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Nesfatin-1 antagonized rotenone-induced neurotoxicity in MES23.5 dopaminergic cells

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ABSTRACT

Nesfatin-1 is a recently identified brain–gut peptide involved in feeding and energy homeostasis. Recently, it has been proved that nesfatin-1 could exert its neuroprotective effect against subarachnoid hemorrhage-induced injury via its anti-apoptotic and anti-inflammatory properties. However, whether it has neuroprotective effect on dopamine neurons is largely unknown. In the present study, we investigated the neuroprotective effect of nesfatin-1 on rotenone-treated MES23.5 dopaminergic cells and illustrated the underlying mechanisms. Our results showed that nesfatin-1 pretreatment could significantly attenuate rotenone-induced cell loss. Further studies showed that the neuroprotective effect of nesfatin-1 against rotenone was mediated by reversing rotenone-induced mitochondrial dysfunction. Nesfatin-1 could rescue rotenone-induced mitochondrial transmembrane potential collapse and restore the function of mitochondrial respiratory chain complex I. In addition, rotenone-induced release of cytochrome C from mitochondria, ROS production and the subsequent caspase-3 activation were also attenuated by nesfatin-1 pretreatment. Our data suggested that nesfatin-1 exerted its neuroprotective effect on dopaminergic cells against rotenone by ameliorating mitochondrial dysfunction and its anti-apoptotic property. This suggested that nesfatin-1 had the potential to be considered as an aid for prevention of Parkinson's disease.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a selective loss of dopaminergic neurons in the nigrostriatal pathway and dopamine (DA) depletion in the striatum [14,33]. These processes are responsible for a clinical syndrome characterized by stiffness, tremor, slowness of movement, and postural instability [13]. Although many factors have been considered to be involved in the etiology of PD, the precise pathogenic mechanisms leading to neurodegeneration of PD are not known [3,22,33]. And more importantly, no treatment in preventing or postponing the progression of PD is satisfying up to now. This has led to the current notion that development of novel anti-parkinsonian drugs is imperative.

Increasing evidence suggested that PD was associated with mitochondrial dysfunction through a variety of pathways, including free-radical generation, inflammation, and mitochondrial respiratory chain complex I dysfunction [7,8,16]. Dysfunction of mitochondrial respiratory chain complex I caused reactive oxygen species (ROS) accumulation as well as ATP depletion and formed a complex cascade leading to neuronal death by apoptosis [9]. Rotenone, a widely used liposoluble pesticide, could readily cross the blood–brain barrier and accumulate throughout the brain. This could impair mitochondrial function, induce the degeneration of the nigrostriatal pathway and ultimately lead to pathological changes resembling PD [11,19,29]. This model would enable scientific re-evaluation of drugs for treating PD and offer a basis for the development of novel therapeutic strategies.

Nesfatin-1 is an 82-amino acid peptide, which was first described as a satiety molecular in regulating feeding behavior in 2006 by Oh-I and colleagues from Maebashi, Japan [23]. Their studies proved that injection of nesfatin-1 decreased food intake of rats in a dose-dependent manner [23]. Following studies further showed that nesfatin-1 played an important role in the food intake, body-weight control and energy homeostasis in both human and rodents [5,18,34]. Recently, it was proved that nesfatin-1 exerted its neuroprotective effect against subarachnoid hemorrhage-induced injury via its anti-apoptotic and anti-inflammatory properties

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[24,32]. Evidence also proved that nesfatin-1 had the capacity to cross the blood–brain barrier without saturation [26,27,31]. This led to the hypothesis that nesfatin-1 might be a new therapeutic strategy to treat PD. Previous studies in our lab have showed that nesfatin-1 could decrease excitability of dopaminergic neurons in the substantia nigra (SN) [15], although the underlying mechanisms remain largely unknown. Therefore, we were interested to know whether this peptide played a role in protecting dopamine neurons against PD. In this study, we made an attempt to define the neuroprotective effect of nesfatin-1 on dopaminergic neurons against rotenone and elucidated the underlying mechanisms by analyzing its effect on rotenone-mediated mitochondrial dysfunction and apoptosis in MES23.5 cells. Our results showed that nesfatin-1 could exert its neuroprotective effect against rotenone via its anti-apoptotic property by ameliorating mitochondrial dysfunction.

Materials and methods

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 (DMEM/F12) was from Gibco (Gibco, Grand Island, NY, USA). The cytochrome C antibody was purchased from Clontech (Clontech, USA). Other chemicals and regents available were from local commercial sources.

Cell culture

MES23.5 cells offered by Dr. Wei-dong Le (Baylor Collegeof Medicine, TX, USA) exhibited several properties similar to primary neurons originating from SN [4]. They were cultured in DMEM/F12 containing Sato's components growth medium supplemented with 5% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin in a humid 5% CO_2, 95% air environment at 37 °C. Nesfatin-1 was dissolved in normal saline solution to the concentration of 10 μmol/L, stored at −80 °C. Then nesfatin-1 was diluted with DMEM/F12 without serum to the final concentrations when used. To study the protective effects of nesfatin-1, cells were pretreated with nesfatin-1 for 30 min and then incubated with rotenone (500 nmol/L) for 24 hrs.

Cell viability assay

MES23.5 cells were seeded in a 96-well plate at a density of 2×10^4 cells/well. After attachment and incubation, cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay relies primarily on mitochondrial metabolic capacity of viable cells and reflects the intracellular redox state. After 6 -hrs or 24-hrs incubation of nesfatin-1 and rotenone, cells were treated with MTT (5 mg/mL) for 4 hrs. The medium was removed and 100 μL of DMSO was added to each well. The formazan dye crystals were solubilized for 10 min, and absorbance was measured by colorimetric assay (TECAN, Austria).

Detection of mitochondrial transmembrane potential ($\Delta\Psi_m$)

The changes of $\Delta\Psi_m$ with various treatments in MES23.5 cells were measured by rhodamine123 using flow cytometry (Becton Dickinson, USA) as described before [30,35]. The uptake of rhodamine123 into mitochondria is an indicator of the $\Delta\Psi_m$. After pretreatment with nesfatin-1 (5×10^{-9} mol/L) for 30 min, cells were treated with rotenone (final concentration 500 nmol/L) in serum-contained DMEM/F12 for the subsequent 24 hrs, and then incubated with rhodamine123 in a final concentration of 5 μmol/L for 30 min at 37 °C. After washing twice with HBS, fluorescent intensity was recorded at 488 nm excitation and 525 nm emission wavelengths. Results were demonstrated as FL1-H (Fluorescence 1-Histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellquest software.

Mitochondrial respiratory chain complexes I (NADH dehydrogenase) activity assay

Mitochondria from MES23.5 cells were isolated using a mitochondria isolation kit according to the manufacturer's instructions. Nicotinamide adenine dinucleotide (NADH) dehydrogenase activity was determined at 340 nm by measurement of the decrease in NADH absorbance that occurs when ubiquinone is reduced to ubiquinol (NADH isconverted to oxidized NAD^+) [6]. The reaction was started by adding the sample to the reaction mixture according to the manufacturer's instructions. NADH dehydrogenase activity was expressed as changes in NADH level in micromoles per minute per milligram of protein.

ROS assay

Intracellular ROS generation was examined using H_2DCF-DA. Cells were incubated in HBS containing H_2DCF-DA (5 μM) for 30 min at 37 °C. The fluorescent signals were measured with 488 nm excitation and 525 nm emission wavelengths (Fluorescence 1, FL1). Results were demonstrated as FL1-H (Fluorescence 1-Histogram); setting of the gated region M1 and M2 as a marker to observe the changing levels of fluorescence intensity using Cellguest software.

Measurement of caspase-3 activation

Caspase-3 activity was measured by flow cytometry using a PE-conjugated monoclonal active caspase-3 antibody apoptosis kit according to the manufacturer's protocol (BD Biosciences, Franklin Lake, New Jersey, USA). Briefly, cells were seeded on 6-well plates and treated as described above. After washing twice with cold phosphate buffered saline (PBS), cells were resuspended in Cytofix solution at a concentration of 1×10^6 cells/0.5 mL and incubated for 30 min. Cells were then washed with Perm/Wash buffer twice and incubated in Perm/Wash buffer with antibody (1:5) for 30 min. After washing with Perm/Wash buffer, cells were resuspended with 0.5 mL Perm/Wash buffer and analyzed by flow cytometry. The extent of apoptosis was determined by counting the numbers of active caspase-3 immunoreactive cells as a percentage of total MES23.5 cells using Cellquest software.

The extraction and isolation of mitochondrial and cytoplasmic protein and western blots analysis

The changes of the release of cytochrome C from the mitochondria in MES23.5 cells with various treatments were measured by western blots. Cells (2×10^7/sample) were collected by a desired method after incubation. The extraction and isolation of mitochondrial and cytoplasmic fraction were performed according to the ApoAlert® Cell Fractionation Kit (Clontech, USA). First, cells were centrifuged at 600 × *g* for 5 min at 4 °C to remove supernatant, and then were resuspended in 1 mL of ice-cold Wash Buffer. Next, cells were centrifuged at 600 × *g* for 5 min at 4 °C again, and were resuspended in 0.8 mL of ice-cold Fractionation Buffer Mix (2 μL Protease inhibitor Cocktail + 1 μL DTT + 1 mL 1 × Fraction Buffer) after removing supernatant. After the incubation on ice for 10 min, cells in an ice-cold dounce tissue grinder were homogenized 50