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Celastrol protects human neuroblastoma SH-SY5Y cells from rotenone-induced injury through induction of autophagy

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ABSTRACT

Celastrol, an active component found in the Chinese herb tripterygium wilfordii has been identified as a neuroprotective agent for neurodegenerative diseases including Parkinson's disease (PD) through unknown mechanism. Celastrol can induce autophagy, which plays a neuroprotective role in PD. We tested the protective effect of celastrol on rotenone-induced injury and investigated the underlying mechanism using human neuroblastoma SH-SY5Y cells. The SH-SY5Y cells were treated with celastrol before rotenone exposure. The cells survival, apoptosis, accumulation of α-synuclein, oxidative stress and mitochondrial function, and autophagy production were analyzed. We found celastrol (500 nM) pre-treatment enhanced cell viability (by 28.99%, $P < 0.001$), decreased cell apoptosis (by 54.38%, $P < 0.001$), increased SOD and GSH (by 120.53% and 90.46%, $P < 0.01$), reduced accumulation of α-synuclein (by 35.93%, $P < 0.001$) and ROS generation (by 33.99%, $P < 0.001$), preserved MMP (33.93 ± 3.62%, vs. 15.10 ± 0.71% of JC-1 monomer, $P < 0.001$) and reduced the level of cytochrome C in cytosol (by 45.57%, $P < 0.001$) in rotenone treated SH-SY5Y cells. Moreover, celastrol increased LC3-II/LC3 I ratio by 60.92% ($P < 0.001$), indicating that celastrol activated autophagic pathways. Inhibiting autophagy by 3-methyladenine (3-MA) abolished the protective effects of celastrol. Our results suggested that celastrol protects SH-SY5Y cells from rotenone induced injuries and autophagic pathway is involved in celastrol neuroprotective effects.

1. Introduction

Parkinson's disease (PD) is the second common neurodegenerative disorder characterized by the selective degeneration of neurons in the substantia nigra. Although the pathogenesis of dopaminergic neuron-degeneration is still elusive, at least three mechanisms have been identified including α-synuclein aggregation, oxidative stress, and mitochondria dysfunction (Schapira and Jenner, 2011). These mechanisms interact with each other, and ultimately lead to the neuronal death. Therefore, an ideal therapy for PD should be able to address these problems at the same time.

Autophagy is a basic homeostatic process for the turnover of cellular contents, which cleans organelles and misfolded proteins through the lysosomal machinery. There are three major routes of autophagy-lysosomal pathway (ALP); macroautophagy (termed as autophagy), chaperone-mediated autophagy (CMA) and microphagy (Pan et al., 2008). Increasing evidence shows that autophagy deregulation is linked to PD (Cheung and Ip, 2011). It has been found that α-synuclein can be degraded by autophagy and CMA (Mak et al., 2010; Vogiatzi et al., 2008). Disruption of autophagy promotes accumulation of α-synuclein (Friedman et al., 2012) while over-expression of autophagy-related gene beclin-1 or autophagy inducer attenuates α-synuclein aggregation and neuronal pathology (Lu et al., 2012; Pan et al., 2010; Spencer et al., 2009). In addition, autophagy plays important roles in mitochondrial integrity and reactive oxygen species (ROS) control. Damaged mitochondria are degraded by autophagy through a process called mitophagy (Canals et al., 2003). Suppression of autophagy with drugs or knockout of autophagy related gene leads to significant increases in dysfunctional mitochondria and ROS (Egan et al., 2011; Kaminskyy et al., 2012; Liu et al., 2010). In contrast, autophagy enhancement protects cells from oxidative stress and mitochondrial dysfunction (Hah et al., 2012; Han et al., 2012; Lv and Zhou, 2012). Taken together, disruption of autophagy leads to accumulation of α-synuclein in neurons (Komatsu et al., 2006; Williams et al., 2006), slows the degradation of damaged mitochondria and results in further oxidative stress (Gottlieb and

Abbreviations: PD, Parkinson's disease; ALP, autophagy-lysosomal pathway; CMA, chaperone-mediated autophagy; 3-MA, 3-methyladenine; LC3, microtubule-associated protein light chain 3; ROS, reactive oxygen species; MMP, mitochondria membrane potential; GSH, glutathione; SOD, Superoxide dismutase.

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Carreira, 2010; Hashimoto et al., 2003; Schneider and Zhang, 2010). On the contrary, enhancement of autophagy can alleviate these injuries. As mentioned above, α-synuclein aggregation, oxidative stress and mitochondria dysfunction are the typical pathologies of PD, up-regulation of autophagy might be an effective neuroprotective way for PD.

Celastrol is an active component of tripterygium wilfordii, and has been used to treat neurodegenerative diseases, autoimmune diseases, and many types of cancer for years due to its anti-oxidative and anti-inflammatory effects. A number of earlier studies demonstrated that celastrol protects against toxins-induced dopaminergic neurons death (Cleren et al., 2005; Faust et al., 2009; Francis et al., 2011), but the mechanisms are not entirely understood. Celastrol is a potent inhibitor of induced lipid peroxidation in rat mitochondria (Sassa et al., 1990). In addition, celastrol can suppress microglial activation, inflammatory cytokines releasing by human macrophages and monocytes, and the nitric oxide generation by iNOS (Allison et al., 2001). Recent studies showed that celastrol can induce autophagy in the cancer cell lines and this autophagy induction might promote cell survival (Wang et al., 2012). Hence, we hypothesize that induction of autophagy is one of the mechanisms contribute to celastrol neuroprotective effect.

In this study, by using rotenone-induced human neuroblastoma SH-SY5Y cellular model, we demonstrate that celastrol protects human neuroblastoma SH-SY5Y cells from rotenone-induced injury through autophagy induction.

2. Materials and methods

2.1. Materials

Celastrol with 98% purity or higher was bought from Shanghai Hotmed Sciences Co. Ltd. (Shanghai, China). Rotenone and autophagy inhibitors 3-methyladenine (3-MA) were ordered from Sigma–Aldrich (St. Louis, MO, USA). Antibodies were purchased from following companies: LC3B, cytochrome C from Abcam Company (Cambridge, MA, USA), α-synuclein from Becton Dickinson Company (Franklin lakes, NJ, USA), β-actin, anti-mouse-IgG and anti-rabbit-IgG from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GSH assay kit and the SOD assay Kit were provided by Nanjing Jiancheng Biocompany (Nanjing, Jiangsu, China). The ROS assay kit was bought from Genmed Scientifics Company (Shanghai, China).

2.2. Cell cultures and drug treatment

SH-SY5Y cells obtained from American type culture collection (Manassas, VA, USA) were cultured in DMEM/F12 medium (Hyclone, Logan, UT), supplied with 10% fetal bovine serum (Hyclone, Logan, UT), in a humidified atmosphere containing 5% CO2 at 37 °C. 3-MA was dissolved in DMEM with heating. Rotenone and celastrol were dissolved in dimethyl sulfoxide (DMSO, final concentration of DMSO was 0.01%). 3-MA (5 mM) were added 1 h prior to celastrol and rotenone treatment. Celastrol was added at indicated concentrations 1 h before rotenone treatment. Rotenone (500 nM) was used for 24 h to induce cell damage. To investigate whether celastrol protect cells from rotenone-induced cell death, cells were divided into four groups: Control-group (treated with vehicle), Rotenone-group (treated with rotenone for 24 h), Celastrol-group (pretreated with celastrol for 1 h followed by exposure to vehicle), and Cel+Rot-group (pretreated with celastrol for 1 h followed by exposure to rotenone). To test whether autophagic pathway is involved in celastrol neuroprotective effect, cells were divided into six groups: Control-group (treated with vehicle), Rotenone-group (treated with rotenone for 24 h), Celastrol-group (pretreated with celastrol for 1 h followed by exposure to vehicle), Cel+Rot-group (pretreated with celastrol for 1 h followed by exposure to rotenone), 3-MA-group (pretreated with 3-MA for 1 h followed by exposure to vehicle), 3-MA+Cel+Rot-group (pretreated with 3-MA for 1 h followed by exposure to celastrol and rotenone).

2.3. Cell viability measured by MTT assay

SH-SY5Y cells were seeded in a 96-well culture plate, 2×10^4 - cells per well. After being treated with different testing agents, the cells were incubated with 5 mg/ml MTT for 4 h at 37 °C. The medium was removed carefully after the incubation. The crystals in each well were dissolved in 150 μl of DMSO and quantified by measuring the absorbance at 490 nm using an automated microplate reader (BIO-TEK, VT, USA).

2.4. Apoptosis analysis

Apoptotic cells were detected using an Annexin V-FITC/PI double staining Kit (Joincare, Zhuhai, China). After being treated with different testing agents, cells were washed in cold phosphate buffered saline (PBS), centrifuged twice at 1500 rpm for 5 min and suspended in 500 μl of binding buffer. FITC-labeled Annexin V (5 μl) and propidium iodide (PI, 5 μl) were added, and incubated with the cells at room temperature for 15 min according to the manufacturer's instruction. Apoptotic cells were measured using a FACScan flow cytometer (Becton Dickinson, NJ, USA). Annexin V-positive, PI-negative cells were scored as early apoptotic cells. Cells that were positive for both Annexin V and PI were considered as late apoptotic cells.

2.5. Measurement of SOD and GSH

SOD was detected using a SOD WST-1 assay kit (Jiancheng, Nanjing, China). GSH was detected using a GSH assay kit (Jiancheng, Nanjing, China). After being treated with indicated drugs, cells were harvested, suspended in PBS, and then fragmented with ultrasonic lipid processors (Sonics, USA). The SOD and GSH in the suspension were analyzed according to the manufacturer's instructions. SOD was measured at 450 nm and GSH was measured at 405 nm using an automated microplate reader (BIO-TEK, VT, USA).

2.6. Measurement of ROS and MMP

ROS was detected using ROS assay kit with DCFH-DA (Genmed, Shanghai, China). MMP was detected using mitochondrial membrane potential assay kit (Beyotime, Jiangsu, China). Cells were harvested, suspended in PBS, and immediately stained with DCFH-DA or JC-1 according to the manufacturer's instructions. After washing twice with ice-cold PBS, the samples were analyzed by flow cytometer (FACScan flow Cytometer, Becton Dickinson, NJ, USA).

2.7. Immunofluorescence staining

SH-SY5Y cells were seeded on cover slips. After indicated treatments, cells on cover slips were fixed with 4% paraformaldehyde for 30 min, washed with PBS and permeabilized with 0.1% Trion-X 100 and 5% BSA in PBS, then incubated with anti-α-synuclein (1:100) at 4 °C overnight, and then incubated with Cy3-conjugated goat-anti-mouse IgG (1:100) at 37 °C for 1 h. The slides were observed under a confocal microscope (Olympus, Tokyo, Japan) and the pictures were analyzed by Image Pro Plus 6.0 (Bethesda, MD, USA).