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Research article

Valproic acid protects against MPP+-mediated neurotoxicity in SH-SY5Y Cells through autophagy

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HIGHLIGHTS

- VPA counteract MPP+-induced autophagic flux impairment in SH-SY5Y Cells.
- Autophagy induced by VPA play a role in neuroprotection.
- Autophagy induced by VPA alleviates apoptosis, reduces ROS production and MMP loss caused by MPP+.

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ABSTRACT

Autophagy is a common physiological activity in cells. Studies show that dysregulation of autophagy is involved in the pathogenesis of Parkinson's disease (PD). As a commonly used anti-epileptic drug, valproic acid (VPA) has shown neuroprotective effects in PD. The aim of this study was to explore whether the autophagy induced by VPA involved in the neuroprotective effects in PD cell model. We found that VPA treatment counteracted MPP+-caused autophagic flux impairment. Forthermore, VPA could alleviates apoptosis, reduce reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) loss caused by MPP+. And we also observed that VPA up-regulated the active caspase-3 and Bcl-2/Bax ratio and inhibited cytochrome c (Cyt c) release from mitochondria to the cytoplasm. However, 3-Methyladenine (3-MA) or bafilomycin A1 (Baf-A1), blockers for autophagy, partially weakened the neuroprotective effect of VPA. Our findings suggest that the neuroprotective effect of VPA on neuroblastoma cells may partially result from inducing autophagy and related to the inhibition of the mitochondrial apoptosis pathway.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease characterized by loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) [1,2]. Due to the low level of dopamine in the striatum, hypokinesia, rigidity and resting tremor are the main symptoms of PD. Even though the etiology of PD has not been fully elucidated, available evidence suggests that oxidative stress [3] and mitochondrial dysfunction [4] may lead to the occurrence and progress of PD.

Autophagy is a destructive mechanism in cells by which aging cellular components or malfunctional proteins are degraded and recycled [5]. In the process of autophagy, autophagosomes are formed with double-membraned vesicles containing targeted cellular components. After fusion by lysosome, the autophagosomes can degrade and recycle all contents. Therefore, autophagy is considered as a response to stress and promotes survival of cells. The dysregulation of autophagy function appears to promote cell death or disease [6]. Recently, increasing evidence shows that autophagy is involved in the pathogenesis of PD [7,8]. In brain samples of PD patients and PD animal models, lysosomal depletion and intracellular autophagosomes have been found in SNpc [9,10], indicating that normal autophagy function is impaired in PD. Recently, research with PD models showed that autophagy activation ther-

Abbreviations: PD, Parkinson's disease; DAergic, dopaminergic; SNpc, substantia nigra pars compacta; VPA, valproic acid; HDAC, histone deacetylase; GABA, gamma aminobutyric acid; MPTP, 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; Baf-A1, Bafilomycin A1; 3-MA, 3-Methyladenine; MMP, Mitochondrial membrane potential; Cyt c, cytochrome c; ROS, reactive oxygen species.

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apy could promote behavioral alterations, reduce accumulation of α-synuclein and attenuate neuron cell death [11–14].

Valproic acid (VPA), also known as valproate and sodium valproate, has been used to treat epilepsy and bipolar mood disorder for decades [15,16]. The therapeutic mechanism of VPA has been attributed to blocking the voltage-dependent sodium channels and increasing brain levels of gamma-aminobutyric acid (GABA). VPA also has histone deacetylase (HDAC) −inhibiting effects, which enable the epigenetic regulation of cell function and promote neuroprotective effects [17]. Researchers have found that VPA may play a neuroprotective role in various abnormal central nervous states, such as stroke [18], traumatic brain injury [19] and neurodegenerative disease [20,21].

As to PD, studies in animal and cell models indicate that VPA may be a promising neuroprotective agent. In a PD cell model, the apoptosis of SH-SY5Y cells induced by rotenone could be reduced by VPA [22]. VPA could also exerts its neuroprotection effect by stimulating the release of glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) from astrocytes [23] and by inhibiting the over-activation of microglia[24]. In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD mouse model, VPA protected SNpc DAergic neurons from loss and prevented striatal dopamine depletion [25]. Using a rotenone-induced PD animal model, Monti B et al. [20] found that α-synuclein decreased in its native form, while mono-ubiquitinated α-synuclein deposition was increased in SNpc, and VPA could counteracte these alterations of α-synuclein.

Recently, evidence showed that VPA induced autophagy in glioma [26] and neuro- blastoma[27] cell lines. Until now, it is not clear whether autophagy induced by VPA participates in neuroprotection and what mechanisms are involved. In this study, a cell model of PD was used to investigate wheather autophagy induction was involved in the neuro- protective effect of VPA and the underlying mechanisms were also explored.

2. Methods

2.1. Cell culture and treatment

Human neuroblastoma cell line (SH-SY5Y) was cultured in DMEM/F12 (Gibco, San Diego, CA, USA) containing 10% fetal bovine serum (Gibco, San Diego, CA, USA) and 1% penicillin and streptomycin in a CO_2 incubator at 37 °C, with 5% CO_2. The cells were cultured to a confluence of 80–90% and then subcultured with 0.25% trypsin (AMRESCO, USA). The cell culture medium was replaced every 2 days. All the experiments were conducted at least three times at cell confluence of 70–80%.

The PD cell models were exposed to MPP^+(1 mM) for 24 h. During the experiment, VPA (0.1–2.0 mM) was added to SH-SY5Y cells 1 h before the addition of MPP^+ and the cells were maintained in an incubator at 37 °C with 5% CO_2. For the study of autophagy, both Bafilomycin A1 (Baf-A1) (10 nM) and 3-Methyladenine (3-MA) (2 mM) were incubated 1 h before MPP^+ was added.

2.2. Cell viability assay

Cell viability was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay method [28]. In brief, cells were seeded at 5 × 103 cell/well in 96-well plates and incubated for 24 h with culture medium. Cells were then treated with medium containing MPP^+ or MPP^+ and VPA for another 24 h. Next, 20ul of MTT (5 mg/ml, Sigma-Aldrich, USA) was added into each well and the plate was incubated for 2 h at 37 °C, 5% CO2. Medium was then carefully removed and 100ul DMSO was added to each well for 30 mins to dissolve formazan crystals. The absorption was spectrophoto-measured at 570 nm using a plate reader.

2.3. Apoptosis analysis

Following FITC-conjugated Annexin V/PI staining (Beyotime Biotechnology, China), detection of apoptosis was performed using Hoechst 33358 staining (Beyotime Biotechnology, China) and flow cytometry (BD, Franklin Lakes, NJ, USA).

Cells were incubated with 10ug/ml Hoechst 33358 stain at 4 °C for 20 mins. After staining, nuclear morphological changes were observed with a fluorescence microscope (Olympus, Japan).

Flow cytometry analysis was performed using an Annexin V-FITC apoptosis detection kit following the manufacturer's instructions. In brief, SH-SY5Y cells were collected after drug treatments, washed with PBS and incubated at 37 °C with FITC Annexin V and PI for 15 mins in darkness. Flow cytometry analysis was conducted within 1 h. At least 10,000 cells were analyzed in each treatment.

2.4. Immunofluorescence microscopy

After drug treatment, cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 15 mins at room temperature (RT). After washing three times with PBS, cells were incubated with Triton-X100 (0.25%) for 10 mins at RT, washed again three times with PBS and incubated with blocking buffer (5% goat serum in PBS) for 1 h at RT. Cells were then incubated with antibody to LC–3B (Cell Signaling Technology, USA) diluted 1:500 in blocking buffer overnight at 4 °C. After three washes in PBS, cells were incubated with Alexa 488-conjugated secondary antibody (Invitrogen, USA) diluted 1:500 in Hoechst 33342 (Beyotime Biotechnology, China) for 1 h at RT. After washing three times with PBS, cells were visualized by epifluorescent confocal microscopy with an Olympus FV3000 microscope (Olympus, Japan).

2.5. Mitochondrial and cytosol proteins preparation

All cell groups were collected and isolated using a Cell Mitochondria Isolation Kit (Beyotime Biotechnology, China) according to the instruction manual. Cytosol and mitochondrial protein were then used for western blot analysis of cytochrome c (Cyt c).

2.6. Western blot analysis

All cell groups were collected and total proteins were extracted using cell lysis buffer for western blot (Beyotime Biotechnology, China) and quantified using a BCA Protein Assay Kit (Beyotime Biotechnology, China) according to the instruction manual. Protein extracts were separated using SDS-PAGE and blotted onto polyvinylidene fluoride membrane (Bio-Rad, USA). Monoclonal anti-β-Tubulin (Sigma, USA), anti-LC3 B (Cell Signaling Technology, USA), anti-P62 (Cell Signaling Technology, USA), anti-caspase 3 (Cell Signaling Technology, USA), anti-actived-caspase-3 (Cell Signaling Technology, USA), anti-bax (Bioworld, China), anti-Bcl-2 (Bioworld, China), anti-VDAC1(Bioworld, China) and anti-Cyt c (Bioworld, China) were used as primary antibodies. HRP-conjugated anti-rabbit or mouse IgG (Bioworld, China) was used as secondary antibodies. The densities of blots were measured using a Tanon 5500 ECL luminescent instrument (Tanon, China). Results were obtained by calculating density using Image-Pro Plus software (Media Cybernetics, USA).