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Oleuropein attenuates hydrogen peroxide-induced autophagic cell death in human adipose-derived stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent progenitor cells with self-renewing properties; thus, transplanting functionally enhanced MSCs might be a promising strategy for cell therapy against ischemic diseases. However, extensive oxidative damage in ischemic tissue affects the cell fate of transplanted MSCs, eventually resulting in cell damage and autophagic cell death. Oleuropein (OLP) is a bioactive compound isolated from olives and olive oil that harbors antioxidant properties. This study aimed to investigate the potential cytoprotective effects of OLP against oxidative stress and autophagic cell death in MSCs. We found that short-term priming with OLP attenuated H_2O_2-induced apoptosis by regulating the pro-apoptotic marker Bax and the anti-apoptotic markers Bcl-2 and Mcl-1. Notably, OLP inhibits H_2O_2 -induced autophagic cell death by modulating autophagy-related death signals, including mTOR (mammalian target of rapamycin), ULK1 (unc-51 like autophagy activating kinase 1), Beclin-1, AMPK (AMP-activated protein kinase), and LC3 (microtubule-associated protein 1a/1b-light chain 3). Our data suggest that OLP might reduce H_2O_2-induced autophagy and cell apoptosis in MSCs by regulating both the AMPK-ULK axis and the Bcl-2-Mcl-1 axis. Consequently, short-term cell priming with OLP might enhance the therapeutic effect of MSCs against ischemic vascular diseases, which provides an important potential improvement for emerging therapeutic strategies.

1. Introduction

The balance between cell survival and cell death is crucial for the maintenance of tissue homeostasis; therefore, both processes must be tightly regulated [1,2]. Oxidative stress is one of the most important causes of cell apoptosis [3,4]. This occurs when the production of oxidizing agents, reactive oxygen species (ROS), and free radicals exceeds the antioxidant capacity of cellular antioxidants in a biological system [5,6].

Oleuropein (OLP) is a bioactive compound isolated from *Olea europaea* L. It harbors a basic phenylethanoid structure characterized by a phenethyl alcohol structure with benzene rings linked by an alcohol group [7]. Recently, scientific interest has been focused on the association between olive phenols and human health. OLP and its derivatives have been considered as “super functional food”, because of their various beneficial properties, including antioxidant, anti-microbial, anti-carcinogenic, and anti-viral properties both *in vivo* and *in vitro* [8–10]. A recent study, OLP has also been found to eliminate NO· and increase NO· synthesis *in vitro* [11]. Additionally, OLP was reported to ameliorate oxidative tissue damage by scavenging free radicals in mice [12]. The potent antioxidant activity of OLP is mainly due to the presence of hydroxyl groups in its chemical structure which could donate hydrogen to avoid oxidation [13]. Recently, we also reported that OLP reduces AngII-mediated oxidative stress and enhances the cellular function of endothelial progenitor cells by activating ERK and Akt/eNOS signaling [14].

Mesenchymal stem cells (MSCs) are multipotent progenitor cells with self-renewing properties and are capable of differentiating into various cell types such as osteocytes, chondrocytes, adipocytes, myocytes, and epithelial cells [15–17]. Current data clearly indicate

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that excessive ROS or exogenous H_2O_2 in MSCs impair self-renewal, reduce the differentiation capacity, and inhibit proliferation [18–20], while short-term cell priming with antioxidants stimulates MSC proliferation [21,22]. However, the mechanism by which MSCs counteract oxidative stress caused by excessive ROS largely remains to be resolved.

Autophagy signals play an important role in cell death decisions and can protect cells by preventing apoptosis. In the process of autophagy, cytoplasmic components are sequestered into newly generated double-membrane autophagosomes, sequentially fused with lysosomes, and then removed through a lysosomal degradation pathway [23]. An example of a cause of autophagic cell death is the increase in reactive oxygen species resulting from autophagic degradation of catalase [24].

The present study aimed to investigate the potential cytoprotective effect of OLP against oxidative stress and autophagic cell death induced by H_2O_2 in hAD-MSCs, and elucidate the mechanism underlying this effect. Our report may provide an important basis for therapeutic strategies by demonstrating that short-term cell priming by OLP may enhance the therapeutic effect of hAD-MSCs against ischemic vascular diseases.

2. Materials and methods

2.1. Cell culture and chemical treatment of hAD-MSCs

Human adipose-derived mesenchymal stem cells (hAD-MSCs) were obtained from CEFO (Cell Engineering for Origin, Seoul, Korea) and cultured in minimum essential medium (MEM)-alpha supplemented with 10% fetal bovine serum (FBS) and 5% penicillin. The cells were placed in a 5% CO_2 incubator with saturated humidity at 37 °C. The hAD-MSCs were washed twice with PBS and the medium was replaced with fresh MEM-alpha supplemented with 10% FBS. To investigate the apoptosis signaling pathway, MSCs were pretreated with OLP (100 μM, Sigma-Aldrich, St. Louis, CA, US) at 37 °C for 24 h, and then treated with H_2O_2 (600 μM, Sigma-Aldrich) for 1 h.

2.2. Western blot analysis

After experimental treatment, cell lysates were prepared from harvested cultures using Proprep (Intron Biotechnology, Gyeonggido, Korea), and the protein concentration was measured using the BCA protein assay (Thermo Scientific, Waltham, MA, USA). Equal amounts of protein (20 μg) from each sample were separated using 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immersed in blocking buffer (5% dry milk in TBS) for 1 h and then incubated overnight at 4 °C with primary antibody. The primary antibody was detected with goat anti-rabbit (1:1000, Cell Signaling Technology) secondary antibody. The protein bands were visualized on an X-ray film and their densities were measured using an imaging software. The antibody sources and dilutions used were as follows:

Antibodies against Bcl-2 (SC-7382, 1:1000), MCL-1 (SC-819, 1:1000), JNK (SC-7345, 1:1000), and *p*-JNK (SC-6254, 1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Bax (2876, 1:1000), AMPK (2535, 1:1000), *p*-AMPK (9571, 1:1000), *p*-ERK (4376, 1:1000), AKT (4691, 1:1000), and *p*-AKT (4060, 1:1000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against LC3 (ab48394, 1:1000) and ULK1 (ab128859, 1:1000) were purchased from Abcam (Cambridge, MA, USA). The antibody against Beclin-1 (NB110-87318SS, 1:1000) was purchased from NOVUS (CA, USA).

2.3. Cell proliferation assay

The proliferation of hAD-MSCs after treatment with OLP and H_2O_2 was evaluated using a BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) according to the manufacturer's instructions. Each experiment was repeated three times.

2.4. Cell viability assay

To determine the concentration of doxorubicin to be used for treating cardiac stem cells, cellular viability was assessed through a WST assay using Ez-cytox (Dail-lab, Seoul, Korea). hAD-MSCs were seeded on 96-well plates. The culture plates were then incubated in a 5% CO_2 humidity chamber at 37 °C for 24 h and then treated with 600 μM H_2O_2 for 1 h. After incubation, the culture media containing OLP and H_2O_2-contained were replaced with WST solution. The plates were then incubated at 37 °C for 2 h. The absorbance of the samples at 450 nm were measured using an absorbance reader (TECAN, Grodig, Austria) and compared with a control to account for background absorbance. All experiments were conducted in triplicate.

2.5. Annexin V-PI staining

To measure early/late apoptotic or necrotic cell death, cells were detected using Calibur flow cytometry (Becton-Dickinson, Fullerton, CA, USA) and an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA). Briefly, hAD-MSCs were harvested after treatment with OLP and H_2O_2 and then treated with Annexin V-FITC/PI for at least 15 min in the dark, as indicated by the manufacturer's instructions.

2.6. TUNEL assay

The TUNEL assay for *in situ* detection of apoptosis was performed using the DeadEnd™ Fluorometric TUNEL System assay kit (Promega) according to the manufacturer's instructions. Cells were plated in 24-well flat-bottom plates at a density of 1×10^5 cells per well and treated with OLP for 24 h. The cells were then treated with H_2O_2 for 1 h and then fixed in 4% paraformaldehyde at 4 °C for 25 min. Fixed cells were then permeabilized in 0.1% Triton X-100 and labeled with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. After rinsing with PBS, nuclei were counterstained with PI (1 μg/mL) for 15 min. The localized green fluorescence of apoptotic cells was detected using confocal fluorescence microscopy (Olympus).

2.7. Autophagic activity detection

The occurrence of autophagy in the hAD-MSCs was evaluated using a Cyto-ID® Green Autophagy Detection Kit (Enzo Life Sciences Inc, Farmingdale, NY, USA) and confocal fluorescence microscopy. In brief, cells were plated on eight-well chamber slides pre-coated with collagen I (BD Biosciences) and then were cultured until 40% confluency. The cells were then incubated with OLP or a control solution and washed twice with 1 × assay buffer and stained with Cyto-ID® detection agent according to the manufacturer's instructions. The localized green fluorescence of apoptotic cells was detected using fluorescent microscope (Leica, Wetzlar, Germany).

2.8. Statistical analyses

All data are presented as mean ± standard error (SE). Statistical significance was determined by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) procedure. P

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