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Nur77 is a promoting factor in traumatic brain injury-induced nerve cell apoptosis

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ABSTRACT

Traumatic brain injury (TBI) poses a serious threat to human health. TBI has a high mortality rate, resulting in a great burden on the affected individual's family as well as society as a whole. The incidence of craniocerebral fractures continues to rise as both the economy and transportation options grow, making it imperative that the mortality and disability rate of craniocerebral trauma be reduced. Nur77 is a transcription factor of the nuclear receptor superfamily. Following stimulation of extracellular apoptosis, Nur77 is involved in a variety of diseases as a powerful pro-apoptotic molecule. Here, we determined the effect and mechanism of Nur77 in TBI-induced nerve cell apoptosis in vitro and in vivo. We found that Nur77 and Bcl-2 protein expression increased as nerve cell apoptosis increased in TBI tissues. Furthermore, inhibition of Nur77 improved nerve cell injury by regulation of Bcl-2 and downstream pathways in vitro and in vivo.

1. Introduction

Traumatic brain injury (TBI) has gradually become a major public health problem. In young patients, TBI is a high risk factor, and is one of the primary elements that inform high yield and high mortality [1]. According to the World Health Organization (WHO), TBI may lead to 5 million deaths each year. In 2012, 3500 people were killed each day in traffic accidents, an increase of 600 per day compared to 2000 statistics. In China, with the increase in urbanization and the wide range of motor vehicle options, injury due to road traffic has greatly increased. TBI has gradually become the primary cause of disability and death among young and middle-aged people, which is aggravated by the burden placed on families [2]. Therefore, in-depth study of the pathogenesis and prevention measures associated with TBI is of great significance for improving the survival rate and quality of life following TBI.

Following TBI, the associated brain injury can be divided into two types: primary and secondary. Primary brain injury occurs immediately after the occurrence of brain injury, and is often inevitable, whereas secondary brain injury occurs several minutes to several days after TBI, and is a systematic pathophysiological change in the whole brain Secondary brain injury refers to a series of neurobiochemical processes triggered by TBI, including depolarization, homeostasis glutamate excitotoxicity, oxygen free radical production, lipid peroxidation, blood-brain barrier damage, brain edema, secondary hemorrhage, tissue ischemia, intracranial pressure, mitochondrial dysfunction, and axon fracture. Inflammatory apoptosis and cell necrosis, a series of complex case processes and cascade reactions, also occur. These biochemical processes cause nerve cell damage, resulting in neurological dysfunction [3–5].

Orphan nuclear receptor Nur77, an early reactive protein, is normally located in the nucleus and is associated with various cellular activities [6]. Nur77 can be transferred from the nucleus to the mitochondria when stimulated, thereby inducing apoptosis [7]. In our present study, we examined the effects and mechanisms of Nur77 and its downstream pathway on the development of nerve cell injury induced by TBI.

2. Materials and methods

2.1. Clinical sample

Glioma tissues were collected from 30 glioma patients and TBI tissues were collected from 25 TBI patients who were treated at the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School, from June 2016 to December 2017. Following collection, the tissues were fixed in 10% formaldehyde until use.

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2.2. Cell and culture

PC12 cells were purchased from ATCC (USA) and incubated at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 5% fetal bovine serum (Gibco, USA) and 5% horse serum (Gibco, USA) as previously described [8]. The medium was changed two times per week, and the cells used in the experiment were in a logarithmic growth period.

2.3. TBI cell model preparation and grouping

The PC-12 cells were divided into four groups: negative control (NC), TBI model (Model), Nur77 inhibitor (cyclosporin A [CsA])-treated cells based on the TBI cell model (Model + Nur77 inhibitor), and Bcl-2 inhibitor (APG-1252)-treated cells based on the TBI cell model (Model + Bcl-2 inhibitor). The NC group was treated normally, the Model group cells were cultured in a 6-well plate and a 200-μl pipette tip was used to detach the cells, the Model + Nur77 inhibitor group was treated as the Model group with the addition of 10 μM CsA (R&D, USA), and the Model + Bcl-2 inhibitor group was treated as the Model group with the addition of 10 μM APG-1252 (Ya Sheng Pharmaceutical Co., Ltd., Jiangsu, China).

2.4. Animal

Male Sprague-Dawley (SD) rats (n = 36) were purchased from the experimental animal center of Nanjing Medical University, with an average body weight of 140–200 g. The rats were divided into four groups of nine animals each: NC group, Model group, Model + Nur77 inhibitor group, and Model + Bcl-2 inhibitor group. Establishment of the TBI model in SD rats was based upon on a previously published method [9]. The specific method used in the present study was as follows: The TBI model of SD rats was produced using the free-landing epidural impact method, and moderate brain injury was produced by dropping a 30 g hammer from a height of 35 cm The Model, Model + Nur77 inhibitor, and Model + Bcl-2 inhibitor group rats were prepared with the TBI model method. The NC and Model groups were injected with 0.9% normal saline, the Model + Nur77 inhibitor group with 1 ml/kg CsA, and the Model + Bcl-2 inhibitor group with 1 ml/kg APG-1252. The injection volume for all groups was identical. After 14 d with food ad libitum, all animals were fasted overnight before euthanasia was performed. Tissues were then collected and divided into two parts; one part was stored at −80 °C and one part was fixed in 10% formaldehyde

2.5. Hematoxylin and eosin (H&E) staining

The tissue sections were incubated at 60 °C in a thermostat box for 30 min, placed in turpentine for 30 min, and washed with distilled water for 5 min. The sections were stained with hematoxylin for 15 min, rinsed with tap water for 2 min, placed in 0.5% hydrochloric acid ethanol for 30 s, and then washed with tap water for 20 min. Sections were then stained with eosin dye for 30 s and then washed with tap water for 30 s. The sections were then concentrated by gradient ethanol dehydration (low to high), soaked in dimethylbenzene, and mounted in neutral gum. The pathological changes in the brain tissue were then observed under an optical microscope.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The brain tissues were embedded in paraffin and cut into 5-μm sections. Cell apoptosis was then measured by TUNEL assay (Roche, USA) according to the manufacturer's instructions and as previously described [10]. Blue nuclear staining indicates normal cells, while brown nuclear staining indicates apoptotic cells (positive cells). The brain tissue nerve cell apoptosis rate (%) = (number of apoptotic cells at high magnification / total number of cardiac myocytes in the field of vision) × 100%.

2.7. Immunohistochemistry (IHC) assay

We used the streptavidin-biotin complex (SABC) method to evaluate relative protein expression and closely follow the manufacturer's instructions for the Nur77, Bcl-2, cytochrome C (Cyto C), and Caspase 3 IHC kits (Abcam, UK). The cells were then observed under an optical microscope, with brown-yellow staining indicating positive cells. The Leica Qwin image signal acquisition and analysis system (Leica Microsystems, USA) was used for processing.

2.8. Determination of cell apoptosis by annexin V/propidium iodide (PI)

When the cells were in logarithmic growth, 0.25% ethylenediaminetetraacetic acid (EDTA) trypsin was added, the digestive state was observed under an optical microscope, and cell culture medium was added to terminate digestion. Cells (2 × 105) were collected, washed 2 times with cold phosphate-buffered saline (PBS) (pH 7.2), centrifuged for 5 min at 1000 r/min, and the supernatant discarded. Cells were then resuspended in 1 ml cold PBS, centrifuged for 5 min at 1000 r/min, the supernatant discarded, and cells resuspended in 100 μl combined buffer solution. Annexin V-fluorescein isothiocyanate (FITC) (5 μl) and PI (1 μl) were added to the cell suspension, incubated for 15 min in the dark, and then mixed with 400 μl combined buffer solution. Machine testing.

2.9. Immunofluorescence staining

A 1-ml cell suspension (1 × 107 cells/L) was added to each well of a 24-well glass plate and incubated overnight. The cells were then washed 3 times with PBS, fixed with PD containing 4% polyoxymethylene for 30 min at room temperature, and again washed with PBS (3 × 10 min). Triton X-100 (0.5%) was then added for 20 min to permeabilize the membrane followed by washing with PBS (3 × 10 min). The cells were then incubated with goat serum working fluid for 30 min at room temperature to block non-specific antigen. The Nur77 primary antibody (1:200; Abcam, UK) was added, the cells incubated in a wet box overnight at 4 °C, washed 3 times with PBS, and then Alexa Fluro 568 goat anti-rat secondary antibody (1:1000) was added and incubated for 1 h in the dark. Cells were then washed with PBS (3 × 10 min) and a 4′,6-diamidino-2-phenylindole (DAPI) glycerol seal stain added. The cells were then observed under a fluorescence microscope and the images scanned by a laser-scanning confocal microscope.

2.10. Western Blotting (WB) assay

Cells were collected and washed 3 times with PBS, lysed, incubated for 5 min on ice, the supernatant collected, and the protein concentration measured. The sample was then added to sample buffer, incubated for 5 min at 95 °C to denature the proteins, and then stored at −80 °C until use. The samples (25 μg protein per sample) were then loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and subjected to electrophoresis at 60 V for 2 h, followed by 120 V for 1 h. After electrophoresis, the proteins in the gel were transferred to a polyvinylidene fluoride PVDF membrane in electromigration buffer at 200 mA constant current for 2 h in an ice bath. The membrane was then incubated with 5% skim milk in Tris-buffered saline-Tween 20 (TBST) shaking at room temperature and then rinsed with TBST for 5–10 min with shaking at room temperature. Nur77 (Abcam, UK), Bcl-2 (Abgent, USA), Cyto C (Abcam, UK), and Caspase 3 (Abcam, UK) antibodies (1:1000) were then added and incubated overnight at 4 °C. The membranes were then washed with TBST