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Resveratrol protects against mitochondrial dysfunction through autophagy activation in human nucleus pulposus cells

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ABSTRACT

Intervertebral disc degeneration (IVDD) is closely related with aging, whereas mitochondrial damage is a common feature of aging that results in cell apoptosis. Resveratrol (RES) is a natural antioxidant that protects against mitochondrial dysfunction in various cells. This study aimed to investigate the protective role of RES against mitochondrial dysfunction and human nucleus pulposus cell (NPC) apoptosis. We found that mitochondrial dysfunction and NPC apoptosis could be induced under oxidative stress by 100 μmol/l of H_2O_2. However, RES tended to attenuate the H_2O_2-mediated cytotoxicity. Therefore, autophagic state was evaluated in NPCs to further reveal the underlying mechanism. Results showed that RES reversed the impaired autophagy induced by H_2O_2, and this increased autophagic flux was confirmed by the addition of bafilomycin A1. Moreover, pretreatment with 3-methyladenine showed that the potential mechanism of RES to prevent deteriorating mitochondrial function and cell apoptosis was related to autophagy activation. Furthermore, MRI and histological detection were employed to provide more solid evidence that RES injection in an IVDD rabbit model effectively retards the degenerative process of the intervertebral discs in vivo. In summary, these results suggested that RES could alleviate mitochondrial dysfunction and cell apoptosis under oxidative stress and may delay the progression of disc degeneration, whose mechanism is associated with an advantageous role of autophagy induced by RES.

1. Introduction

Intervertebral disc degeneration (IVDD) is an aging-related disease, which is clinically related to disc herniation and low back pain [1]. IVD comprises the largest avascular tissue in the body, of which the inner hydrated nucleus pulposus (NP) changes the most during degenerative process [2]. Mitochondrial dysfunction is a common feature of aging that contributes to cell damage and death [3]. Recent studies have reported that the pathomechanism of IVDD is closely associated with reactive oxygen species (ROS) and oxidative stress [4]. Mitochondrion is the major organelle that generates ROS, which is also the target organelle of damage by ROS. Therefore, we hypothesized that attenuating mitochondrial dysfunction mediated by oxidative stress can enhance cellular homeostasis of NPCs to protect against apoptosis.

Resveratrol (RES) is a natural polyphenol richly found in red wine and recognized for its health benefits [5]. Our previous work has indicated that RES could inhibit apoptosis of degenerative human NPCs via SIRT1 activation [6]. However, the potential mechanism remains unclear. Increasing evidence has demonstrated that the health benefits of RES are mainly attributed to its antioxidant properties [7]. In vivo animal studies have indicated that RES improves mitochondrial damage caused by a high-calorie diet [8]. Till date, no study has focused on the role of RES in regulating mitochondrial function in human NPCs. Autophagy, a mechanism of orderly recycling of dysfunctional cellular organelles or macromolecules to ensure cellular homeostasis, has been previously implicated in IVDD [9]. Furthermore, our previous work has suggested that the transient increase in autophagy represents a compensatory response to mitochondrial dysfunction [10]. Therefore, the present study aimed to investigate the effect of RES on mitochondrial dysfunction and apoptosis under H_2O_2 treatment and to determine whether autophagy was responsible for RES-protected mitochondrial function in human NPCs.

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2. Materials and methods

2.1. NPCs isolation and culture

Human NP tissue samples were obtained from patients who underwent discectomies. Written informed consent was obtained from all tissue donors, and the experimental protocol was approved by the Ethics Committee of Chongqing Medical University.

NPCs were isolated and cultured, as previously described [6]. In brief, NP tissues were gently separated from the harvested IVD samples through microscopy according to their morphological difference. This was followed by 0.25% trypsin and 0.2% type II collagenase (Sigma, USA) digestion at 37 °C for 3–5 h. After centrifugation, the supernatant was aspirated, and NPCs were collected and cultured in a complete DMEM/F12 medium supplemented with 10% fetal bovine serum (Gibico, USA) and 1% penicillin–streptomycin. NPCs were cultured under hypoxic conditions with a gas mixture of 1% O_2, 5% CO_2, and 94% N_2 at 37 °C in a humidified incubator. Third-passage NPCs maintained in a monolayer were used throughout the experiments.

2.2. Cell treatment

Different concentrations of H_2O_2 with or without RES (50 μmol/l) were added in the medium and incubated for 24 h to induce oxidative stress in NPCs. Then, the NPCs were pretreated with 2 mmol/l of 3-methyladenine (3-MA; Sigma, USA) for 1 h to inhibit autophagic level. In addition, Bafilomycin A1 (100 nmol/l) was used to analyze the autophagic flux, which was also applied 1 h prior medium change.

2.3. Cell viability assay

CCK-8 assays were performed to detect the viability of NPCs according to the manufacturer's instructions and to further determine the sublethal concentration of H_2O_2. Briefly, 1×10^4 cells/well were seeded in 96-well plates and incubated with different concentrations of H_2O_2 for 24 h. Then, 10 μl of CCK-8 solution was added to 90 μl of culture media in each well and continuously incubated for another 2 h in the incubator at 37 °C. Finally, the absorbance of the sample from each well was measured using an automicroplate reader (BD, USA).

2.4. Mitochondrial membrane potential detection

The fluorescent probe JC-1 (Beyotime, China) was used to determine the mitochondrial membrane potential (MMP). Briefly, treated NPCs were washed in PBS buffer and incubated with 5 μg/ml of JC-1 at 37 °C for 15 min. The stained cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, CA). Depolarized MMP resulted in decreased red and increased green fluorescence. Results were expressed as the ratio of red/green fluorescence intensity values.

2.5. Reactive oxygen species detection

The intracellular production of ROS was evaluated by DCFH-DA (Beyotime, China), which would be oxidized into a fluorescent green dichlorofluorescein (DCF) by reacting with ROS. Briefly, treated NPCs were washed and incubated with DCFH-DA (10 μg/ml) in 1 ml of DMEM/F12 at 37 °C for 30 min. Then, NPCs were washed and analyzed by flow cytometry at an excitation wavelength of 488 nm and emission wavelength of 525 nm. The mean fluorescence intensity (MFI) of 1×10^4 cells was recorded; these fluorescent levels represented the percentage of cells positive for ROS production.

2.6. ATP content detection

Intracellular ATP content in NPCs was determined by a bioluminescent ATP assay (Beyotime, China). Briefly, whole-cell extracts were lysed by a somatic cell ATP-releasing reagent. After mixing with ATP detection solution containing luciferase, bioluminescence was measured using a Wallac Victor 1420 multilabel counter (Welch Allyn, Finland). Results were normalized to cellular protein concentration, which was determined by an enhanced BCA protein assay kit (Beyotime, China).

2.7. Apoptotic incidence detection

Apoptotic incidence was detected by flow cytometry using annexin V/PI double staining (BD biosciences, USA). Briefly, 1×10^4 NPCs were incubated with 5 μl of annexin V and 5 μl of PI for 30 min at room temperature; then, cells were analyzed by flow cytometry within 1 h. Apoptotic cells were counted as the sum of the early [annexin V (+)/PI (−)] and late [annexin V (+)/PI (+)] apoptotic phases.

2.8. Western blot analysis

NPCs were lysed using modified RIPA buffer (Beyotime, China) supplemented with 1 mmol/l of PMSF protease on ice following the manufacturer's protocol. Each protein sample (50 μg) was resolved by SDS-PAGE (12%) and transferred to PVDF. After protein transfer, the membranes were blocked by blocking buffer (Abcam, UK) for 1 h at 37 °C and then incubated overnight with primary anti-LC3, P62 (1:1000; Sigma, USA), and β-actin (1:5000; Beyotime, China) at 4 °C. The membranes were incubated with the respective secondary antibodies for 1 h at 37 °C after washing, and the bands were visualized using an ECL-Plus detection kit (Pierce, USA). The abundance was quantified by densitometry using Quantity One software (Bio-Rad, USA).

2.9. Confocal microscopic analysis

NPCs were transfected with GFP-LC3 adenoviral particles (HanBio, China) to visualize the autophagosome. Briefly, cells were infected with adenovirus at a MOI of 50 for 6 h and then replaced with fresh complete medium for another 24 h culture. Autophagy was evaluated by counting the number of green fluorescent puncta of autophagosomes under laser confocal microscopy (Hitachi, Japan).

2.10. In vivo animal experiments

The animal research was performed in accordance with the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals. All experimental protocols were approved by the Ethics Committee of Chongqing Medical University.

An IVDD rabbit model was established by annulus fibrosus puncture method [11]. Briefly, 15 mature New Zealand rabbits of either gender (3–3.5 kg) were randomly divided into three groups: group I was the sham-operated group, group II was the puncture-operated group, and group III was a combination of puncture operation and RES injection groups. After administering general anesthesia using pentobarbital sodium (30 mg/kg), a small sagittal skin incision was made from L2 to L6 to locate the disc position. Subsequently, in group II, L3/4 and L4/5 lumbar discs were punctured using a 18 G needle with a depth of 5 mm and rotated in the axial direction by 180° for 1 min at each level. In group I, only a

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