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NPIASA a Novel Peptide Prevents Sh-Sy5y Neuroblastoma Cells Against Rotenone-Induced Mitochondrial Dysfunction, Oxidative Stress and Apoptosis

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Abstract

The present research was designed to explore the neuroprotective effect of NPIASA against rotenone-induced mitochondrial dysfunction, oxidative stress and apoptosis in a SH-SY5Y human neuroblastoma cellular model. The cells were divided into four experimental groups (control, rotenone (100 nM), NPIASA (5) + rotenone (100 nM), NPIASA (5) alone treated) based on 3-(4, 5-dimethyl 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In SH-SY5Y cells, rotenone induced cytotoxicity, oxidative stress and mitochondrial dysfunction whereas pre-treatment of NPIASA attenuated the rotenone toxicity. Besides, rotenone induced the cytotoxicity by up-regulating caspases -3, -6, -8, -9 expressions and down regulating Bcl2 expression. NPIASA pre-treatment reversed the toxicity effects induced by rotenone in cells. Collectively, our results proposed that NPIASA mitigated the rotenone-induced oxidative stress, mitochondrial dysfunction and apoptosis. However, additionally pre-clinical studies are warranted in rodents to use NPIASA as a revitalizing therapeutic agent for PD in future.

Keywords: Parkinson's disease; Peptide; Alpha-synuclein aggregation; SH-SY5Y cells and apoptosis

Introduction

Parkinson's disease (PD) is a neurodegenerative disease, caused due to degeneration of dopaminergic neurons in the brain particularly substantia nigra which leads to motor disorder. It is characterized by rigidity, resting tremor and bradykinesia. It has been estimated that a major proportion of global deaths and disabilities (~25%) are caused by brain-associated disorders. Though various causes including genetic mutation and environmental toxins are associated with PD, the route cause for neuronal degeneration in PD remains unresolved. In-depth studies have been recorded that several environmental factors, genetic mutations, molecular and cellular events such as abnormal protein folding (alpha-synuclein aggregation), immense load of oxidative stress, mitochondrial dysfunction and impaired apoptotic machinery are the possible factors in the pathogenesis of the disease.

Among the gathered shreds evidence of environmental neurotoxins, rotenone was found to play a vital role in developing PD. Epidemiological reports state that prolonged use/exposure of neurotoxin was connected with the onset of PD in later days. It crosses the cell membrane, penetrates the mitochondrial outer membrane where it binds and inhibits mitochondrial complex I. Mitochondrial complex I is an enzyme essential for the respiratory oxidation chain which promotes the mitochondria to release ROS and cytochrome C to the cytoplasm thereby it stimulates the oxidative stress. Due to the presence of these properties, it was widely used in the research field to induce cell line models of PD.

Presently, available therapies for PD include combination of Levadopa (precursor of dopamine) with entacapon (COMT inhibitor) or selegiline (MAO inhibitor), tranylcypromine or isocarboxazid or orrasagiline. These pharmacological mediations suppress the symptoms temporarily, but none of them prevent the pathogenesis of PD. Other approaches like stem cell therapy, neural transplantation, and deep brain stimulation of the globuspallidus and sub-thalamic nucleus are only at the preliminary level. Accordingly, rationalization of therapeutic measure might bring in the basal improvement in preventing, progression and retrogression of PD.

In modern years, research on short peptides has advanced considerably. The possible mechanism peptides exhibits are antimicrobial, anti-inflammatory, antithrombotic, antioxidant, inhibition of protein aggregation and interaction with microtubules which all these characteristic features might prevent neurological diseases.

As a result of extensive research conducted at biotech and pharmaceutical companies today peptide-based drugs have emerged as a major class of therapeutics.

The available treatments for managing PDs are insufficient, and the number of drugs approved is limited due to high failure rates of lead compounds in clinical trials. The use of short peptide inhibitors as therapeutic drug candidates is also highlighted.

Comparing the available drugs for PD treatment, peptides have numerous advantages over small molecules, such as high biological activity, site specific, membrane penetration ability biocompatible, no side effects, easily available. and cost-effective. Hence, this study was aimed to elucidate the neuroprotective action of NPIASA (novel peptide) against rotenone-induced toxicity in SH-SY5Y dopaminergic cells and to investigate the possible mechanism underlying its basic neuroprotection.

Materials and Methods

Materials

NPIASA (Novel peptide inhibiting alpha-Synuclein aggregation) was purchased from BioDesk- Banglore, 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT), rotenone, rhodamine-123 (Rh- 123), 2′7′-dichlorofluorescein diacetate (DCFH-DA), 4, Hoechst staining, acridine orange (AO), ethidium bromide (AO/EtBr), Dulbecco's modified Eagle medium (DMEM): nutrient mix F-12 (1:1), antibiotic/antimycotic agent, fetal bovine serum (FBS), EDTA, DMSO, trypsin-EDTA, 100X antimycotic and antibiotic solution obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-Bax, Bcl-2, Cytochrome c (cyt-c), caspases-3, -6, -8, -9, and anti-β-actin antibodies were obtained from cell signalling (Beverly, MA, USA).

Cell culture

SH-SY5Y human neuroblastoma cell line was purchased from National Centre for Cell Sciences (NCCS) Pune, India. Cells grown in DMEM F12 Hams (1:1) medium supplemented with 1% antimycotic and antibiotic solution and 10% fetal bovine serum, maintained in a humidified atmosphere at 37°C, 95% air, 5% CO2 incubation. Cell culture medium was changed thrice in a week. All experiments were performed after one-day incubation. The NPIASA was added 2hrs before rotenone treatment.

MTT Assay

To assess the neuroprotective effects of NPIASA, SH-SY5Y cells seeded at a density of 3x103 cells per well in 96 plates; the cell viability was analyzed using the conventional MTT reduction assay (Dhanalakshmi et al., 2015). Briefly, after 24 h incubation cells were treated to various concentrations of rotenone (0, 0.5, 5, 50, 100 and 200 nM) and NPIASA (0, 0.5, 1, 2.5, 5, 10, 20, 40, 60 and 80 µM). To assess therapeutic efficacy of NPIASA against rotenone toxicity, cells were pre-treated with various concentrations of NPIASA (0, 0.5, 1, 2.5, 5, 10, 20, 40, 60 and 80 µM) for 2 h and subsequently rotenone (effective dose) was added to the cells after 24 h followed by the addition of MTT (2mg/ml PBS) added to each well, and the cells incubated at 37°C for 4 h. The supernatants were aspirated carefully, and 100 µl of DMSO added to each well to dissolve the precipitate, and the absorbance was measured at 490 nm with a microplate reader.

Experimental design

- Group I: Untreated control cells
- Group II: Rotenone (100 nM)
- Group III: NPIASA (5 µM) + Rotenone (100 nM)
- Group IV: NPIASA (5 µM)

Measurement of intracellular ROS levels assay

Intracellular ROS levels were determined by using the DCFH-DA - fluorescence dye which can penetrate the intracellular matrix of cells, there it is oxidized to fluorescent dichlorofluorescein (DCF) as previously reported (Nataraj et al., 2016). SH-SY 5Y cells (1x 105) were seeded in six-well plates and were pre-treated with NPIASA 5 µM for two h and then incubated with rotenone 100 nM and maintained in a CO2 incubator for 24 h. Followed by 25 µM DCFH-DA for 30 mins at 37ºC, washed twice with PBS and finally the fluorescent intensity measured in fluorescence microscopy with an excitation wavelength of 485 nm and an emission wavelength of 535 nm respectively using Shimadzu Rf- 5301PC Spectrofluorimeter. A fluorescence microscope was used to capture images.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined by using the mitochondrial specific fluorescent dye - Rh-123. Cells were seeded in 6-well plate (1x 105) and were treated with NPIASA 5 µM for two h and rotenone 100 nM for 24 h, followed by incubation of Rh-123 (5 mmol/ml) for 15 minutes (Jayaraj et al., 2013). Then washed twice with PBS and fluorescence was quantified by using blue filter (450-490 nm) and fluorescence intensity was measured by using spectrofluorometer at 535 nm.

Results

NPIASA Protected SH-SY5Y cells against Rotenone-Induced cytotoxicity

The viability of cells were measured in SH-SY5Y cells after (0 – 200 nM) rotenone treatment for 24 hours. Significant and dose-dependent cytotoxicity were observed when treated with 0 – 200 nM rotenone ($p<0.05$) shown in Figure.1. At a dose of 100 nM, it caused ~ 50% of cell death as compared with control and considered as inhibitory dose and chosen for the following experiments.

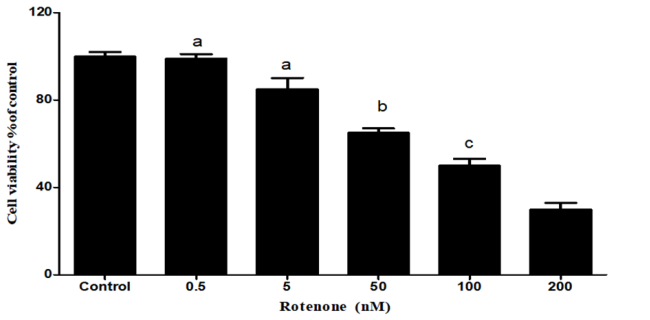

Figure1: Effect of NPIASA on rotenone induced cytotoxicity.

MTT assay was practised to evaluate whether NPIASA could antagonize rotenone-induced cytotoxicity in vitro in SH-SY5Y cells. Results of the research showed that NPIASA pre-treatment (0, 0.5, 1, 2.5, 5, 10, 20, 40, 60 and 80 µM) reduced toxicity caused by rotenone and maximum protection was offered at 5 µM concentration and was taken as neuroprotective dose and increased the viable cells when compared to the cells with alone rotenone treatment. However, the viability of cells increased up to 80% after NPIASA 5 µM pre-treatment when compared with rotenone alone treated ($p<0.05$).

3.2 To evaluate the antioxidative effects of NPIASA, ROS levels were estimated. The generation of ROS levels was high in Rotenone 100 nM ($p<0.05$) treated SH-SY5Y cells when compared with control cells. Pre-treatment with (5µM) NPIASA ($p<0.05$) markedly decreased ROS formation. Treatment with 5µM NPIASA alone did not significantly affect the ROS generation levels when compared with control cells.

3.3 Loss of Mitochondrial membrane potential (MMP) is a critical step and earlier symptoms in mitochondrial-mediated apoptotic signalling. Accordingly, we measured the fluorescence emitted by

Rhodamine -123 both in experimental and control groups which steadily penetrates the healthy cells, stains mitochondria and exhibits high fluorescent
intensity. Rotenone 100 nM exposure significantly diminished the intracellular green fluorescence ($p<0.05$), indicating the mitochondrial membrane depolarization. However, incubation with NPIASA 5µM significantly attenuated rotenone-induced mitochondrial membrane depolarization ($p<0.05$), which is revealed by an increase in fluorescence intensity These results suggest that NPIASA may prevent apoptosis by maintaining mitochondrial function.

3.4 AO/EB a dual staining method was performed to evaluate the distinctive apoptotic characteristic of the morphology of control and treated SH-SY5Y cells. These methods distinguish the viable and non-viable cells with uniform green nuclei and orange to green nuclei. SH-SY5Y cells treated with rotenone-induced the formation of orange/red luminescent significantly apoptotic cells ($p<0.05$) whereas pre-treatment of 5µM NPIASA, increased the cell viability significantly and decreased apoptotic cell death ($p<0.05$) as compared to rotenone alone exposed SH-SY5Y cells.

3.5 NPIASA attenuates the rotenone-induced nuclear Fragmentation in SH-SY5Y cells – Hoechst staining

Hoechst 33258 staining was performed to affirm the cellular apoptosis. The nuclei of rotenone-treated SH-SY5Y cells appeared hyper-condensed, as well as fragmentation of chromatin shown in. The rotenone-treated cells exhibited increased % of condensed nuclei when compared with control ($p<0.05$). However, pre-treatment with NPIASA significantly inhibited the characteristics of these nuclear morphological changes ($p<0.05$) when compared with rotenone alone treated cells. This investigational report recommended that NPIASA protected SH-SY5Y cells against rotenone-induced nuclear fragmentation.

3.6 Western blot analysis was performed to determine the effect of the NPIASA on the expression of pro and anti-apoptotic proteins Bax, Bcl2 and caspases family which were vital members of cytoplasmic proteins involved in apoptosis. The protein expressions of Caspases – 3, 6, 8, 9, Bax, cytosol cyto-c, were increased significantly and mitochondrial cyto-c, Bcl2 were decreased dramatically in rotenone-treated SH-SY5Y cells compared to the control cells in the densitometric analysis. Pretreatment with 5µM NPIASA gradually reinstate the imbalanced expressions of these proteins when compared with rotenone alone treated cells. NPIASA alone treated cells showed no significant changes when compared with the control cells.

Discussion

In the present investigation, we presented the data showing that NPIASA could significantly attenuate rotenone-induced neurotoxicity in SH-SY5Y cells. In the performed study, the death rate of SH-SY5Y cells was found to be 50% on the exposure of rotenone (100 nM) which corroborates with previous studies . Pretreatment with NPIASA notably and dose-dependently attenuated the toxic effects against rotenone-induced apoptosis. Previous investigational report stated that Ghrelin peptide also protected rotenone-induced neuronal death in MES23.5 hybridized neuroblastoma cell line which is corroborating with our results.

Garnered documentation has manifested the vital role of mitochondrial dysfunction in the pathogenesis of PD. Mitochondria form the principle intracellular sources of ROS generation which may be a prime target of oxidative stress Low levels of ROS needed for intercellular cell proliferation and redox balance for the normal physiological condition. Several signalling pathways were modified by excessive formation of ROS, leading to damage of vital intercellular molecules in mitochondria and causes cell death. Accordingly, protection against mitochondrial dysfunction forms a broad pathway as a target for PD therapy. In our research, we investigated whether NPIASA could exert the protective effect on rotenone-induced (an environmental neurotoxin) mitochondrial dysfunction and illustrated the possible mechanisms. The cytotoxicity of rotenone mainly mediated through elevated generation of ROS levels. After entering into mitochondria, it selectively binds and inhibits NADH CoQ10 reductase (Complex I) which results in shunting of electrons via ETC-II. The accumulated electron reacts with O2 and elevates the ROS generation 5–7 times more than basal levels. In this research, DCF-DA fluorescence showed that rotenone-induced toxicity elevated the levels of intracellular ROS. Our research results correlates with the previous.

The excess generation of ROS levels will lead to oxidative stress, which results in many non-communicable diseases i.e., neurodegenerative diseases. Naturally available antioxidants have been found to hold the capacity to beneficially prevent the damage caused by ROS. Antioxidant peptides have been widely investigated for their significant prevention and improvement of the disease conditions of non-communicable chronic degenerative diseases.

Peptides have the ability to scavenge radicals. The peptides with notable antioxidant capacity are typically short chains with 4–6 residues. Evidently, molecular weight lower than (<1 kDa) was the useful characteristic for the presence of antioxidant activity of peptides. The peptides showing molecular weight 1–4 kDa showed the highest antioxidant activities. Another research showed the antioxidant effect of peptides could be increased notably by the presence of any one among three aromatic amino acids (Trp, Tyr and Pre) . The amino acids such as Val, Gly, Lys, and, Ile may be responsible for forming a favorable hydrophobic environment for peptide molecules. According Cys was found to be the most active antioxidant amino acid and whose activities may be due to the formation of strong disulfide bonds with the precursor molecules. In the amino acid sequence of the peptide, the presences of hydrophilic and basic amino acids (His, Pro and Lys), hydrophobic amino acids (Val, Leu and Phe), and aromatic amino acids (Tyr and Phe) in the peptide sequences are trusted to contribute to its inclusive high antioxidant activity. Pre-treatment with NPIASA in SH-SY5Y cell line before rotenone treatment decreased the level of ROS to control levels. NPIASA of molecular weight 570 Da contains VCSVY- 5 amino acids, the presence of two hydrophobic amino acids (V- Valine), one sulphydryl amino acid (C-Cysteine), one hydrophilic aminoacid (S-serine) and one aromatic amino acid (Y-Tyrosine) might be the reason for having its strong antioxidant activity.

Mitochondria, an complex organelle mainly play a vital role in the ATP production, neuronal cell viability, ROS generation and engaged in oxygen consumption. Disruption in mitochondrial function might form a significant pathogenic event that causes neurodegeneration. Complex I inhibition, leads to excess ROS generation, which causes mitochondrial membrane potential loss and the release of cytochrome C (pro-apoptotic proteins) to the cytosol from mitochondrial inter membrane space, where cytochrome C provoke the progression of the apoptotic pathway. Rhodamine-123 fluorescence employed as a probe for measurement of mitochondrial transmembrane potential. Rotenone exposure significantly decreased the rhodamine-123 fluorescence

indicating the loss of mitochondrial transmembrane potential. Previous reports by Menke et al. stated that rotenone exposure diminished mitochondrial transmembrane potential in SH-SY5Y cell line supported our reports too. Our investigational data showed that the pre-treatment of NPIASA in SH-SY5Y before rotenone administration moderately decreased the rhodamine-123 fluorescence and returned to control levels.

Conclusion

The effects of NPIASA on the apoptosis were determined by various apoptotic proteins expression markers. Bcl-2 and Bax were the two important apoptotic-related proteins involved in regulating programmed cell death either by inhibiting (Bcl-2, Bcl-XL) or inducing (Bax, Bid) apoptosis. Bcl-2 hindering apoptosis by suppressing mitochondrial membrane depolarization while Bax promotes apoptosis by instigating mitochondrial membrane depolarization and cyt-c releas. Previous investigational reports by states that administration of rotenone significantly favoured the apoptotic process by the increased level of Bax protein expression and decreased the level of Bcl-2 protein expression. Our results are concordant with the previous studies. Besides, rotenone-treated SH-SY5Y cells exhibited increased levels of caspase -3, -6, -8, -9 expressions. Findings of our research stated that NPIASA arrested rotenone-induced apoptosis by elevating the expression of Bcl-2, and diminishing expression of caspase -3, -6, -8, -9 , Bax and cyt-c .

States that neuronal cell death by apoptosis plays major role in the origin and progression of PD, which is indicated by nuclear condensation, cell shrinkage, DNA fragmentation, and membrane blabbing. In the present research, these apoptotic process were detected by using dual and Hoechst 33258 staining. The rotenone-treated SH-SY5Y cells showed increased flouresence in dual staining, increased percentage of nuclear condensation and fragmentation in Hoechst 33258 staining which indicates increased apoptosis. NPIASA pre-treatment attenuated the rotenone induced apoptosis, the protein expression studies and staining experiments determined that NPIASA significantly debilitated these feature of apoptosis that revealed its neuroprotection against rotenone- induced apoptosis.

In summary, we manifested that NPIASA could significantly attenuated the rotenone induced neurotoxicity in SH-SY5Y cells via modifying the mitochondrial dysfunction, reduction of ROS levels, stabilizing the mitochondrial membrane potential, thereby decline the cyt-c release. Besides, this it inhibited caspase -3, -6, -8, -9 expressions and apotosis. This furnished experimental basement for the build out of therapeutic drug for PD, recommending that novel peptide NPIASA may be a therapeutic strategy against rotenone an environmental toxin for the prophylactic and/or supportive therapies of PD.

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