Neuroscience Letters 547 (2013) 65–69

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Alpha-synuclein overexpression induced mitochondrial damage by the generation of endogenous neurotoxins in PC12 cells

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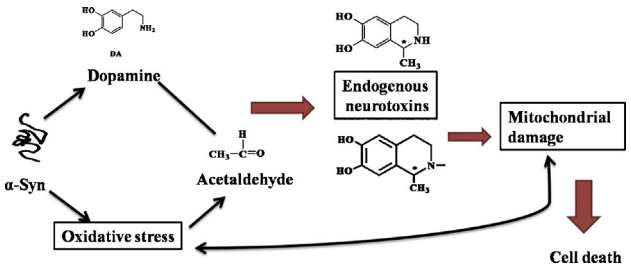
HIGHLIGHTS

- Alpha-synuclein induced oxidative stress and increased DA content.
- Alpha-synuclein induced production of endogenous neurotoxins.
- Endogenous neurotoxins become a bridge between α-synuclein and cell death.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 February 2013
Received in revised form 17 April 2013
Accepted 3 May 2013

Keywords:
α-Synuclein
Salsolinol
*N*M-salsolinol
Mitochondrial dysfunction

ABSTRACT

Alpha-synuclein is one of the important components of Lewy body which involved in neuropathology of Parkinson's disease (PD). The relationship between α-synuclein and cell death is still unclear. In the study, PC12 cell, stably over expressing α-synuclein model was used, and we investigated the level of intracellular oxidative stress, dopamine and endogenous neurotoxin. The results showed that the level of oxidative stress and intracytoplasmic dopamine (DA) was increased in cells over expressing α-synuclein compared with normal PC12 cells. Simultaneously, additional generation of endogenous neurotoxins 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) and 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolin (*N*M-salsolinol) was detected and this phenomenon was exacerbated after exposed to H_2O_2 for 24 h, but mitigated when treated with dopamine synthesis inhibitors. The presence of endogenous neurotoxins exacerbated α-synuclein induced mitochondrial damage. These results suggest that the endogenous neurotoxins may become a bridge between α-synuclein and cell death.

1. Introduction

The relationship between the two pathological features of PD, the death of dopaminergic (DAergic) neurons and the presence of Lewy bodies (LBs) [4], is still unknown. Alpha-synuclein as the major components [21] of LBs has been confirmed by immune electron microscopy to co-localize with tyrosine hydroxylase (TH) [17] which suggests α-synuclein may play an important role in the regulation of DA biosynthesis. Research data show that over expression of α-synuclein in the rat nigrostriatal system induced cellular oxidative stress and lead to a loss of 30–80% of the nigral DAergic neurons and a 40–50% reduction of striatal DA [7]. Along this line, primary cultures of embryonic mouse epencephalon, over expressing wild-type or mutant human α-synuclein (Ala53Thr) show a 27 or 49% loss of DAergic neurons respectively, with no impairment in viability of other cells in the culture [24]. These results indicate that nigral DAergic neurons are vulnerable to high level of α-synuclein.

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahy-dropyridine) as neurotoxin can cause the dopamine neurons degeneration in PD [1]. Salsolinol (1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline) and *N*M-salsolinol (1(*R*),2(*N*)-imethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) are two endogenous neurotoxins similar to MPTP and involved in PD. In human brain, salsolinol can be

Abbreviations: PD, Parkinson's disease; salsolinol, 1-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline; *N*M-salsolinol, 2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahy-dropyridine.

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http://dx.doi.org/10.1016/j.neulet.2013.05.012

endogenously synthesized from dopamine and acetaldehyde by Pictet–Spengler reaction [8,14], and then transferred into *N*M-salsolinol by N-methyltransferase in vitro [15] and in vivo [10]. N-methyltransferase activity has been found in rat striatum [10]. *N*M-salsolinol was taken up into the nerve terminal by a dopamine-transport system, oxidized into $DMDHIQ^+$ and caused oxidative stress contribute to the progress of apoptosis in DAergic cells [12,13]. Previous studies showed that oxidative stress can increase the endogenous synthesis of salsolinol and *N*M-salsolinol in vitro and in vivo [3,22]. It could be due to the product of lipid peroxidation – the acetaldehyde induced by oxidative stress in DAergic cells [5].

In our study, over expression α-synuclein not only caused additional generation of DA and also increased level of oxidative stress, and this consequence will lead to the supernumerary production of salsolinol and *N*M-salsolinol. Both neurotoxins could give rise to mitochondrial damage and contribute to cell death.

2. Materials and methods

2.1. Stable α-synuclein overexpression cell line and cells viability assay

Differentiated PC12 cells were grown to ∼80% confluence in Dulbecco's modified Eagle's medium (GIBCO) with 5% fetal bovine serum (FBS, GIBCO), 10% horse serum and 100 U/mL penicillin–streptomycin (SIGMA) and transfected with *pc*DNA3.1/mychis::α-synuclein vector [23] using FuGENE® HD transfection reagent (ROCHE). After 24 h, 500 μg/mL G418 (SIGMA) was added for 15 days. The drug-resistant single cell clones were further selected in the 96-well plate. Alpha-synuclein levels were evaluated by Western blot analyses. G418 (100 μg/mL) was used for PC12 over-expressed α-synuclein. Cells viability was determined by MTT assay. Briefly, cells were seeded at 5×10^3 per well in a 96-well flat bottom plate. After different treatment, 0.5 mg/mL MTT (SIGMA) was added for another 4 h, and DMSO was incubated before the absorbance was measured at 570 nm (THERMO Lab systems MK3).

2.2. LC–MS/MS analysis of dopamine, salsolinol and NM-salsolinol

The cells were collected and resuspended in PBS, and then lysed by sonication for 40 s (2 s on, 1 s off). The protein concentration was determined and 0.1 M perchloric acid was used to remove the protein. The sample for LC–MS/MS was filtered through 0.22 μm membrane. LC–MS/MS analysis used Discovery® HS F5 (2.1 mm × 150 mm, 3 μm) column at 30 °C. The mobile phase was consisted of methanol–water (25/75, v/v) with 10 mM ammonium formate (pH 3.5) and delivered at flow rate of 0.15 mL/min. The injection volume was 20 μl. MS (Agilent 6460, USA) was run in the multiple-reaction-monitoring mode (MRM). MS was operated in the positive mode with a capillary voltage of 3500 V. The nebulizer gas pressure was 35 psi, drying gas flow was 6 l/min and drying gas temperature was 300 °C. The $[M+H]^+$ ions pairs were used for salsolinol (*m*/*z* 180.1/117.1), dopamine (*m*/*z* 137/91) and isoproterenol (*m*/*z* 212.1/117.1) *N*M-salsolinol (*m*/*z* 194/145).

2.3. Western blot

Cells were lysed in RIPA buffer and mixed with loading buffer (200 mM Tris–HCl pH 6.8, 50% glycerol, 2% SDS, 20% β-mercaptoethanol, 0.04% bromophenol blue), boiled for 5 min. The protein (50 μg) was separated on 12% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA, USA), blocked with 5% nonfat milk in TBS–Tween buffer for 1.5 h at room temperature, and incubated overnight at 4 °C with the primary antibody against β-actin (1:2000, ProteinTech, IL, USA) and α-syncline (1:2000, ProteinTech, IL, USA). The membrane was washed with TBS–Tween buffer for 60 min (10 min for each), and then incubated for 1 h in horseradish peroxidase-conjugated goat antimouse IgG at room temperature. After extensive washing, the bands were visualized with enhanced chemiluminescence's reagents (THERMO) and exposed to X-ray film. The densitometry of the bands was analyzed by Bio-Rad imaging system Quantity One®.

2.4. Intracellular reactive oxygen species production assay and mitochondrial membrane potential detection

The cellular level of malondialdehyde (MDA) and H_2O_2 was measured to indicate oxidative stress level in cells. MDA level was measured based on thiobarbituric acid-based colorimetric method. The amount of H_2O_2 was determined based on hydrogen peroxide colorimetric method. Briefly, Sample was prepared by collecting cells after centrifugation, and resuspended in PBS buffer containing protease inhibitor solution. Cells were lysed by sonication for 40 s (2 s on, 1 s off). Protein concentration was determined with Bradford method. The MDA level was measured using malondialdehyde assay kit (Northwest Life Science Specialties, WA, USA), and the H_2O_2 level was measured by hydrogen peroxide colorimetric assay kit (Northwest Life Science Specialties, WA, USA). Mitochondrial membrane potential (MMP) was detected using a cell-permeable, mitochondrial-specific fluorescent probe JC-1 (BD Pharmingen, MD, USA) [18]. Cells were seeded on the glass bottom confocal dish and grown to ∼80% confluence. After 15 min loading by JC-1 working solution (1 μg/mL), cells were washed twice with 1× assay buffer and observed under the microscope. Fluorescence images were collected by Leica TCS SP5 laser scanning confocal microscopy. Fluorescence intensity was detected by spectrofluorometer (Horiba, Kyoto, Japan) with excitation/emission wavelength of 488 nm/534 nm (green fluorescence) and 514 nm/594 nm (red fluorescence) respectively, and the ratio of green/red fluorescence intensity was recorded.

2.5. Statistics analysis

Data was analyzed as mean ± SEM from at least three independent experiments. Statistical significance was evaluated using *t*-test analysis. The differences were considered statistically significant at a *P*-value <0.05 (*) or <0.01 (**).

3. Results

α-Synuclein stably over expressed in PC12 cell was named PC12-α. The level of α-synuclein was determined by Western blot method. α-Synuclein protein level was increased 20% in PC12-α cell (Fig. 1A and B). Cells were exposed to H_2O_2 (0–500 μM), and cell viability was determined using MTT assay. As shown in Fig. 1C, H_2O_2 increased the cytotoxicity in a dose-dependent manner in PC12 and PC12-α cell. PC12-α cell (overexpression α-synuclein) was significantly more susceptible to oxidative stress.

In order to indicate the level of oxidative stress in cells, the intracellular level of malondialdehyde (MDA) and H_2O_2 was measured by HPLC–MS method. Level of H_2O_2 and MDA was increased in PC12-α cells compared with PC12 cells (Fig. 2A and B). It was implicated that α-synuclein might enhance the oxidative stress in PC12-α cell. Intracellular dopamine of PC12 cells and PC12-α cell was detected by HPLC–MS as well. The level of dopamine was increased in PC12-α cell (Fig. 2C). The results suggested that α-synuclein promotes increasing level of oxidative stress and intracellular dopamine content.

The level of endogenous neurotoxin salsolinol and *N*M-salsolinol in PC12 and PC12-α cell was measured by HPLC–MS. The