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Ghrelin protects retinal ganglion cells against rotenone via inhibiting apoptosis, restoring mitochondrial function, and activating AKT-mTOR signaling

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ABSTRACT

Ghrelin, a 28-amino acid peptide hormone, has protective effects on neuronal cells. The present study aimed to examine the neuroprotective effects of ghrelin on the rat retinal ganglion cells in the rotenone-induced in vitro model of Parkinson's disease (PD). Cell viability and cell apoptosis were determined by MTT assay and flow cytometry, respectively. Mitochondrial functions were detected by mitochondrial complex I activity assay and mitochondrial membrane potential (MMP) assay. The mRNA and protein expression levels were determined by qRT-PCR and western blot, respectively. Rotenone significantly suppressed cell viability and increased cell apoptosis, also decreased the mitochondrial complex I activity as well as MMP in rat retinal ganglion cell line (RGC-5). Growth hormone secretagogue receptor (*Ghsr*) siRNA transfection significantly suppressed the expression of *Ghsr* in RGC-5 cells. Ghrelin treatment attenuated the effects of rotenone-induced changes in cell viability, cell apoptosis and mitochondrial functions in RGC-5 cells. Post-transcriptional suppression by *Ghsr* siRNA transfection and treatment with GHS-R antagonist, YIL781, both significantly attenuated the effects of ghrelin in RGC-5 cells. Rotenone decreased the protein levels of Bcl-2 and increased the protein levels of Bax, cleaved caspase-3 and cleaved caspase-9, and this effect was reversed by ghrelin treatment. Ghrelin also prevented the inhibitory effects of rotenone on the AKT-mTOR signaling. The effects of ghrelin on the rotenone-induced changes in apoptosis-related protein levels and AKT-mTOR signaling were attenuated by *Ghsr* siRNA transfection and treatment with YIL781 in the RGC-5 cells. In addition, both rapamycin and AKT inhibitor IV pre-treatment significantly attenuated the effects of ghrelin on rotenone-induced changes in cell viability and cell apoptosis. In conclusion, ghrelin by acting on the GSH-R to protect rat retinal ganglion cells against rotenone via inhibiting apoptosis and restore mitochondrial functions in RGC-5 cells, and this effect was partially associated with the AKT-mTOR signaling pathway in RGC-5 cells.

1. Introduction

Parkinson's disease (PD) is primarily a motor disorder that results from dopaminergic neurons degeneration in the substantia nigra (Bonet-Ponce and Singleton, 2017). Apart from the role of dopamine in motor function, dopamine has been also shown to be a major neurotransmitter in the retina (Lee et al., 2014). Studies have demonstrated that visual functions such as absolute sensitivity, temporal sensitivity, spatial contrast sensitivity and color vision are impaired in PD (Ekker et al., 2017). Recent clinical studies by using optical coherence tomography technology also showed a reduced thickness in the retinal nerve fiber layer (Inzelberg et al., 2004; Stemplewitz et al., 2015), suggesting the neurodegeneration of retina in PD. Unfortunately, the underlying mechanisms of neurodegeneration of retina during the progression of PD are largely unknown.

Rotenone, a kind of fat-solubility environmental toxin, is commonly used as an insecticide. Due to the inhibitory effects of rotenone on the mitochondrial complex I activity (a key mitochondrial enzyme for the oxidation respiratory chain) (Xiong et al., 2012), the chronic exposure of rotenone has been suggested to be a risk factor for the development of PD (Fleming, 2017). Retinal ganglion cells (RGCs) in the retina have a very rich supply of mitochondria, and maintaining the normal mitochondrial function is essential for the survival of retinal ganglion cells. Mitochondrial dysfunctions have been shown to be closely linked

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to the cell death of RGCs. In the Leber's hereditary Optic Neuropathy study, mitochondrial complex I deficiency was found to cause inflammation and RGC cell death in the Ndufs4 mouse (Yu et al., 2015). In the rat model of glaucoma, cumulative mtDNA damage and mutations are linked to the progressive loss of RGCs (Wu et al., 2015). In the rotenone-induced rodent model of PD, longitudinal in vivo imaging with detection of apoptotic retinal cells and optical coherence tomography technologies revealed increased RGCs apoptosis and a transient swelling of the retinal layers at day 20 of the rotenone onset (Normando et al., 2016). In addition, rotenone was also found to induce an increase in cell death and oxidative stress in RGCs, and these changes were prevented by the co-treatment of memantine, an uncompetitive NMDAR blocker (Rojas et al., 2008). In the in vitro study, rotenone-induced cell death of RGC-5 cells was caspase-independent and involved the JNK and p38 pathways, and the effect was attenuated by treatment with specific green tea flavonoids (Kamalden et al., 2012). In this regard, finding novel targets for the prevention of rotenone-induced RGC apoptosis/death may represent a good strategy for the development novel therapeutics for the treatment of retinopathy in PD patients.

Ghrelin, a 28-amino acid peptide hormone, is produced by X/A cells of the oxyntic glands of the gastric fundus in the stomach (Conn and Bowers, 1996). Apart from the distribution of ghrelin in the stomach, ghrelin was also found in the gastrointestinal tract, pancreas, ovary, adrenal cortex, brain, liver, adipose tissues and heart tissues (Hellstrom, 2009). Ghrelin exerts its biological functions including growth hormone secretion, appetite and food intake, gastric secretion and gastrointestinal motility, glucose homeostasis, cardiovascular functions via acting on the growth hormone secretagogue receptor (*Ghsr*) (Pradhan et al., 2013). Recent studies also reported the neuroprotective effects of ghrelin in diverse experimental models of traumatic brain injury, spinal cord injury, ischemia, epilepsy, Alzheimer's disease and PD (Dos Santos et al., 2013). Studies showed that $Ghsr^{-/-}$ mice showed significantly greater loss of substantia nigra dopaminergic neurons compared with wild-type controls in the an MPTP-induced PD model, and the neuroprotective mechanisms including the reduction in apoptosis, suppression of microglial activation and inhibition of local inflammatory responses in the substantia nigra (Bayliss et al., 2016). Though the neuroprotective and antioxidant effects of ghrelin has been shown in the experimental glaucoma model (Can et al., 2015), the potential neuroprotective effects of ghrelin on the retinopathy in PD have not been examined so far. In this regard, the present study examined the in vitro neuroprotective effects of ghrelin on the rat retinal ganglion cell line (RGC-5 cells) in the rotenone-induced model of PD, and the underlying molecular mechanisms of the neuroprotective effects of ghrelin in RGC-5 cells were also explored.

2. Materials and methods

2.1. Cell lines and cell culture conditions

The rat retinal ganglion cell line (RGC-5) was obtained from ATCC (Manassas, USA). RGC-5 cells were cultured in 1 mg/ml glucose DMEM (Sigma, St.Louis, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA), and 100 U/ml penicillin and 100 μg/ml streptomycin and were cultured in a humidified incubator with 5% CO_2 at 37 °C.

2.2. SiRNAs, chemicals, siRNAs transfection and drug treatments

Ghsr siRNA (sense: 5′- CCACAAACAGACAGUGAAGUU-3′; antisense: 5′- CUUCACUGUCUGUUUGUGGUU-3′) and scrambled siRNA (sense: 5′- CAACAACGAAGCGACAUAAUC-3′; antisense: 5′- UUAUGUCGCUU CGUUGUUGUC-3′) were obtained from Ribobio (Guangzhou, China). Rotenone, ghrelin and the GHS-R antagonist (YIL-781) were purchased from Sigma. For the siRNA transfection study, RGC-5 cells were seeded in 6-well culture plates and cultured without antibiotics for 24 h, and *Ghsr* siRNA or scrambled siRNA was transfected into cells by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions, and 24 h after transfection, cells were processed for further experimentation. For the rotenone treatment study, after culturing the cells for 24 h, cells were exposed to the treatment with different concentrations of rotenone (1, 3, 10 and 30 μM) or the vehicle of rotenone (control) for 24 h, and cells were then processed for further experiment. For the ghrelin + rotenone group, after culturing the cells for 24 h, cells were pretreated with ghrelin (100 nM) for 1 h, and then the cells were exposed to rotenone (30 μM) for 24 h before further experimentation. For the *Ghsr* siRNA + ghrelin and rotenone group, 24 h after *Ghsr* siRNA transection, cells were pretreated with ghrelin (100 nM) for 1 h, and then cells were exposed to rotenone (30 μM) for 24 h before further experimentation. For the YIL781 + ghrelin and rotenone group, cells were pre-incubated with YIL781 (300 nM) for 30 min before treatment with ghrelin (100 nM) for 1 h, and then cells were exposed to rotenone for 24 h before further experimentation. For the mTOR inhibitor and AKT inhibitor assay, cells were pre-incubated with the mTOR inhibitor (rapamycin, 10 μM; Sigma) or AKT inhibitor (AKT inhibitor IV, 10 μM; Sigma) for 30 min before treatment with ghrelin (100 nM) for 1 h, and then cells were exposed to rotenone for 24 h before further experimentation.

2.3. Cell viability assay (MTT assay)

For the MTT assay, cells in 96-well plates were subjected to the different treatments (see Section 2.2), and then cells were incubated with a final concentration of 0.5 mg/ml MTT (Sigma) for 1 h at 37 °C. Medium was then removed and the blue formazan crystals was solubilized by 100 μl DMSO, and the cell viability was detected at a wavelength of 490 nm by a microplate reader (Bio-Tek, Winooski, USA).

2.4. Cell apoptosis analysis (flow cytometry)

The cell apoptosis after different treatments (see Section 2.2) were detected by flow cytometry technique. Briefly, the treated RGC-5 cells were harvested and washed twice with phosphate buffered saline (PBS). Cells were then incubated with Annexin-V and propidium iodide for 15 min in the dark at room temperature, and the cell apoptosis was analyzed by a FACScan flow cytometry (Becton-Dickinson, Franklin Lakes, USA).

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from treated RGC-5 cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was then reversely transcribed to cDNA by using random primers and a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed using a standard protocol from Power SYBR Green (Takara) on an ABI 7500 instrument. *Gapdh* was used as internal control for the *Ghsr* expression. The primers for *Ghsr* were: forward: 5′-TGGGTGTCCAGCGTCTTCTTCTTT-3′, reverse: 5′-CAAACACCACCACA GCAAGCATCT-3′; the primers for *Gapdh* were: forward: 5′- CAACTCC CTCAAGATTGTCAGCAA-3′, reverse: 5′- GGCATGGACTGTGGTCA TGA-3′. The relative expression of *Ghsr* was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6. Western blot assay

Total proteins from RGC-5 cells were extracted by using the lysis buffer containing the protease inhibitor (Roche, Basel, Switzerland). The protein concentrations were detected by the BCA kit (Bio-Rad, Hercules, USA). The proteins were separated by electrophoresis on a SDS-polyacrylamide gels, and proteins were then transferred to the PVDF membrane. The membrane was blocked with 1.5% skimmed milk