Toxicology Letters 283 (2018) 58–68

Contents lists available at ScienceDirect

Toxicology Letters

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

6-Hydroxydopamine induces autophagic flux dysfunction by impairing transcription factor EB activation and lysosomal function in dopaminergic neurons and SH-SY5Y cells

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ARTICLE INFO

Keywords:
6-Hydroxydopamine
Autophagic flux
Transcription factor EB
Lysosome
Apoptosis

ABSTRACT

Autophagy deregulation has been implicated in Parkinson's disease (PD), yet the role of autophagy in neuronal survival remains controversial. In this study, we comprehensively investigated the time-course of autophagy-related markers in 6-OHDA-induced Parkinsonian rat models and assessed its effect on the state of autophagic flux both in vivo and in vitro. We observed an early activation of autophagy followed by autophagic flux impairment, which was confirmed with autophagy inhibitor chloroquine in vivo and Ad-GFP-mCherry-LC3-infected SH-SY5Y cells in vitro. In addition, 6-OHDA not only remarkably reduced the expression level of lysosome-associated membrane protein 1 (Lamp1), but also impaired the hydrolase activities of lysosomal proteases. Transcription factor EB (TFEB), a key transcription factor controlling lysosome biogenesis, was also significantly downregulated by 6-OHDA and its nuclear translocation was inhibited as well, which could account for the impaired lysosomal function. Promoting lysosome biogenesis through TFEB overexpression could protect SH-SY5Y cells against 6-OHDA-induced neurotoxicity. The above findings demonstrated that autophagic flux dysfunction was closely associated with 6-OHDA-induced neurotoxicity and highlighted the importance of functional lysosomes and homeostatic autophagic flux in developing therapeutic agents for PD.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases with a global prevalence of 0.3% (Pringsheim et al., 2014). So far, the clinical diagnosis of PD is mainly based on the presence of motor symptoms, including bradykinesia, rigidity and rest tremor (Ascherio and Schwarzschild, 2016). Loss of dopaminergic neurons in the substantia nigra and subsequent depletion of dopamine in the striatum is the cardinal mechanism of the motor features in PD, and several studies have revealed that dramatic loss of dopaminergic neuron starts before the onset of motor symptoms (Dijkstra et al., 2014; German et al., 1989; Iacono et al., 2015). Developing disease-modifying treatments to slow or prevent the progression of neurodegeneration has been a major therapeutic challenge for PD.

Macroautophagy, hereafter referred to as autophagy, is the substantial pathway that removes long-lived proteins and organelles. It is a highly dynamic process where cytosolic contents targeted to be recycled are enclosed in a double membrane to form an autophagosome, which fuses with lysosomes and the contents are further digested upon exposure to a set of lysosomal hydrolases (Nixon, 2013). Functional autophagy is revealed by the term autophagic flux, which reflects the rate of protein degradation through autophagy (Lumkwana et al., 2017). Mounting evidence suggest that an autophagy defect plays a crucial role in the development of PD (Menzies et al., 2015). Defects of autophagy have been reported in PD-derived samples, including post-mortem brain tissues, induced pluripotent stem cells (iPSC)-derived dopaminergic neurons and fibroblasts (Chu et al., 2009; Dehay et al., 2012; Sanchez-Danes et al., 2012). Accumulation of autophagosome-like structures has been observed in post-mortem PD brain samples (Chu et al., 2009; Dehay et al., 2010). As the abundance of autophagosomes is determined by both autophagosome synthesis and degradation rate, the autophagosome pool size cannot reflect the actual

Abbreviations: PD, Parkinson's disease; iPSC, induced pluripotent stem cells; 6-OHDA, 6-hydroxydopamine; DAT, dopamine transporter; ROS, reactive oxygen species; TUNEL, terminal deoxynucelotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; CCK-8, cell counting kit-8; MOI, multiplicity of infection; CQ, chloroquine; TEM, transmission electron microscopy; DAPI, 4,6-diamidino-2-phenylindole; RT-PCR, real-time polymerase chain reaction; ANOVA, one-way analysis of variance; TH, tyrosine hydroxylase; SN, substantia nigra; Lamp1, lysosomal-associated membrane protein 1; CTSB, cathepsin B; CTSD, cathepsin D; TFEB, transcription factor EB; LMP, lysosomal membrane permeabilization; MDA, malondialdehyde; MiT/TEF, microphthalmia-transcription E; HLH, helix-loop-helix; CLEAR, Coordinated Lysosomal Enhancement and Regulation; BafA, bafilomycin A

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https://doi.org/10.1016/j.toxlet.2017.11.017
Received 19 September 2017; Received in revised form 8 November 2017; Accepted 17 November 2017
Available online 21 November 2017
0378-4274/

status of autophagic flux (Loos et al., 2014). Hence, it is necessary to assess the autophagic flux from the aspects of autophagosome formation and cargo clearance independently. And an understanding of the role autophagic flux in the pathogenesis of PD will facilitate the future development of therapeutic approaches.

6-Hydroxydopamine (6-OHDA) is a common neurotoxicant for generating experimental models of PD with merits of low complexity, low cost and high reproducibility (Le et al., 2014). It has high affinity for catecholaminergic transporters like dopamine transporter (DAT), which facilitates its transportation into dopaminergic neurons (Le et al., 2014). Once 6-OHDA accumulates within the cell, it undergoes rapid auto-oxidation and produces a number of free radicals, including hydrogen peroxide, superoxide and hydroxyl radicals. These reactive oxygen species (ROS) can further lead to lipid peroxidation, cytoskeleton disorganization, DNA defects and mitochondrial membrane potential collapse (Bernstein et al., 2011; Bove and Perier, 2012). In addition, 6-OHDA itself inhibit mitochondrial respiratory chain enzymes, which is also responsible for its neurotoxicity (Bove and Perier, 2012). Recent studies have demonstrated that 6-OHDA can activate autophagy both in vivo and in vitro, but the role of autophagy in 6-OHDA-induced neurotoxicity is still a matter of debate (Lin and Tsai, 2017; Zhang et al., 2016). Herein, an understanding of how 6-OHDA affects the overall autophagic flux might reconcile the conflicting roles of autophagy. In the current study, with 6-OHDA-induced in vivo and in vitro Parkinsonian models, we investigated the effect of 6-OHDA on autophagic flux and its role in 6-OHDA-induced neurotoxicity.

2. Materials and methods

2.1. Chemicals and antibodies

Apomorphine (#A4393), 6-OHDA (#H116), chloroquine (CC6628) and mouse anti-tyrosine hydroxylase (TH, #1299) monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Lamp 1 monoclonal antibody (#ab24170) was purchased from Abcam (Cambridge, MA, USA). Antibodies against LC3B (#2775S) and cleaved caspase 3(#9661) were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against TFEB (A303-673A) was obtained from Bethyl Laboratories (Montgomery, TX, USA). Antibodies against p62 (#18420-1-AP), Bax (#50599-2-Ig), Bcl-2 (#12789-1-AP) and hitsone H3 (17168-1-AP) were obtained from Proteintech Group, Inc. (Chicago, IL, USA). Anti-GAPDH (#Ab103-01) antibody was purchased from Vazyme Biotech Co. (Nanjing, China). The HRP-conjugated anti-mouse (#ZB-2305) and anti-rabbit (#ZB-2301) antibodies were obtained from ZSGB-BIO (Beijing, China). The terminal deoxynucelotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was conducted with in situ cell death detection kit (Roche, Germany).

2.2. Cell culture

Human neuroblastoma cells (SH-SY5Y) were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology (shanghai, China) and grown in DMEM/F12 culture medium (Biological Industries) supplemented with 10% fetal bovine serum (Biological Industries) and 100 units/ml of penicillin/streptomycin (Biological Industries) at 37 °C in humidified atmosphere containing 5% CO_2.

2.3. Cell viability assay

Cell counting kit-8 (CCK-8, Dojindo Molecular Technologies) was applied to assay cell viability according to the manufacture's instruction. Briefly, 100 μl DMEM/F12 containing 10 μl CCK-8 was added to each well at the end of treatment and incubated at 37 °C for 1 h. Live cells were counted according to the optical density detected by a microplate reader at 450 nm.

2.4. Adenovirus infection

The adenovirus Ad-mCherry-GFP-LC3B was purchased from Beyotime (Beijing, China). SH-SY5Y cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 10 and treated with experimental conditions as indicated.

2.5. Cell transfection

2×10^5 cells in 2 ml growth medium were seeded in each well of a 6-well plate to reach confluency of 70%. 6 μl jetPRIME reagent (Polyplus Transfection) was mixed with 2 μg plasmid pcDNA-TFEB to form transfection complexes and added to each well. After incubation for 4 h, the transfection medium was replaced with fresh medium.

2.6. Animals

Male Sprague–Dawley rats (200–220 g) were obtained from Beijing HFK Bioscience Cooperation, China. Rats were housed in the standard environment with constant temperature of 23 ± 3 °C and relative humidity of 55 ± 3% on a 12 h light/dark cycle and were free access to food and water and. All animal care and procedures were approved by Shengjing Hospital Medical Ethics Committee in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

A total of 96 rats were evaluated in the current study. There were 13 rats in the control group and the 1 day-post 6-OHDA treatment group, 10 rats in other groups of different time points after 6-OHDA treatment, and 5 rats in the sole Chloroquine (CQ) treatment group and the CQ plus 6-OHDA treatment group. To be more concrete, 5 rats in each group were guaranteed for Western blot or cathepsin activity study; 5 rats in each group were evaluated for histological study; 3 rats in the control group or in the 1 day-post 6-OHDA treatment group were evaluated under the transmission electron microscopy (TEM).

2.7. 6-Hydroxydopamine treatment

Rats were injected with 6-OHDA as previously described before (He et al., 2017). Briefly, rats were anaesthetized with sodium pentobarbital (65 mg/kg, i.p.). Unilateral lesion of the nigrostriatal pathway was obtained by stereotaxic injection of 6-OHDA (15 μg in 3 μl of saline with 0.02% ascorbic acid) in the right striatum at the following coordinates: 0.7 mm anterior to the bregma, 3.0 mm lateral right to the midline suture, and 4.5 mm ventral to the dura; 0.2 mm posterior to the bregma, 2.6 mm lateral right to the midline suture, and 6.0 mm ventral to the dura (Gong et al., 2012; He et al., 2017). The rats in the control group received vehicle only (0.02% ascorbic acid in 0.9% saline) at the same coordinates according to the same procedure. For short-term study, brain tissues were collected at 6 h, 12 h and 1 day post injection; and for long-term study, brain tissues were collected at 4, 7, 21, 35 days post injection. To study the effect of 6-OHDA on autophagic flux in vivo, brains were collected 7 days post injection.

2.8. Chloroquine treatment

Chloroquine was dissolved in PBS (10 mg/ml). In order to monitor autophagic flux in vivo, rats were treated with chloroquine (10 mg/kg, i.p.) 2 h before sacrifice as described by Yao et al. (Yao et al., 2015).

2.9. Behavioral testing

Apomorphine-induced rotation was measured at the first, third and fifth week after 6-OHDA lesion. Rats were placed in a chamber (1×1 m^2) and received i.p. injection of 0.5 mg/kg apomorphine. After they were acclimated for 10 min, asymmetry rotational data were continuously recorded for 30 min. All procedures were performed in a double-blind manner.