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Ghrelin protects MES23.5 cells against rotenone via inhibiting mitochondrial dysfunction and apoptosis

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ABSTRACT

Ghrelin is an endogenous ligand for the growth hormone secretagogue (GHS) receptor and has several important physiological functions. Recently, particular attention has been paid to its neuroprotective effect. Rotenone is used to investigate the pathogenesis of Parkinson's disease (PD) for its ability to inhibit mitochondrial complex I. The current study was carried out to investigate the neuroprotective effects of ghrelin against rotenone in MES 23.5 dopaminergic cells and explored the possible mechanisms underlying this protection. Our results showed that rotenone induced significant decrease in cell viability which was counteracted by ghrelin treatment. In addition, rotenone challenge reduced mitochondrial membrane potential, inhibited the activity of mitochondrial complex I and depressed cytochrome C release from mitochondria. This mitochondrial dysfunction was reversed by ghrelin treatment. Furthermore, our results demonstrated that ghrelin protected MES23.5 cells against rotenone-induced apoptosis by inhibiting activation of caspase-3. Overall, our findings showed ghrelin provided protective effects on MES23.5 dopaminergic cells against rotenone via restoring mitochondrial dysfunction and inhibiting mitochondrial dependent apoptosis.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease characterized by resting tremor, rigidity and bradykinesia. The primary pathological changes of PD is the loss of dopaminergic neurons of the substangia nigra pars compacta (SNpc) (Fahn, 2003). Although genetic mutations contribute to the development of PD, mitochondrial dysfunction, protein mishandling, inflammatory response and environment factors were reported to play an important role in the pathogenesis of sporadic PD for most of the cases (Modgil et al., 2014). Among the risk factors, accumulating evidence showed that exposure to the environmental neurotoxin rotenone increased the probability of developing PD (Sanders and Greenamyre, 2013). Rotenone is a kind of fat-solubility environmental toxin which is used as the insecticide. Epidemiological study showed that a long time rotenone exposure was associated with the one set of PD (Tsui et al., 1999). Rotenone can pass through the cell membrane and the mitochondrial outer membrane. And then it inhibits the activity of mitochondrial complex I, an enzyme essential for the oxidation respiratory chain (Friedrich et al., 1994; Hollingworth et al., 1994). And this leads to the release of ROS and cytochrome C from mitochondria to cytoplasm which induce oxidative stress and apoptotic cascade of cells (Bedner et al., 1999; Zamzami et al., 1996). Braak and colleagues have proposed a hypothesis for PD progression that rotenone might initiate PD pathogenesis by inducing a-synuclein pathology from enteric nervous system and olfactory bulb to the midbrain (Braak et al., 2003). Due to these properties, it was widely used to induce both cell and animal models of PD (Alam and Schmidt, 2002; Betarbet et al., 2000; Hoglinger et al., 2003; Sherer et al., 2003).

Ghrelin is a 28-amino acid peptide found in 1999 (Kojima et al., 1999). It's the only endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Bednarek et al., 2000; Kojima and Kangawa, 2005). GHS-R has two types: GHS-R1a and GHS-R1b. It was reported that ghrelin exerted its bioactivities by binding to GHS-R1a (Howard et al., 1996). Ghrelin is mainly secreted by stomach, and it could also be secreted by pancreas, hypothalamus and pituitary etc. (Cowley et al., 2003; Kojima et al., 1999). The main function of ghrelin is promoting appetite and adiposity and regulating the energy metabolism (Korbonits et al., 2002; Lin et al., 2004; Lindeman et al., 2002; Theander-Carrillo et al., 2006; Tritos et al., 2003). Recently, accumulating evidence supported that ghrelin exerted the function of neuroprotection and anti-apoptosis (Baldanzi et al., 2002; Chung et al., 2007; Kim et al., 2004; Kim et al., 2005; Nanzer et al., 2004). It is reported that ghrelin protected hypothalamus neuron from oxygen and glucose deprivation (Chung et al., 2007; Kim et al., 2005). Our previous studies also demonstrated that ghrelin antagonized 1-methyl-4-phenyl-

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1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity (Dong et al., 2009; Jiang et al., 2008). The mechanisms underlying this protection were mediated by its anti-oxidant effect by inhibiting the production of ROS and anti-apoptosis effect by decreasing the Bcl-2/Bax ratio. However, whether ghrelin could exert the protective effect against environmental neurotoxin rotenone-induced neurotoxicity is not clear. The aim of this study was to investigate the cytoprotective potential of ghrelin against rotenone-induced toxicity in MES23.5 dopaminergic cells and elucidated the possible mechanisms underlying this neuroprotection.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis. MO, USA). Dulbecco's modified Eagle's medium/Nutrient Mixture-F12 (Ham; DMEM/F12) was from Gibco (Gibco, Grand Island, NY, USA). The mitochondria isolation kit was from Clontech (Clontech, USA). Mitochondrial respiratory chain complexes I activity colorimetric assay kit were from Genmed Scientifics (Genmed Scientifics Inc, Arlington, Massachusetts). The phycoerythrin (PE)-conjugated monoclonal active caspase-3 antibody apoptosis kit was from BD Bioscience Company (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). Hoechst 33,258 was from Beyotime (Beyotime, Jiangsu, China).

2.2. Cell culture

MES23.5 cells were offered by Dr. Wei-Dong Le (Baylor College of Medicine, TX, USA). It is a dopaminergic cell line hybridized from murine neuroblastoma-glioma N18TG2 cells with rat mesencephalic neurons, which exhibits several properties similar to the primary neurons originated in the substantia nigra (Crawford et al., 1992). Cells were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% FBS, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 °C, in a humid 5% CO_2, 95% air environment. For experiments, cells were seeded at a density of $1 \times 10^5/cm^2$ in the plastic flasks. 96-well plates were used for MTT assay and 6-well plates were used for other experiments. Rotenone was prepared with DMSO, the final concentration of DMSO in cells was 0.1% and the same concentration was used as a vehicle control. To study the protective effects of ghrelin, cells were pretreated with ghrelin (10^{-9} mol/L) for 20 min, and then ghrelin was washed away after 20 min preincubation and new ghrelin was added to incubate with rotenone (500 nmol/L) for 24 h.

2.3. Methyl thiazolyl tetrazolium (MTT) assay

To assess the neuroprotective effects of ghrelin, MES23.5 cells were seeded in 96-well plates at 2×10^4 cells/well in 150 μL culture medium. After 24 h, cells were pre-incubated with ghrelin (10^{-9} mol/L) dissolved in DMEM/F12 without serum supplement for 20 min, and then treated with rotenone (500 nmol/L) for another 24 h. MTT was added (5 mg/mL) to culture medium for 4 h at 37 °C, and cell viability was assessed at 494 nm and 630 nm with a spectrophotometer (Tecan, Grodig, Austria).

2.4. Mitochondria extraction

ApoAlert® Cell Fractionation Kit (Clontech) was used to extract mitochondria. After centrifuging at 600 g for 5 min at 4 °C, cells were resuspended in 1 ml ice-cold wash buffer. After that, cells were centrifuged at 600 g for 5 min at 4 °C. Then remove supernatant and resuspend cells in 0.8 ml ice-cold fractionation buffer mix. After incubating on ice for 10 min, cells were homogenized in an ice-cold dounce tissue grinder. Next, the homogenate was transferred to a 1.5 ml microcentrifuge tube, and centrifuged at 700 *g* for 10 min at 4 °C. Then, the supernatant was transferred to a fresh, 1.5 ml tube, and centrifuged at 10,000 *g* for 25 min at 4 °C. Finally the supernatant was collected as the cytosolic fraction, and the pellet was resuspended in 0.1 ml fractionation buffer mix as the mitochondrial fraction. COXIV expression in mitochondria and β-actin expression for cytoplasm were used as control.

2.5. Mitochondrial respiratory chain complexes I (NADH dehydrogenase) activity assay

Mitochondrion was isolated as described above and was normalized for protein. NADH dehydrogenase which is located in the inner mitochondrial membrane could catalyze the transfer of electrons from NADH to coenzyme Q (CoQ). NADH dehydrogenase activity was determined at 340 nm using spectrophotometer by following the decrease in NADH absorbance that occurred when NADH was converted to oxidize NAD^+. The reaction was started by the incubation of the reaction mixture according to the manufacturer's instructions (Genmed Scientifics Inc, Arlington, Massachusetts). Then samples were added to the reaction mixture. The specific activity of complex I was calculated by subtracting the nonsensitive activity from the total activity. Results were expressed as changes in NADH level in micromoles per minute per milligram of protein.

2.6. Measurement of mitochondrial transmembrane potential ($\Delta\Psi m$)

After pretreatment with ghrelin (10^{-9} mol/L) for 20 min, cells were treated with rotenone (500 nmol/L) without serum for a further 24 h, and then incubated with rhodamine 123 at a final concentration of 5 μmol/L for 10 min at 37 °C. Fluorescence intensity was recorded at 488 nm excitation and 525 nm emissions by flow cytometry.

2.7. Western blot analysis

Mitochondria fraction and cytosolic fraction were separated by 8% sodium dodecyl sulfate polyacrylamide gel and then transferred to polyvinylidene difluoride membrane. Blots were probed with anti-cytochrome C monoclonal antibody (Clontech, 1:200). Blots were also probed with anti-β-actin monoclonal antibody (Sigma, 1:5000) and anti-COXIV monoclonal antibody (Clontech, 1:500) as a loading control.

2.8. Active caspase-3 assay

Caspase-3 activity was measured by flow cytometry using a PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Bioscience). After washing twice with cold phosphate-buffered saline, cells were resuspended in Citofix/Cytoperm™ (0.5 mL) at a density of 1×10^6 cells/0.5 mL and incubated on ice for 20 min. Cells were then washed twice with Perm/Wash buffer and incubated with antibody (100 μL Perm/Wash buffer plus 20 μL antibody per sample) for 30 min. After one wash with Perm/Wash buffer, cells were resuspended in 0.5 mL Perm/Wash buffer and analyzed by flow cytometry at 523 nm excitation and 658 nm emissions. Apoptosis was evaluated as the percentage of caspase-3-positive cells in the total number of cells using CellQuest software (BD Bioscience).

2.9. Hoechst 33,258 staining

MES23.5 cells were seeded on sterile cover glasses placed in a six-well plate at a density of 1.0×10^4 cells per square centimeter. Cells in different groups were fixed and washed twice with phosphate buffer solution (PBS) and then stained with Hoechst 33,258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China). Cells were then examined immediately and photographed under a fluorescence microscope (Olympus, Japan) with an excitation wave length of 330–380 nm. Apoptotic cells were defined on the basis of nuclear morphological changes, such as chromatin condensation and