Neuroscience Letters 566 (2014) 11–16

Contents lists available at ScienceDirect

Neuroscience Letters

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

Flavin-containing monooxygenase, a new clue of pathological proteins in the rotenone model of parkinsonism

Boyu Li, Yuhe Yuan, Wanqing Zhang, Wenbin He, Jinfeng Hu, Naihong Chen*

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

HIGHLIGHTS

- We generated rotenone model in primary midbrain dopaminergic neurons.
- We examined the relationship between FMO, parkin and neuron apoptosis.
- FMO and parkin was decreased in rotenone model, accompanied by caspase 3 activation.
- FMO dysfunction was a reason for the apoptosis of DA neurons in rotenone model.
- Parkin function was compromised in neuro-pathological states.

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form
12 November 2013
Accepted 20 November 2013

Keywords:
Parkinson's disease
Rotenone
Flavin-containing monooxygenase
Parkin
Apoptosis

ABSTRACT

Parkinson's disease (PD) is a major age-related neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra par compacta (SNpc) and accumulation of aggregated alpha-synuclein in brain areas. Rotenone is a neurotoxin that is routinely used to model PD, thus to help us understand the mechanisms of neural death. Flavin-containing monooxygenase (FMO), usually known as an important hepatic microsomal enzyme like cytochrome P450, was found to play a role in the brain recent years. In our study we aimed to find out the role that FMO might play in PD pathology. Thus we successfully generated rotenone model in primary midbrain dopaminergic neurons and identified the apoptosis of neurons caused by rotenone. We found that in rotenone model of Parkinsonism, the expression/protein level of parkin and FMO1 were decreased accompanied by the activation of caspase 3. Blocking FMO activity by FMO inhibitor methimazole directly caused activation of caspase 3, meanwhile parkin protein level was decreased. Our data indicated that FMO, whose dysfunction could be a reason for the apoptosis of dopaminergic neurons in rotenone model, might be a new clue of pathological proteins in rotenone model of parkinsonism. Meanwhile, it was suggested that parkin function was compromised in neuro-pathological states, thereby further adding to the cellular survival stress.

1. Introduction

Parkinson's disease (PD) is a major age-related neurodegenerative disorder, accompanied primarily by motor symptoms, such as resting tremor, rigidity, and bradykinesia. It is pathologically characterized by loss of dopaminergic neurons in the substantia nigra par compacta (SNpc) and accumulation of aggregated alpha-synuclein in brain stem, spinal cord, and cortex [1]. About 10% of PD cases are familial PD, estimated to be caused by mutant proteins such as alpha-synuclein, parkin, DJ-1, etc. [2]. Others are sporadic PD, caused by uncertain factors, like pesticides [3]. However, the pathology of PD is not clear currently. It has been reported that mitochondrial impairment, oxidative stress, alpha-synuclein aggregation, dysfunctional protein degradation, glutamate toxicity, calcium overloading, inflammation and loss of neurotrophic factors are involved in the development of PD.

Rotenone is a pesticide which has been widely used in agriculture [4]. Recent years epidemiological studies found that exposure to rotenone was linked to a higher risk of PD [5,6]. It was found that long term, systemic administration of rotenone produced selective degeneration of dopaminergic neurons [3] and accurately recapitulated many features of PD in rats [7,8], including: accumulation and aggregation of endogenous, wildtype alpha-synuclein; alpha-synuclein- and polyubiquitin-positive Lewy bodies and Lewy neuritis; apomorphine-responsive behavioral deficits; etc. Rotenone is a powerful inhibitor of complex I in the mitochondrial respiration [9]. Observations showed that a defect in mitochondrial complex I activity may induce the apoptosis of dopaminergic

* Corresponding author. Tel.: +86 10 63165177.
E-mail address: chennh@imm.ac.cn (N. Chen).

0304-3940/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2013.11.036

cells, which may contribute to the neurodegenerative process in PD [10]. Studies demonstrated that rotenone induced apoptosis of dopaminergic neurons in substantia nigra and behavioral impairments in rats [11].

Flavin-containing monooxygenase (FMO) was firstly described by Dr. Daniel Ziegler and colleagues at the University of Texas at Austin [12]. FMO oxygenates drugs and xenobiotics containing a soft-nucleophile, usually nitrogen or sulfur. Like cytochrome P450 (CYP), FMO utilizes the reducing equivalents of NADPH to reduce one atom of molecular oxygen to water, while the other atom is used to oxidize the substrate [13]. Six FMO genes have been reported in human. FMO1-5 are functional, with an amino acid sequence homology of 55–60%. The structure or functional properties of the FMO family are still relatively unknown. Generally, the human FMO oxygenates nucleophilic heteroatom-containing chemicals and drugs and converts them into harmless, polar, readily excreted metabolites. Sometimes, however, FMO bioactivates chemicals into reactive materials that can cause toxicity [14]. FMO was usually known as a metabolic enzyme in the liver, now people realized that FMO was important to the brain. Parkin, usually known as an E3 ubiquitin ligase, is important for the survival of the neurons that degenerate in PD. Parkin mutations are the most common cause of autosomal recessive early-onset parkinsonism in familial disease, including the autosomal recessive juvenile disease. The frequency of the mutation is estimated at 50% in families with autosomal recessive early-onset parkinsonism [15]. The caspase 3 protein is a member of the cysteine-aspartic acid protease (caspase) family [16]. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspase 3, together with caspase 6 and caspase 7, is an executioner caspase responsible for cleaving numerous substrates, which ultimately leads to cell death. Caspase 3 has been generally regarded as a classical apoptotic marker. Studies found that parkin was decreased accompanied by the activation of caspase 3 in dopaminergic neurons in substantia nigra in rotenone rat model [11].

In our study, we aimed to find out the role that FMO might play in rotenone model of parkinsonism. In view of that the pathological feature of PD is the loss of dopaminergic neurons in the SNpc of midbrain, we generated rotenone model in primary midbrain dopaminergic neurons, identified the expression/protein levels of parkin and FMO1 in apoptotic neurons, and explored the relationship between FMO and apoptosis by using FMO inhibitor methimazole.

2. Materials and methods

2.1. Drugs and reagents

Rotenone and methimazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reactive oxygen species assay kit was purchased from Beyotime (China). The AnnexinV-FITC kit was purchased from BD Biosciences (Heidelberg, Germany). Anti-TH mouse monoclonal antibody was purchased from Cell Signaling Biotechnology (Hertfordshire, England). Anti-parkin rabbit polyclonal antibody was purchased from Bioworld technology (St. Louis, MN, USA). Anti-caspase 3 rabbit polyclonal antibody, horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG and FITC-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescent (ECL) substrate was purchased from Pierce (Rockford, IL, USA). Neurobasal medium and B27 were purchased from Gibco (Burlington, Ont. USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The ReverTra Ace qPCR RT Kit and SYBR Green Realtime PCR Master Mix were purchased from TOYOBO (Osaka, Japan).

2.2. Primary cell culture

Primary cultures of midbrain neurons were obtained using a modification of the procedure of Banker and Cowan [17]. The day before plating, plastic culture dishes containing glass coverslips were treated with poly-L-lysine (100 mg/mL). On the day of plating, Sprague-Dawley (SD) rat embryos at embryonic day 18 were sacrificed. The midbrain were then dissected out and incubated for 15 min in an enzymatic solution (0.125% trypsin EDTA in Hanks's solution), washed three times in minimal essential medium (MEM), and triturated to dissociate cells. Four hundred microliters of cell suspension (diluted to 1×10^6 cells/mL) was added to poly-lysine coated dishes and cultured in neurobasal medium supplemented with B27. 10 μmol/L Ara-C was added to the cultures at the 4th day to prevent glial proliferation and the medium was changed every 4–5 days. The study was conducted following NIH Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996).

2.3. Immunofluorescence analysis

Neurons cultured for 7 days were rapidly washed with PBS and fixed by 4% paraformaldehyde. After fixation, the cells were permeabilized with 0.2% (v/v) Triton X-100 and then blocked with 3% goat serum. The cells were then incubated at 4 °C overnight with a 1:100 dilution of TH antibody. Then the neurons were incubated for 30 min with a 1:100 dilution of FITC-conjugated goat anti-mouse IgG and then Hochest 33342. Immunoreactivity was monitored using a Leica TCSNT confocal fluorescence microscope (Germany).

2.4. Apoptosis analyzed by Annexin V-FITC/PI assay

After exposure to rotenone (100 nmol/L), neurons were harvested by centrifuging (2000 rpm, 10 min) and washed with cold PBS twice, and then resuspended in 200 μL binding buffer with about 1×10^6 cells. Then 10 μL of Annexin V-FITC was added. Cells were gently oscillated and incubated for 15 min at room temperature in the dark. After adding 300 μL binding buffer and 5 μL propidium iodide (PI) to each tube, cells were analyzed by flow cytometry. Stained cells were analyzed *via* a FACSCalibur and the CellQuest software (Becton Dickinson, USA). The *x* axis (FL1) reflects Annexin V–FITC fluorescence; the *y* axis (FL2) reflects PI fluorescence. Lower left quadrant shows cells which are negative for both Annexin V-FITC and PI, lower right shows Annexin V positive cells which are in the early stage of apoptosis, upper left shows only PI positive cells which are dead, and upper right shows both Annexin V and PI positive, which are in the stage of late apoptosis or necrosis. Late apoptosis/necrosis rate was presented in the statistical figure.

2.5. Reactive oxygen species (ROS) detection

The intracellular ROS level was detected by DCFH-DA probe following the manufacturers' instructions. Cultured midbrain neurons were washed twice with serum-free medium and loaded with 10 μM DCFH-DA for 20 min at 37 °C in the dark. Then the cells were washed with serum-free medium twice and examined by fluorescent microplate reader. Fluorescence was measured using excitation at 485 ± 7.5 and emission at 535 ± 12.5 nm.

2.6. Western blotting analysis

After treatment, the cells were washed with PBS and lysed in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L PMSF, 50 μg/mL leupeptin, 1 μg/mL pepstatin A, 20 μg/mL aprotinin, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na_3VO_4, 50 mmol/L NaF and 20 mmol/L