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MIF protects against oxygen-glucose deprivation-induced ototoxicity in HEI-OC1 cochlear cells by enhancement of Akt-Nrf2-HO-1 pathway

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ABSTRACT

Ischemia and oxidative stress play crucial roles in the pathophysiology of sudden sensorineural hearing loss (SSNHL). Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine and serves an important role in hearing function. The present study was designed to evaluate the effect of MIF on oxygen-glucose deprivation (OGD)-induced ototoxicity and to elucidate its molecular mechanism. In HEI-OC1 auditory cells, OGD reduced cell viability and increased supernatant lactate dehydrogenase (LDH) and MIF in a time-dependent manner. However, the reduced cell viability exerted by OGD was attenuated by antioxidant and MIF. Luciferase reporter assay demonstrated that MIF could activate NF-E2-related factor 2 (Nrf2), and real-time PCR showed increased mRNA expressions of Nrf2 and two Nrf2-responsive genes, including heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1). MIF also suppressed oxidative stress induced by OGD, as demonstrated by decreased MDA and increased GSH in cellular supernatant. Inhibition of Nrf2 using siRNA suppressed HO-1 protein expression, the protective effect on OGD-induced injury and decrease in oxidative stress by MIF. Moreover, MIF prevented OGD-induced reduction of Akt1 phosphorylation at Ser473. LY294002, an inhibitor of PI3K/Akt signaling, attenuated the enhancement of Nrf2 protein and protective effect of MIF in OGD-treated cochlear cells. We demonstrate that MIF protects cochlear cells against OGD-induced injury through activation of Akt-Nrf2-HO-1 pathway.

1. Introduction

Sudden sensorineural hearing loss (SSNHL) is a sudden, unexplained sensorineural hearing loss that occurs within 72 h, with hearing loss $\geq$ 20 dB in at least two adjacent frequencies [1]. The clinical manifestations of unilateral SSNHL are very similar to those of clinical ischemic vascular diseases such as transient ischemic attack or transient schizophrenia [2]. When the organ is ischemic, the cochlea changes from aerobic to anaerobic metabolism, resulting in a decrease in cellular ATP. Once reperfusion is achieved after ischemia, reactive oxygen species cause damage to the cochlear cells and result in loss of cochlear function [3]. In-depth study of the pathogenesis of cochlear ischemia-reperfusion is of great significance in protecting cochlear damage and improving the hearing recovery. In fact, activation of endogenous antioxidant defense systems has a protective effect on the ischemia-reperfusion cochlea [4].

Macrophage migration inhibitory factor (MIF) is a neurotrophic factor with high expression in the inner ear, and can promote the growth and survival of auditory neurons. MIF knockout mice showed impaired hearing, reduced number of sensory hair cells, and accelerated age-related hearing loss [5]. MIF is known to protect cells from oxidative insult, and was identified as a specific binding protein of BTZO-1, an antioxidant response element (ARE) activator. MIF protein bounds to BTZO-1 and then protects cells and organs from oxidative insults via ARE activation in animal models with oxidative stress [6]. However, the role of MIF in the pathogenesis of cochlear ischemia-reperfusion injury remains unexplored.

Therefore, this study was intended to explore the mechanisms involved in the protective effects of MIF on oxygen-glucose deprivation (OGD) injury of cochlear cells. We found that pretreatment with MIF could protect the cultured cochlear cells against OGD injury and suppress oxidative stress. The results revealed that Akt-

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Nrf2-HO-1 pathway was essential for the protective effects of MIF.

2. Materials and methods

2.1. Drugs and treatments

Recombinant mouse MIF was purchased from R&D Systems ((1978-MF-025; Chantilly, VA, USA). Myeloperoxidase (MPO) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies against Nrf2 (catalog no. 12721P), HO-1 and p-Akt1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

The mouse cochlear auditory nerve cell lines HEI-OC1 were obtained from American Type Culture Collection (Manassas, VA) and cultured in high-glucose DMEM (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO), 2 mM L-glutamine (Invitrogen-GIBCO). Cells were cultured in a 5% CO_2 humid atmosphere at 33 °C.

2.3. OGD model

OGD were used as an in vitro model of cochlear ischemia and reperfusion injury. Briefly, cochlear cell line HEI-OC1 was immersed into glucose-free DMEM (Gibco) without FBS. Then, they were placed into an anaerobic chamber (Mitsubishi Gas Chemical Company, Japan). After OGD 3, 5 and 7 h, cells were replaced with normal DMEM medium containing 10% FBS and returned to the incubator under normoxic condition (33 °C, 5% CO2) for 24 h. The control cells were treated similarly except for exposure to OGD.

2.4. Cell viability assay

The effect of MIF on cell viability of cochlear cells was measured by MTT assay. HEI-OC1 cells were plated in a 96-well culture plates at a density of 1×10^4 cells per well. Cells were pretreated with MIF (0, 50, 100, 200 ng/mL) for 1 h, followed by OGD for 3, 5 and 7 h. After recovery for 24 h, cells were incubated with MTT solution (5 mg/mL) at 37 °C for 4 h. After removing supernatant by centrifugation at 1000*g* for 10 min, the formazan pellet was dissolved with 100 μL DMSO in each well. The cell viability was assessed by measuring the absorbance at 570 nm wavelength using a ELISA plate reader (Ricso RK201, Shenzhen Ricso Technology Co., Ltd, Shenzhen, Guangdong, China). The cell viability for each group was normalized to that of the controls. All experiments were repeated at least three times.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The content of LDH, MIF, MDA and GSH levels in the cellular supernatant were evaluated by ELISA kits (Abcam, Cambridge, MA, USA). The supernatant of cell culture and different levels of standard were added into the 96-well plate (100 μL/well), and incubated at 37 °C for 90 min. The antibodies (100 μL/well) were added and incubated for 60 min. After washing for 3 times, the optical absorption at 450 nm was measured using a microplate reader (Ricso RK201, Shenzhen Ricso Technology Co., Ltd, Shenzhen, China).

2.6. Luciferase reporter assays

The Nrf2 ARE sequence was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and amplified by PCR. The forward primer was 5′-CAC CGT GAC TCA GCA ATT CAC CGT GAC TCA GCA ATT CAC CGT GAC TCA GCA ATT-3′, and the reverse primer was 5′-TCG AAA TTG CTG AGT CAC GGT GAA TTG CTG AGT CAC GGT GAA TTG CTG AGT CAC GGT GGT AC-3′. The Nrf2 ARE were inserted into the psiCHECK-2 vector downstream of luciferase reporter gene. For luciferase assay, the HEI-OC1 cells were seeded into 24-well plates, and transfected with constructed reporter plasmids (0.2 μg) or NC mimics (empty vector) by a luciferase assay kit (Promega), according to the manufacturer's protocol. After 48 h, the luciferase activities were measured using Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany).

2.7. siRNA transfection

The Nrf2 siRNAs plasmid was synthesized by Shanghai GenePharma Co., Ltd, using the following primers, forward: 5′-CGU GAA UCC CAA UGU GAA ATT-3′, reverse: 5′-UUU CAC AUU GGG AUU CAC GTT-3′. HEI-OC1 cells were grown in 24-well plates and transiently transfected with 0.5 μg of Nrf2 or control siRNA constructs using lip2000 transfection kit (Invitrogen, USA), according to the manufacturer's protocol. After incubation at 33 °C and 5% CO_2 for 36 h, cells were further treated with OGD and MIF.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

HEI-OC1 cells were pretreated with MIF (50, 100, 200 100 ng/mL), followed by OGD for 5 h and recovery for 24 h. Total RNA was extracted using Trizol® reagent (Life Technologies, Carlsbad, CA, USA). and was used as template (2 μg) for reverse transcription reaction to synthesize complementary DNA (cDNA) using reverse transcription using the Superscript III enzyme (Life Technologies). The cDNA was further used as a template to determine mRNA levels of Nrf2, HO-1 and NQO1 using SYBR Green reagent (TaKaRa, Japan) in the StepOne Plus Real-time PCR System (Applied Biosystems, USA). The PCR condition was set as follows: initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Nrf2 primer: forward, 5′- TCT TGG AGT AAG TCG AGA AGT GT-3′; reverse, 5′- GTT GAA ACT GAG CGA AAA AGG C -3′. HO-1 primer: forward, 5′-AAG CCG AGA ATG CTG AGT TCA-3′; reverse, 5′-GCC GTG TAG ATA TGG TAC AAG GA-3′. NQO1 primer: forward, 5′-ATG GGA GGT GGT CGA ATC TGA-3′; reverse, 5′-GCC TTC CTT ATA CGC CAG AGA TG-3′. GAPDH primer: forward, 5′-GGA GTC CAC TGG CGT CTT C-3′; reverse, 5′-GCT GAT GAT CTT GAG GCT GTT G-3′. The 2-ΔΔCt method was used to calculate mRNA expression, which was normalized to GAPDH expression. All reactions were performed in triplicate.

2.9. Western blotting

The cytoplasmic and nuclear protein samples were extracted by the protein extraction kit (Nanjing KeyGen, China), and protein quantification was determined according to the manufacturer's instruction. The supernatants (50 g protein) were separated by SDS-PAGE gel, transferred to PVDF membranes and blocked with 10% non-fat dry milk for 1 h at room temperature. Then the PVDF membranes were incubated with primary antibody against Nrf2, HO-1 and p-Akt1 (Ser473) overnight at 4 °C. After being washed with TBS-T for 3 times, they were incubated with secondary antibody for 1 h at room temperature. The immunoblots were visualized using a chemiluminescent detection system (ECL, Amersham Life Sciences, Buckinghamshire, UK), and were scanned for calculating the grey values. Relative expression was expressed as the target protein grey value normalized to β-actin.

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