Original Article

Neuroprotective effect of hyperoside on human PC12 cells against the oxidative damage

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Abstract: We aim at investigating the protective effect of hyperoside (HY) on human PC12 cells against oxidative stress. HY (0, 2, 10, 50 μM) was used to pretreat human PC12 cells for 12 h. Medium containing H₂O₂ was used for establishing oxidative damaged cell model. Flow cytometry was employed to identify the cell apoptosis and ROS level. BAX, Bcl-2, and caspase 3 gene and protein expressions were identified by RT-PCR and western blot assay. HY treatment can effectively reduce the oxidative damage of human PC12 cells. Cell viability of treated cells was higher than that of damaged groups. HY also decreased cell apoptosis and ROS level in H₂O₂ insulted PC12 cells. BAX, Bcl-2 and caspase 3 genes and protein expression in HY treated PC12 cells were regulated notably. In summary, HY can protect human PC12 cells against the oxidative damage via regulation of AKT/JNK signaling, which can be served as a new agent for neurological diseases.

Keywords: HY, PC12 cells, oxidative damage

Introduction

Free radicals and oxidative stress are two critical elements which are involved in the onset, pathogenesis and progression of various kinds of diseases, including neurodegenerative disorders like Huntington’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, stroke, Alzheimer’s disease and progressive multiple sclerosis [1, 2]. Oxidative stress is depicted by an increase in the accumulation of reactive radicals such as superoxide radical, H₂O₂ and hydroxyl radical (ROS) giving rise to an imbalance between reactive oxygen free radical species (ROS) and the innate cell’s ability to scavenge the reactive species. This in turn can cause damages to cell components (proteins, lipid membranes and nucleic acids) leading to cell death [3, 4]. Cells can protect themselves from damages, injury and apoptosis induced by oxidative stress through their internal antioxidant defense mechanisms [5]. It is believed that substances which possess antioxidant activity could have possible effective therapeutic options in treating disorders caused by ROS because of their ability to boost the preventive effects of these antioxidant defense enzymes as well as scavenge free radicals.

Traditional Chinese medicine has been applied for various disease therapies in China for thousands of years. Cuscutae semen is the dry root of Cuscuta australis and Cuscuta chinensis, which has been used for tonifying kidney and strengthening essence in Chinese [6, 7]. In addition, it has also been long used for drinking [8]. According to Chinese pharmacopoeia (2005, 2010), Cuscutae semen shows favorable capability on neurological diseases [7]. Bioactive compounds including alkaloids, anthraquinones, hyperoside, flavonoids, glycosides, sterols, tannic acid and saccharides are secondary metabolites found in Cuscutae semen [9-11]. However, little investigation was performed on screening the specific compounds closely related to the treatment effect of Cuscutae semen on neurodegenerative disorders. We previously obtained hyperoside (HY) [2-(3,4-dihydroxyphenyl)-3-(β-D-galactopyranosyloxy)-5,7-dihydroxy], and HY exhibited significant ability in PC12 cell viability promotion.
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In the present study, we demonstrated the protective ability of HY against hydrogen peroxide-induced damage to PC12 cells and clarified the possible mechanism involved in. Our study may provide new thoughts for the neurodegenerative disorders treatment.

Methods and materials

Cell culture

Human PC12 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI1640 medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 lg/ml streptomycin) at 37°C in a humid atmosphere containing 5% CO₂.

Hyperoside preparation

HY with a purity of 98.78% was obtained as a canary yellow needle-shaped crystal (Nanjing Zelang Medical Technological Co. Ltd., Nanjing, China). It was dissolved in an appropriate amount of dimethylsulfoxide (DMSO) and diluted to the desired concentrations before utilization, with the final concentration of DMSO kept below 0.5%.

Cell viability

Standard tetrazolium bromide (MTT) assay was used to assess cell viability. Briefly, cells (5 × 10³ cells/well) were seeded in 96-well plates. With HY (0, 2, 10 and 50 μg/ml) treatment for 2 h, PC12 cells were insulted with hydrogen peroxide (H₂O₂, 200 μM) for 24 h, and 50 ml MTT (Sigma) solution (2 mg/ml in PBS) was added to each well and the plates were incubated for additional 4 h at 37°C. The medium was then removed and the cells were incubated with 200 μl dimethyl sulfoxide (DMSO) in the dark for 30 min to dissolve violet crystals. The absorbance was read at 570 nm on an automatic microplate reader with DMSO as the blank. All assays were performed in quintuplicate and repeated at least three times.

Cell apoptosis assay

With HY (0, 2, 10 and 50 μg/ml) treatment for 2 h, hydrogen peroxide (H₂O₂, 200 μM) insulted PC12 cells were stained with annexin V-fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Detection of reactive oxygen species (ROS)

Detection of ROS was performed by flow cytometric analysis as described previously. In brief, cells (5 × 10⁴ cells/well) were cultured in 24-well plate with HY (0, 2, 10 and 50 μg/ml) treatment for 2 h, and hydrogen peroxide (H₂O₂, 200 μM) insulted PC12 cells after a period of exposure (12 h). Cells were washed with PBS and resuspended in complete medium followed by incubation with 0.5 μM dihydrorhodamine 123 (Sigma) for 30 min at 37°C. ROS fluorescence intensity was determined by cytometry with excitation at 490 nm and emission at 520 nm.

Reverse transcription and real-time PCR

Total RNA was isolated using Trizol reagent (Gibco®life Technology, Carlsbad, CA, USA). Reverse transcription reactions were performed as described. Real-time PCR was performed on ABI 7500 (Applied Biosystem, Foster City, CA, USA) thermal cycler. using a standard SYBR Green PCR kit (Thermo Fisher Scientific) protocol The relative mRNA expression of target gene compared with GAPDH were calculated using the 2⁻ΔΔCt method. The primers for each gene were listed as following: 5'-AGACCGAAGTCCGGACAGGC-3' and 5'-GAGACCACACGTCCCTGTTG-3' for Bcl-2 (product: 113 bps); 5'-CCGACTGTGCCTCTGCTC-3' and 5'-GGCCTCAGCCCATCTTCTC-3' for Bax (product: 132 bps); 5'-AACAAGCGACTGATGGACAGAG-3' and 5'-ACAAAGCGACTGATGGACAGAG-3' for Caspase 3 (product: 161 bps); 5'-GCGACTGTGCCTCTGCTC-3' and 5'-GAGACCACACGTCCCTGTTG-3' for GAPDH (product: 191 bps).

Western blot

Treated and untreated PC12 cells were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bredford, USA). The blots were blocked with 5% skim milk, followed by incubation with primary antibodies. Antibodies against Bcl-2, Bax and caspase 3 were purchased from Abcam. Antibodies against p-AKT, AKT, p-JNK, JNK and...
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GAPDH were purchased from Santa. Blots were then incubated with goat anti-mouse secondary antibody (Beyotime, Shanghai, China) or goat anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Millipore).

Statistical analysis

The data were analyzed using SPSS computer software Version 16.0. The data for multiple comparisons were performed by one-way ANOVA followed by LSD t-test. A value of $P < 0.05$ was considered statistically significant and all results per presented as the means ± SD.

Results

HY stimulated PC12 cells proliferation

For the purpose of evaluating the pro-proliferation ability of HY on PC12 cells, cell viability was detected after treated with different concentrations of HY. The Figure 1 showed that HY significantly increased the cell proliferation of PC12 cells in a time- and dose-dependent manner compared with the control group. However, treatment with the high concentrations of HY (100 and 200 μg/ml) did not significantly increase the cell viability compared with 50 μg/ml HY treatment. As a result, the doses of 5, 10 and 50 μg/ml were chosen for further investigations.

HY protected PC12 cells against H$_2$O$_2$-induced apoptosis

Oxidative damage to human neurocyte is one of the inducing factors causing neurodegenerative disorders. Our Annexin V/PI staining results showed that treatment of PC12 cells with H$_2$O$_2$ (200 μM) resulted in significant increase of apoptotic rates compared with the control group (Figure 2). Pretreated with HY (2, 10 and 50 μg/ml) for 2 h, the apoptotic rates of PC12 cells were notably decreased in comparison with the H$_2$O$_2$ treated group. The results indicated the protective effects of HY against H$_2$O$_2$-induced apoptosis of PC12 cells.

HY decreased the ROS level in H$_2$O$_2$-insulted cells

The breakdown of the mitochondrial membrane potential is an early stage of the apoptotic process. ROS generation is linked to mitochondria. Fluorescence probe DCFH-DA was used to determine the levels of ROS production in PC12 cells. As shown in Figure 3, cells exposed to H$_2$O$_2$ caused a significant increase in the intracellular accumulation of ROS compared with the control group. HY treatment markedly attenuates the intracellular ROS accumulation in H$_2$O$_2$-insulted cells in a dose-dependent manner.

HY regulated PI3K/AKT and MAPK signaling in H$_2$O$_2$-insulted PC12 cells

PI3K/AKT and MAPK signaling play a crucial role in cell apoptosis, proliferation, differentiation and various cellular functions. Phosphorylation of AKT shows protective effect in cell apoptosis, while phosphorylation of JNK stimulates the process of apoptosis. In the present study, western blot assay was performed to evaluate the phosphorylation of AKT and JNK. In Figure 4A and 4B, p-AKT/AKT in PC12 cells treated with H$_2$O$_2$ was descended dramatically, and p-AKT/AKT in groups pretreated with different doses of HY (2, 10 and 50 μg/ml) was
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Figure 2. Effects of HY on H₂O₂-induced apoptosis of PC12 cells. PC12 cells treated with different dose of HY (0, 2, 10, and 50 μg/ml) for 2 h, then exposed to H₂O₂ (200 μM) for 24 h. Annexin V assay was used for apoptosis detection. Data were presented as mean ± SD, n = 6, **P < 0.01, versus control, *P < 0.05, **P < 0.01 versus H₂O₂ treated PC12 cells.
Figure 3. Effects of HY on ROS level of PC12 cells. PC12 cells treated with different dose of HY (0, 2, 10, and 50 μg/ml) for 2 h, then exposed to H$_2$O$_2$ (200 μM) for 6 h. FCM was used for ROS detection. Data were presented as mean ± SD, n = 6, **P < 0.01, versus control, *P < 0.05, **P < 0.01 versus H$_2$O$_2$ treated PC12 cells.
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Figure 4. Effect of HY on p-AKT and p-JNK expression in H₂O₂-insulted PC12 cells. A and B. PC12 cells treated with different dose of HY (0, 2, 10, and 50 μg/ml) for 2 h, then exposed to H₂O₂ (200 μM) for 6 h. Western blot was performed to identify the protein levels of p-AKT, AKT, p-JNK and JNK in PC12 cells, and GAPDH was also detected as the control of sample loading. Data were presented as mean ± SD, n = 3, **P < 0.01, versus control, *P < 0.05, ***P < 0.01 versus H₂O₂ treated PC12 cells.

Figure 5. Effect of HY on Bcl-2, Bax and caspase 3 expression in H₂O₂ insulted PC12 cells. A and B. PC12 cells treated with different dose of HY (0, 2, 10, and 50 μg/ml) for 2 h, then exposed to H₂O₂ (200 μM) for 3 h. Real-time PCR was employed for Bcl-2, Bax and caspase 3 mRNA expression analysis. C-E. When treated with H₂O₂ for 6 h, protein level of Bcl-2, Bax and caspase 3 was detected by western blot. Data were presented as mean ± SD, n=6, **P < 0.01, versus control, *P < 0.05, ***P < 0.01 versus H₂O₂ treated melanocytes.

increased by 83.3%, 103.1% and 236.5%, respectively compared with that of H₂O₂ treated group. On the contrary, p-JNK/JNK expression was depressed with HY treatment (Figure 4A and 4B).

HY mediated expressions of Bcl-2, Bax and caspase 3 in H₂O₂-induced PC12 cells

Relative expression of Bcl-2/Bax and caspase 3 expression play crucial role in the process of
cell apoptosis. mRNA expression and protein level of Bcl-2/Bax and caspase 3 were measured by RT-PCR and western blot. In Figure 5A, Bcl-2 expression was down regulated and Bax was up regulated in PC12 cells with \( H_2O_2 \) treatment. HY effectively increase the relative mRNA expression of Bcl-2/Bax. Figure 5C and 5D showed that relative expression of Bcl-2/Bax was descended by \( H_2O_2 \) treatment, while HY treatment effectively up regulated the Bcl-2/Bax level in a dose-dependent manner.

As is shown in Figure 5B, mRNA expression of caspase 3 was enhanced in PC12 cells exposed to \( H_2O_2 \), while HY (2, 10 and 50 \( \mu g/ml \)) dose-dependently reduced caspase 3 mRNA expression by 16.5%, 59.8% and 81.7%, respectively. In addition, similar results were observed in the protein levels of caspase 3 detected by Western blotting (Figure 5C and 5D).

Discussion

Repair of injured PC12 cells is one of the most important driving forces for the neurodegenerative disorders treatment. As Chinese pharmacopeia (2005, 2010) recorded, Cuscutae semen shows favorable capability on the neurodegenerative disorders treatment [7, 12]. However, the pharmacodynamic material basis of Cuscutae semen in the neurodegenerative disorders treatment remains unknown. In the present study, we screened 6 compounds from Cuscuta australis and HY exhibited outstanding effects on the induction of melanogenesis in human PC12 cells.

PC12 cells are supposed to be more vulnerable to the damaging effects of oxidative stress than other cells [13, 14]. Oxidative stress is also one of inducing factors causing neurodegenerative disorders. In the present study, it was shown that HY significantly reduced the apoptosis of cultured human PC12 cells treated with \( H_2O_2 \). PI3K/AKT and MAPK signaling are reported as important regulators of cell apoptosis. Phosphorylation of AKT shows protective effects in cell apoptosis, while phosphorylation of JNK MAPK stimulates the process of apoptosis [15, 16]. \( H_2O_2 \)-treatment significantly decreased the phosphorylation of AKT, but increased the phosphorylation of JNK. Pretreatment with HY could partially reverse the effects of on the phosphorylation of AKT and JNK. These data demonstrated that HY could protect human PC12 cells against \( H_2O_2 \) induced apoptosis via regulating PI3K/AKT and JNK signaling.

Mitochondrial dysfunction caused by oxidative stress can result in the decease of MMP level [17, 18]. In the present study, MMP level of \( H_2O_2 \)-insulted PC12 cells pretreated with HY was notably increased in comparison with that of \( H_2O_2 \) only treated PC12 cells. The loss of MMP causes an increase in the permeability of the mitochondrial membrane, followed by the release of pro-apoptotic molecules such as cytochrome c. Cytochrome c releasing from mitochondrial interacts with ATP, Apaf-1 and Caspase 9, and subsequently activates Caspase 3, which consequently elicits caspase-dependent apoptotic cell death [19, 20]. In our study, Caspase 3 mRNA and protein expression in \( H_2O_2 \)-insulted PC12 cells with HY pretreatment were significantly decreased compared to that in \( H_2O_2 \)-insulted PC12 cells. These results indicated that HY show protective capability for human PC12 cells from oxidative damage by block mitochondrial apoptotic pathway.

Taken together, we propose that HY protects PC12 cells against oxidative damage by activating AKT, inhibiting JNK phosphorylation and suppressing mitochondria apoptosis signaling, which could provide insight towards neurodegenerative disorders therapy. HY might be a useful therapeutic agent in the treatment of neurodegenerative disorders.

Disclosure of conflict of interest

None.

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