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Overexpression of conserved dopamine neurotrophic factor (CDNF) in astrocytes alleviates endoplasmic reticulum stress-induced cell damage and inflammatory cytokine secretion

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ABSTRACT

Astrocyte damage and the disorders of cytokine secretion induced by endoplasmic reticulum stress (ERS) are crucial pathological processes in ischemic injury of the central nervous system (CNS), (e.g., ischemic reperfusion injury of the brain and spinal cord). ERS stimulates damage to astrocytes and the release of pro-inflammatory cytokines, which deteriorates CNS injury. This current study investigates whether the overexpression of conserved dopamine neurotrophic factor (CDNF) alleviates ER stress-induced cell damage and inflammatory cytokine secretion. We found that primary astrocytes showed both a successful transduction and a significant overexpression of CDNF protein following lentivirus application. Our results show that the percentage of LDH released as a result of ER stress was significantly lower in astrocytes with an overexpression of CDNF than in the control groups without CDNF overexpression, indicating that CDNF alleviates ER stress-induced astrocyte damage. The secretion and mRNA expression levels of pro-inflammatory cytokines were increased by tunicamycin, and this stimulation was significantly suppressed by an overexpression of CDNF, demonstrating that CDNF plays an important role in astrocyte inflammation and functioning by resisting ER stress. These findings suggest that primary astrocytes can be efficiently transduced with CDNF lentiviral vectors and that the overexpression of CDNF in astrocytes shows the potential to alleviate cell damage and proinflammatory cytokine secretion, which may represent a promising strategy for neuroprotection in the CNS.

1. Introduction

Astrocytes play an important role both in physiological and pathological process in the central nervous system (CNS) [1,2]. They respond swiftly to subtle changes in the microenvironment and secrete an array of pro-inflammatory and anti-inflammatory cytokines, chemokines, and trophic factors to modify the ambient microenvironment [3,4]. Astrocyte-derived factors are important in neuronal survival, neurogenesis and neuron repair [5,6]. Astrocyte damage and the disorders caused by cytokine secretion are crucial pathological processes in CNS ischemic injury (e.g., brain and spinal cord ischemic reperfusion injury).

In ischemic reperfusion injury, endoplasmic reticulum stress (ERS) is caused by hypoxic-ischemia, glucose starvation, ATP depletion, oxidative stress and Ca^{2+} homeostasis disorders. Astrocyte damage and apoptosis is then induced by ERS through the accumulation of unfolded or misfolded proteins in a subcellular organelle mainly referred to as a protein-folding factory known as the ER [7–10]. Tunicamycin, a naturally occurring antibiotic and a trigger of the ER stress response (ERSR), is used to imitate the condition of an astrocyte under ERSR, which will then cause cell damage and inflammatory responses *in vitro* [11].

Previous studies demonstrated that there is no effective therapy to treat CNS ischemic reperfusion injury; however, conserved dopamine neurotrophic factor (CDNF), a vertebrate-specific paralogue of the human mesencephalic-astrocyte-derived neurotrophic factor (MANF) [12], may provide a potential method. Previous evidence suggests that MANF is an ER stress response protein and is able to protect cells against ER stress-induced cell death *in vitro* [13]. However, the role of CDNF and the function of astrocytes in the ER stress response is not clear; therefore, we investigated the effect of overexpression of CDNF on ERS-induced cell damage and inflammatory cytokine secretion in astrocytes *in vitro*.

2. Materials and methods

2.1. Animals

Wistar rats were obtained from the Laboratory Animal Center of Shandong University. All animals were kept under controlled light/

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dark conditions (12/12 h), temperature (23 °C), and humidity (60%). In the handling and care of all animals, the International Guiding Principles for Animal Research were followed, as stipulated by the World Health Organization (1985) and as adopted by the Laboratory Animal Center of Shandong University. All efforts were made to minimize pain and the number of animals used.

2.2. Rat astrocyte isolation and culture

Primary astrocytes were prepared from neonatal Wistar rats post-natal day one, as previously described [14]. Briefly, the cortical tissues freed of meninges and blood vessels were mechanically dissociated, and the cell suspension was seeded at a density of 1×10^6 cells/ml in Dulbecco's modified Eagle medium (5.5 mmol/L glucose) (DMEM, Gibco, Grand Island, NJ, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NJ, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA). When the culture reached confluency, the microglia and oligodendrocytes were removed by an orbital shaker; the remaining cells, a majority of them astrocytes, were replated. Before each experiment, the plated cells were incubated with serum-free DMEM medium for 1 h.

2.3. Immunocytochemistry

Primary astrocytes were subcultured on sterile glass coverslips for 12–16 h, washed in PBS, fixed in 4% formaldehyde for 10 min at 37 °C, permeabilised in 0.2% Triton-X 100 for 10 min at room temperature, washed in PBS, and then blocked in 1% BSA in PBS for 20 min at room temperature. The cells were then incubated with primary antibodies (mouse monoclonal anti-GFAP 1:100, Abcam) overnight at 4 °C. Cells were then washed in PBS and incubated with secondary antibodies (Texas-Red-conjugated anti-mouse IgG 1:1000, Invitrogen) in PBS for 40 min at 37 °C. Images were captured using an EVOS fluorescence microscope (EVOS, AMG, WA, USA).

2.4. Recombinant CDNF lentiviral vector design and production

The lentivirus was produced from co-transfection with three plasmids (pMD2G, pSPAX, and the transfer plasmid vector plenti-his, kindly provided by Prof. Tang DQ of Shandong University). CDNF was amplified from a cDNA3.1-human CDNF plasmid constructed in our previous study [15] using the following primers: CDNF forward primer 5′-TAGGATCCATGTGGTGCGCGAGCCCAGT-3′, CDNF reverse primer 5′-TACTCGAGTCAGAGCTCTGTTTTGGGGT-3′. Recombinant lentiviral vectors plenti-CDNF were harvested and concentrated using the Lenti-X Concentrator (Clontech, TaKaRa, CA, USA) 72 h following co-transfection of the pMD2.G (3.4 μg), pSPAX (6.6 μg), and the transfer plasmid plenty-his (10 μg) into 293T cells cultured in DMEM (10% FBS). Transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA) with the manufacturer's recommendations. Lentiviral vectors plenti-his were also produced as a control group using the plasmid plenti-his instead of plenti-CDNF.

2.5. In vitro lentiviral vector transduction

Astrocytes were seeded in 6-well plates (Corning, Corning, NY) at a density of 5×10^4/well, and 1 ml Dulbecco's modified Eagle medium (5.5 mmol/L glucose) with 10% FBS was added to each well. Primary astrocytes were cultured for 24 h. Viral multiplicity of infection (MOI) was detected by Lenti-X GoStix (Clontech, TaKaRa, CA, USA). Primary astrocytes transduced with 5 MOI of the plenti-CDNF/plenti-his lentivirus were used in the following studies as the morphology of these cells was most similar to the non-transduced astrocytes. Following incubation at 37 °C in 5% CO_2 for 24 h, the virus-containing medium was removed and replaced with 1 ml of fresh culture medium per well. As plenti-CDNF does not contain a GFP-reporting gene, the transduction efficiency should be determined by measuring the CDNF protein using a Western blot.

2.6. Western blot analysis

Five days following lentiviral vector transduction, plenti-CDNF-expressing astrocytes were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 1 mM PMSF. The extract was centrifuged at 12,000×*g* for 5 min at 4 °C to remove cell debris. Protein concentration was determined by a BCA protein assay kit according to the manufacturer's instructions. Equivalent amounts of protein (10 μg) for each sample were separated by 10% Acrylamide-SDS–PAGE using 5% stacking and 12% separating gels and were subsequently transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). Primary antibodies (goat anti-CDNF, 1:1000 dilution, R&D Systems, Inc., Minneapolis, MN, USA) and a rabbit anti-goat immunoglobulin (IgG)-horseradish peroxidase (HRP) secondary antibody (1:30,000 dilution, Cell Signaling Technology, Danvers, MA) was applied. Equal amounts of protein-loading were confirmed by re-probing the membranes with the mouse anti-β-actin-HRP (1:10,000 dilution, Abcam). Immunoblots were visualized by chemiluminescence (Pierce Biotechnology, Rockford, IL) with exposure to autoradio-graph film (X-OMAT AR; Eastman Kodak, Rochester, NY).

2.7. Interventions of astrocytes after lentivirus transduction

Five days after the lentiviral vector transduction, the experimental plenti-CDNF-transfected astrocytes and the control plenti-his-transfected astrocytes were seeded in 6-well plates (Corning, Corning, NY) at a density of 5×10^4/well, and 1 ml Dulbecco's modified Eagle medium (5.5 mmol/L glucose) with 10% FBS was added to each well. After 24 h, the medium was removed and replaced with 1 ml of fresh culture medium without FBS and was allowed to sit in culture for the next 24 h. The cells were then treated with either 50 ng/ml of tunicamycin or a vehicle for the following studies [16]. Tunicamycin was dissolved in phosphate-buffered saline (PBS; pH 7.4), and the same volume of PBS was used as the vehicle.

2.8. LDH release assay

Five days after lentivirus transduction, plenti-CDNF and plenti-his-transfected astrocytes and non-transfected cells were treated with tunicamycin (50 ng/ml) for 5 h, which was expected to cause ER-stress. To measure the extent of damage to the cells, the stable cytosolic enzyme resulting from cell lysis, lactate dehydrogenase (LDH) was measured in the cell culture medium using an LDH-Cytotoxicity Assay Kit II (BioVision, CA, USA). Simply stated, the clear medium (100 μl/well) was transferred into an optically clear 96-well plate, then 100 μl of LDH Reaction Mix was added to each well, mixed and incubated at room temperature for 30 min. The absorbance at 450 nm was measured by a microplate reader spectrophotometer (Multiskan Ascent).

2.9. ELISA

After 5 h of the treatment of tunicamycin, cytokine (IL-1ß, IL-6, and TNF-α) levels in astrocyte culture medium were determined by ELISA, as described by the manufacturer (R&D Systems). ODs were determined using a Spectromax 190 microplate reader (Molecular Devices) at 450 nm. Cytokine concentrations in the