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Mild hypothermia protects hippocampal neurons from oxygen-glucose deprivation injury through inhibiting caspase-3 activation

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ABSTRACT

Mild hypothermia (MH) is thought to be one of the most effective therapeutic methods to treat hypoxic-ischemic encephalopathy (HIE) after cardiac arrest (CA). However, its precise mechanisms remain unclear. In this research, hippocampal neurons were cultured and treated with mild hypothermia and Ac-DEVD-CHO after oxygen-glucose deprivation (OGD). The activity of caspase-3 was detected, in order to find the precise concentration of Ac-DEVD-CHO with the same protective role in OGD injury as MH treatment. Western blot and immunofluorescence staining were conducted to analyze the effects of MH and Ac-DEVD-CHO on the expressions of caspase-3, caspase-8, and PARP. The neuronal morphology was observed with an optical microscope. The lactic acid dehydrogenase (LDH) release rate, neuronal viability, and apoptotic rate were also detected. We found that MH (32 °C) and Ac-DEVD-CHO (5.96 μMol/L) had equal effects on blocking the activation of caspase-3 and the OGD-induced cleavage of PARP, but neither had any effect on the activation of caspase-8, which goes on to activate caspase-3 in the apoptotic pathway. Meanwhile, both MH and Ac-DEVD-CHO had similar effects in protecting cell morphology, reducing LDH release, and inhibiting OGD-induced apoptosis in neurons. They also similarly improved neuronal viability after OGD. In conclusion, caspase-3 serves as a key intervention point of the key modulation site or regulatory region in MH treatment that protects neuronal apoptosis against OGD injury. Inhibiting the expression of caspase-3 had a protective effect against OGD injury in MH treatment, and caspase-3 activation could be applied to evaluate the neuroprotective effectiveness of MH on HIE.

1. Introduction

Hypoxic ischemic encephalopathy (HIE) is a common complication of cardiac arrest and refers to a cerebral injury resulting from inadequate oxygen supply to the brain [1,2].

Clinical and experimental studies have both shown that mild hypothermia (MH) improves neurological outcomes in HIE after cardiac arrest [3–6]. The protective mechanisms of MH on HIE are complex. Many studies have found that MH improves the neurological function of patients with HIE by reducing neuronal apoptosis [7,8].

Apoptosis is the result of a biochemical cascade and caspase proteases are major participants in the apoptotic program [9]. Caspases can be categorized into two groups: upstream initiators and downstream executioners. Caspase-3 is an executioner that implements apoptosis [9,10]. It is involved in multiple apoptotic signals, mediates apoptosis by destroying a broad spectrum of cellular substrates, and activates the degradation of DNA, which is the terminal phase of cell death [10,11]. Our previous research showed that mild hypothermia can inhibit the activation of oxygen-glucose deprivation (OGD)-induced caspase-3 injury in a rat hippocampal neuron model [12], while the role of caspase-3 in the protective molecular mechanism of mild hypothermia against OGD injury remains uncertain.

By comparing the protective effects of mild hypothermia and Ac-DEVD-CHO (a caspase-3 inhibitor) against OGD, our current study was designed to determine whether inhibiting the activation of caspase-3 is involved in the main and common molecular mechanism of mild hypothermia in protecting hippocampal neurons from OGD injury.

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2. Materials and methods

2.1. Cell culture

Hippocampal neurons in primary culture were used for this study. Suckling rats born within 1d to 3d (provided by the Experimental Animal Center of Sun Yat-sen University) were selected. After disinfection, the rat cerebrum tissues were quickly removed and placed into a culture dish containing PBS. Under an operating microscope, hippocampal tissues were separated and placed in a centrifuge tube containing Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium (U.S. Gibco Company) and were gently scattered. The mixture was subsequently centrifuged at 1000 rpm for 5min, and the supernatant liquid was removed. It was resuspended with DMEM/F12 containing 10% fetal bovine serum (Hyclone Company, US), inoculated into a 6 cm culture dish at a density of 1 × 106 cells per dish and then incubated in an incubator containing 5% CO_2 at 37 ˚C. After 24 h, the medium was completely replaced with one containing 2% B27 (Gibco Company, US). On the third day, cytosine arabinoside at a final concentration of 5 μmol/L was added for 24 h to inhibit the proliferation of glial cells. The culture liquid was completely changed every three days. On the 8th day, immunochemical fluorescence was used to identify neuronal microtubule-associated protein 2 (MAP-2).

2.2. Grouping

The experimental cells were randomly divided into six groups (n = 6): the control, mild hypothermia (MH), Ac-DEVD-CHO, OGD, mild hypothermia + OGD (MH + OGD), and Ac-DEVD-CHO + OGD groups. The duration of OGD was 2 h. The mild hypothermic temperature was 32 ˚C and lasted for 24 h after OGD. The cells were placed into a common incubator (37 ˚C ± 0.1 ˚C, 19% O_2, 5% CO_2) or the mild hypothermic incubator (32 ˚C ± 0.1 ˚C, 19% O_2, 5% CO_2) according to different groups. Next, 0–10 μM Ac-DEVD-CHO (Calbiochem) was added to the neurons after OGD and incubated for 24 h when needed. All neurons were detected 24 h after reoxygenation.

2.3. OGD and reoxygenation

Cell cultures were subjected to OGD injury using a previously described protocol [12]. In brief, culture medium was replaced with a glucose-free balanced salt solution (BSS) containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 26 mM $NaHCO_3$, 0.8 mM $MgCl_2$, and 1.18 mM NaH_2PO_4. OGD cells were then transferred to an anaerobic chamber (PLAS & LABS, MI, USA) equilibrated for 10 min with a continuous flux of gas (95% N_2/5% CO_2) and humidified at 37 ˚C for 2 h. After OGD, cultures were carefully washed with 10 mM glucose in DMEM, then incubated in incubators at either 37 ± 0.1 ˚C or 32 ˚C ± 0.1 ˚C in 95% O_2/5% CO_2 (reperfusion) for 24 h. Control cells were incubated in DMEM with 10 mM glucose in a normoxic incubator for the same period of time.

2.4. Caspase-3 activity assay

We extracted the total protein from the neuronal cytoplasm and determined the caspase-3 concentration. Next, 200 μg of caspase-3 was collected and treated with the caspase-3 colorimetric determination reagent kit (Biovision). The absorbance of each well was detected at 405 nm with an enzyme-labeling measuring instrument. Caspase-3 activity (OD of the test group/OD of the control) was detected 24 h after reoxygenation. The different concentrations of Ac-DEVD-CHO (0-10μMol/L), together with their corresponding caspase-3 activities were used to construct a linear regression equation. Finally, we selected the concentration of Ac-DEVD-CHO that displayed a caspase-3 activity equal to that of the mild hypothermia group as its final concentration.

2.5. Western blot analysis

Cell lysates were diluted in 5 × SDS buffer and denatured at 100 ˚C for 3 min. Proteins were electrophoresed on a 12% SDS–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane using a Bio-Rad transblot apparatus. After incubation at room temperature for 1 h in blocking solution (5% nonfat dry milk), membranes were incubated overnight at 4 ˚C with rabbit anti-caspase-3 (1:1000, Sigma, USA), rabbit anti-PARP (1:1000, Sigma, USA), and rabbit anti-caspase-8 (1:1000, Sigma, USA) antibodies. After three washes in TBST, the immunoblots were incubated with goat alkaline phosphatase-labeled anti-rabbit antibody (1:1000, Cell Signaling Technology, USA). All data were normalized to the levels of actin used as a loading control, and the amount of immunoreactivity was expressed relative to the corresponding control.

2.6. Immunofluorescence staining

Hippocampal neurons were fixed *in vitro* with 4% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized with PBS plus 0.5% Triton X-100 for 15 min. The cells were incubated with PBS containing 5% goat serum for 45 min at room temperature and then washed again three times with PBS. Fixed neurons were incubated with anti-MAP2 (APPLYGEN, Biotech, Beijing, China), anti-cleaved caspase-3 antibody (1:500, Sigma, St Louis, MO, USA), and anti-cleaved caspase-8 antibody (1:500, Sigma, St Louis, MO, USA) for 2 h at room temperature. They were then incubated for 1 h with FITC-conjugated secondary antibodies (1:1000, Beyotime Biotech, Jiangsu, China). The nuclei were stained with DAPI (1:1000, Sigma, St Louis, MO, USA) and the slides were left to dry off over night at room temperature. Immunostaining was examined using a laser confocal microscope (Leica, Heidelberg, Germany).

2.7. Lactic acid dehydrogenase (LDH) release assay

After OGD, cytoplasmic LDH was partially released into the culture liquid. A higher LDH release rate is related to increased cell injury. After OGD and reoxygenation for 24 h, the medium was collected and LDH activity was determined using a commercially available assay kit (Beyotime Biotech, Jiangsu, China) according to the manufacturer's protocol [13,14]. First, 50 μL of the culture liquid was taken to detect extracellular LDH activity. Then, the cell lysate was added into the culture dish with Triton-100, and the supernatant liquid was collected to detect the total cell LDH activity. LDH release rate (%) = extracellular LDH activity/total LDH activity.

2.8. Cell viability assay

In order to assess neuronal viability, a quantitative colorimetric MTT assay was employed. Briefly, cells were seeded into 96-well plates (1 × 104 cells per well) and 24 h after reoxygenation, 20 μL of MTT solution (5 mg/mL, U.S., Amresco Company) was added into each well. Then, the neurons were incubated at 37 ˚C for 4 h in a humidified atmosphere. Finally, the absorbances at 490 nm were measured for each well using an ELISA 96-well plate reader (Bio-Rad Laboratories, CA, USA). Results are expressed as the percentage of viable cells detected following OGD compared to the normoxic control plates.

2.9. Apoptotic rate assay

A flow cytometer was used to assess the apoptotic rate of neurons. After reoxygenation for 24 h, the various groups of cells were collected and resuspended with phosphate buffer solution. Annexin V–FITC (Austria Bender Medsystems Company) and PI (America Sigma Company) were added and uniformly mixed. The apoptotic rate was then detected by flow cytometry (America Becton Dickinson Company).