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Overexpression of HIPK2 attenuates spinal cord injury in rats by modulating apoptosis, oxidative stress, and inflammation

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ABSTRACT

HIPK2 is considered to be a tumor suppressor. It also has been implicated in several functions such as apoptosis and inflammation that are linked to spinal cord injury (SCI). However, whether HIPK2 ameliorates the neurological pain of SCI remains unclear. Here, we investigated the effects of HIPK2 on neurological function, oxidative stress, levels of inflammatory cytokines and expression of Bcl-2/Bax in an SCI model. Firstly, we evaluated the therapeutic effects of HIPK2 on neurological pain in the SCI rat using the Basso, Beattie and Bresnahan scores and H & E staining. Overexpression of HIPK2 significantly elevated the levels of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), and reduced the mRNA expression of Nogo-A and RhoA in SCI rats. Furthermore, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays showed that overexpression of HIPK2 significantly reduced the number of apoptotic cells. Overexpression of HIPK2 also decreased expression of Bax and Caspase-3 and elevated expression of Bcl-2 in the SCI model, indicating that HIPK2 exhibited its protective activity by inhibiting SCI-induced apoptosis. Then, we measured the serum concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX). We also determined the mRNA and protein levels of nuclear factor-κB p65 unit, tumor necrosis factor-α (TNF-α), and interleukin (IL)-1β. HIPK2 overexpression reduced oxidative stress and the levels of inflammatory cytokines compared with SCI control animals. Additionally, acetylation of HIPK2 was reduced in SCI rats. Overexpression of HIPK2 could enhance autophagy by elevating the expression of Beclin-1 and LC3-II while autophagy is regarded as a beneficial regulator to improve spinal cord injury. Together, overexpression of HIPK2 improved contusive SCI induced pain by modulating oxidative stress, Bcl-2 and Bax signaling, and inflammation, and also regulating autophagy.

1. Introduction

Spinal cord injury (SCI) can result in debilitating paralysis and other complications, and understanding SCI is receiving increased attention [1]. Nerve tissue degeneration and necrosis, glial scar formation and cavity formation are results of SCI. Cardiovascular dysfunction and other health concerns can be secondary results [2,3]. Currently, there is no effective pharmacological treatment for SCI due to its complexity. Therefore, it is necessary to clearly understand the mechanisms underlying SCI so that effective treatments can be developed.

Oxidative stress is caused by the imbalance between reactive oxygen species in the body and the antioxidant system [4]. Because oxidative stress can be a secondary response to SCI, it has attracted more and more attention [4]. It has been reported that oxidative stress plays an important role in limiting recovery from SCI, and downregulation of oxidative stress can significantly improve the prognosis of SCI [5,6]. Many studies indicate that oxidative stress and inflammatory cytokines, such as TNF-α, IL-1β and p65, perform essential roles in the process of SCI-induced apoptosis [7]. Additionally, Bax and Bcl-2 are the apoptotic and anti-apoptotic regulators in the development of SCI, respectively [8,9]. Inhibition of neuronal apoptosis by overexpression of Ad-HIF-1α or other therapeutic approaches promoted angiogenesis by regulating the Bax/Bcl-2 signaling in SCI animals [10–12].

Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase that is localized mainly in the nucleus [13]. HIPK2 is considered to be a tumor suppressor, and it controls a series of biological functions including DNA damage, apoptosis, hypoxia and proliferation and invasion of cancer cells [14–16]. The proapoptotic function of HIPK2 is restricted by reversible acetylation [17]. However, the neuroprotective effects of HIPK2 on SCI have not been described. Thus, we aimed to investigate the mechanisms underlying the protective role of HIPK2 in oxidative stress, inflammation, and regulation of

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the SCI-induced Bcl-2 and Bax pathway in rat models.

2. Methods and materials

2.1. Animals and cell culture

Adult male Sprague-Dawley (SD) rats (about 260 g) were purchased from the Model Animal Research Center of Nanjing University. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). These rats were given free access to food and water and were housed at 2325 °C under a 12 h light/dark cycle (relative humidity 40–60%). The rats were randomly divided into three groups (10 per group): an uninjured group (no SCI), an SCI control group and an Ad-HIPK2-infected SCI group. After the adenovirus injection for 2 days, an SCI model was established in a previous report using Allen's advanced method [18]. SCI control animals were injected with sterile saline containing Ad-control (Ad-con). The other rats were injected with an equal volume of Ad-HIPK2 two days before the contusion injury. Except for the described antibodies, commercial kits and chemicals in this study, all of the other materials were purchased from Sigma (Shanghai, China).

PC12 cells were obtained from the American Type Culture Collection (ATCC, Maryland, USA). The cells were grown at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, California, USA) supplemented with 10% fetal bovine serum (Gibco), 100 mg/L streptomycin and 0.1 U/L ampicillin. Twenty-four hours prior to transfection, 6×10^5 PC12 cells were seeded onto 6-well plates overnight. When the cells were at 70% confluency, 4×10^3 plaque-forming units (PFU) was used for a well (six-well plates) (Invitrogen, Carlsbad, CA, USA) diluted with DMEM without serum. After transfection with Ad-HIPK2, Ad-HIPK2 10KR, and Ad-HIPK2 10KQ for 12 h, the cells were incubated with 300 μM H_2O_2 for 24 h. The CCK-8 assay (Sigma, Shanghai, China) was performed to determine the viability of PC12 cells according to the manufacture's instruction.

2.2. Adenovirus preparation, plasmid construction, and siRNA

We constructed a plasmid for overexpressing HIPK2 full length. Recombinant adenoviruses expressing mouse HIPK2 (Ad-HIPK2), HIPK2 10KR (Ad-HIPK2 10KR), HIPK2 10KQ (Ad-HIPK2 10KQ) or SCI control (Ad-con) were generated according to the manufacturer's instructions of the pAd-Easy system with a GFP tag (Invitrogen, California, USA). Ad-vector was used as a negative control. In this section, lipofectamine™ 2000 transfection reagent was purchased from Invitrogen. The adenovirus constructs were diluted in 200 μL PBS and injected via tail vein at a dose of 4×10^{10} PFU per rat, and PC12 cells were incubated with 4×10^3 PFU for a well (six-well plates). Meanwhile, the PC12 cells were transfected with plasmid containing HIPK2 full length and siRNA for HIPK2 using lipofectamine™ 2000 reagent, and the siRNA for HIPK2 was purchased from GenePharma (Shanghai, China).

2.3. Behavioral analysis

All of the rats were evaluated by two experienced examiners in a double-blind method. The Basso, Beattie and Bresnahan (BBB) locomotor rating scale was used to determine the changes in motor function. BBB scores range between 0 (no observable hind-limb movements) and 21 (normal gait) [19].

2.4. Histological examination

Rats were anesthetized and sacrificed one day after injury. The tissues from rats were fixed in 4% formalin buffer. The fixed specimens were processed to paraffin blocks, sectioned, and subject to hematoxylin-eosin (H & E) staining for histological analysis using standard protocols. The H & E staining was performed as previously described [20].

2.5. TUNEL assay and annexin V/PI double-staining analysis of apoptosis

Apoptotic cells in the spinal cord tissues of rats were determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) as per the manufacturer's instructions (Sigma). The stained sections were observed under a microscope (Olympus, Germany).

Apoptotic cells were determined using an annexin V-FITC-labeled apoptosis detection kit and flow cytometry following the manufacturer's instructions (Sigma). Briefly, PC12 cells (2×10^6 cells/mL) were seeded in 100 mm dishes and cultured overnight in DMEM medium containing 10% FBS. HIPK2 was overexpressed or knocked down by HIPK2 plasmid and siHIPK2 respectively in PC12 cells for 48 h, and then the cells were incubated with H_2O_2 for 60 min. These cells were harvested in cold PBS and collected by centrifugation for 10 min at 800 × *g*. the cells were then resuspended at a density of 2×10^6 cells/mL in binding buffer (HEPES buffer, 10 mM, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and stained simultaneously with FITC-labeled annexin V (25 ng/mL; green fluorescence) and propidium iodide (PI) (50 ng/mL) in the dark. The PI, a marker of cell viability, was provided as a 50 mg/mL solution (Sigma). These samples were analyzed with a fluorescence-activated cell sorter (FACS) and flow cytometer (Becton Dickinson), and the collected data was analyzed with Cell Quest software. Annexin V-positive and PI-negative cells were considered as apoptotic. Double-stained cells were recorded as being either necrotic or as late apoptotic.

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

The reverse transcription polymerase chain reaction (RT-PCR) and the quantitative real-time PCR (Q-PCR) were performed as previously described [21]. Total RNA was extracted from tissues using TRIZOL reagent. Both TRIZOL reagent and the SYBR green PCR system were from Invitrogen. The cDNA was obtained by reverse transcription in a 20 μL reaction containing 2 μg of total RNA, oligo (dT), and reverse transcription premix. Normal amplification consisted of 28–30 cycles as follows: denaturing at 94 °C for 30 s, annealing at 56 °C for 1 min and extending at 72 °C for 30 s, followed by a final 10 min incubation at 72 °C. The PCR was performed using a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Hercules, USA). PCR products were electrophoresed on 2% agarose gels and visualized using a Tanon 2000 imaging system (Tanon, Changxing, China).

The Q-PCR reactions were performed with the SYBR green PCR system in an ABI 7500 thermal cycler (Applied Biosystems, X). The cycling conditions were as follows: 95 °C for 3 min; followed by 40 cycles involving denaturing at 95 °C for 10 s, annealing at 60 °C for 5 s and extension at 72 °C for 10 s. Expression of mRNAs was normalized to β-actin mRNA level, which was used as an internal control. The primers used were as follows: HIPK2, sense, 5′-GCC AAT CCC GAA GTC TCC AT-3′, antisense, 5′-AGA GCT TGC TGG AAA GGG TC-3′; BDNF, sense, 5′-CCA ATC GAA GCT CAA CCG AAG A-3′, antisense, 5′-GGC GGT TTC CTT CTC CAA GC-3′; GDNF, sense, 5′-GGA GAC CGG ATC CGA GGT G-3′, antisense, 5′-GCG CTT CGA GAA GCC TCT TA-3′; caspase-3, sense, 5′-TGG AAA GCC GAA ACT CTT C-3′, antisense, 5′-AGG AAT AGT AAC CAG GTG CTG-3′; Bcl-2, sense, 5′-AGC ATG CGA CCT CTG TTT GA-3′, antisense, 5′-TCA CTT GTG GCC CAG GTA TG-3′; Bax, sense, 5′-AGG ACG CAT CCA CCA AGA AG-3′, antisense, 5′-CAG TTG AAG TTG CCG TCT GC-3′; TNF-α, sense, 5′-CATCCGTTCTCTACCCAGCC-3′, antisense, 5′-AATTCTGAGCCCGGAGTTGG-3′; IL-1β, sense, 5′-GAC TTC ACC ATG GAA CCC GT-3′, antisense, 5′-GGA GAC TGC CCA TTC TCG AC-3′; p65, sense, 5′-CAT GGA TCC CTG CAC ACC TT-3′, antisense, 5′-CTC AGC