comparisons in multigroup analysis. A P value of <0.05 was considered statistically significant.