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The anti-hypertensive drug reserpine induces neuronal cell death through inhibition of autophagic flux

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ABSTRACT

Reserpine is a well-known medicine for the treatment of hypertension and schizophrenia, but its administration can induce Parkinson's disease (PD)-like symptoms in humans and animals. Reserpine inhibits the vesicular transporter of monoamines and depletes the brain of monoamines such as dopamine. However, the cellular function of reserpine is not fully understood. In this report, we present one possible mechanism by which reserpine may contribute to PD-like symptoms. Reserpine treatment induced the formation of enlarged autophagosomes by inhibiting the autophagic flux and led to accumulation of p62, an autophagy adapter molecule. In particular, reserpine treatment increased the level of α-synuclein protein and led to accumulation of α-synuclein in autophagosomes. Treatment with rapamycin enhanced the effect of reserpine by further increasing the level of α-synuclein and neuronal cell death. *Drosophila* raised on media containing reserpine showed loss of dopaminergic neurons. Furthermore, cotreatment with reserpine and rapamycin aggravated the loss of dopaminergic neurons. Our results suggest that reserpine contributes to the loss of dopaminergic neurons by interfering with autophagic flux.

1. Introduction

Autophagy (specifically, macroautophagy) is an evolutionary conserved catabolic pathway that is responsible for degrading and recycling long-lived proteins and organelles [1]. Autophagy is induced by both extracellular stress conditions (e.g., nutrient starvation, hypoxia, high temperature, and microgravity) and intracellular stress conditions (e.g., damaged organelles) [1,2]. Autophagy is a highly dynamic process. The targeted components are enclosed by the sequestering phagophore (or a sequestering membrane) to form an autophagosome, which fuses with a lysosome to form an autolysosome for degradation via lysosomal hydrolases [3]. Autophagy can be monitored using microscopic and biochemical methods; in particular phosphatidyl ethanolamime (PE)—modified Atg8/LC3 (Atg8-PC/LC3-II) indicates the appearance of autophagosomes, which can be analyzed by fluorescence microscopy or Western blotting [3,4]. The dynamic process of autophagy, such as conversion of autophagosome into autolysosomes, is termed autophagic flux and can be interrupted by lysosomal inhibitors such as Bafilomycin A1, which is therefore a valuable tool for analysis of autophagic flux [5].

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by akinesia, bradykinesia, tremor, rigidity, and postural abnormalities [6]. PD is associated with the selective and progressive loss of dopaminergic neurons and the presence of Lewy body inclusions in neurons of the substantia nigra [7]. α-synuclein is crucial for PD pathogenesis, and inefficient clearance of α-synuclein leads to cellular toxicities [8,9]. Recent studies showed that α-synuclein can be degraded by autophagy as well as by the ubiquitin proteasome system, and α-synuclein protein can be detected in autophagic vesicles [9,10].

Because the molecular mechanism of PD is not clearly understood, PD has been extensively studied using animal and cellular models. Reserpine treatment was one of the earliest animal models for PD research, and the efficacy of L-DOPA, the first-line medicine

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for PD, was verified using reserpine-pretreated mice [11,12]. In addition, chronic administration of reserpine to patients can induce PD-like symptoms such as lethargy, depression, and motor dyskinesia [13,14]. Reserpine is also reported to decrease the level of tyrosine hydroxylase (TH) and the number of TH+ cells in the substantia nigra [15]. Reserpine inhibits the vesicular monoamine transporter (VMAT2), and depletes the brain monoamines such as dopamine by interfering with storage capacity [11]. However, the mechanism by which reserpine induces PD-like symptoms is not fully understood.

In this report we aimed to find autophagy modulating agents and identified reserpine as a negative modulator of autophagy. We found that reserpine treatment inhibited the autophagic flux and increased the level of α-synuclein. We also found that the number of dopaminergic neurons in *Drosophila* was decreased by reserpine treatment. These results suggest that regulation of autophagy might be the additional function of reserpine that induces PD-like symptoms.

2. Materials and methods

2.1. Cell culture and trypan blue assay

HEK293, HCT116, and PC12 cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). A HEK293 stable cell line expressing GFP-LC3 was generated as described previously [16] using a GFP-LC3 plasmid provided by T. Yoshimori [17]. Transfection of HEK293 and PC12 cells was performed using lipofectamine (Invitrogen, Carlsbad, CA, USA). Cell viability was measured using the trypan blue assay. Briefly, cells were seeded in a 24-well plate and then treated with different concentrations of reserpine for the indicated time periods. Viable cells were counted using a Bio-Rad TC10 automated cell counter (Hercules, CA, USA). Reserpin, bafilomycin A1, and rapamycin were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Western blotting

For protein immunoblot analysis, polypeptides in whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane filters. Detection was conducted with a 1:2000 or 1:5000 dilution of primary antibody using an enhanced chemiluminescence (ECL) system. Images were acquired using a Chemidoc-it 410 imaging system (UVP, Upland, CA) and LAS4000 system (GE Healthcare, Uppsala, Sweden). The antibody for LC3 was purchased from Novus biological (Littleton, CO, USA), antibodies for AMPK, phospho-AMPK, Erk, and phospho-Erk were from Cell Signaling Technology (Beverly, MA, USA), and the antibody for p62 was from Sigma–Aldrich. The plasmid encoding α-synuclein was purchased from Addgene (Cambridge, MA, USA).

2.3. Immunofluorescence and confocal microscopy

Cells were grown on sterilized glass coverslips. After drug treatment, the cells were fixed with 4% paraformaldehyde. For immunostaining, cells were blocked with 10% goat serum in PBS and stained with a 1:500 dilution of primary antibody in PBS, and then reacted with a 1:1000 dilution of Alexa 488- or Alexa 568-conjugated secondary antibody (Invitrogen). Finally, the slides were washed three times with PBS, stained with DAPI, and mounted in mounting medium (Vector, Burlingame, CA, USA). Images were captured with a Carl Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochem, Germany). GFP-mRFP-LC3 (ptfLC3) constructs were purchased from Addgene.

2.4. Drosophila strains

The tyrosine hydroxylase (TH)-GAL4 fly line was a gift from S. Birman. The UAS-GFP strain was obtained from the Bloomington Stock Center. We crossed these strains to obtain progenies expressing GFP in DA neurons (TH > GFP).

2.5. Quantification of dopaminergic neurons

Male TH > GFP flies were raised on standard fly media containing reserpine and/or rapamycin for 10 days. Fly brains were obtained and fixed with 4% paraformaldehyde as described previously [18]. GFP-positive neurons in dorsolateral region 1 (DL1) clusters from 10 brains of each genotype (n = 20) were observed in a blind fashion to eliminate bias using an LSM 700 confocal microscope (Carl Zeiss).

3. Results

3.1. Reserpine induces autophagosome formation

Autophagy is involved in many human diseases such as neurodegenerative diseases and cancers. We screened for novel autophagy-inducing compounds using HEK293 cells stably expressing GFP-LC3 (GFP-LC3 cells). GFP-LC3 cells were treated with the various compounds for 24 h and compounds that induced cytoplasmic punctuates were identified using a fluorescent microscope. This screening revealed that reserpine, a well-known anti-hypertensive and anti-schizophrenia drug, induces autophagosome formation in GFP-LC3 cells. We next treated GFP-LC3 cells with various concentrations of reserpine (0, 1, 2.5, 5, 10, and 20 μM) and examined the cytoplasmic pattern of GFP-LC3 protein. The cytoplasmic punctuates were evident at a concentration of 5 μM reserpine, and treatment with 10 and 20 μM reserpine resulted in enlarged autophagosomes in the cytoplasm (Fig S1A). In particular, we often observed ring-shaped autophagosomes that appeared to include large materials (Fig S1A, 10 μM).

Autophagosomes can be formed either by activation of the autophagy process or by inhibition of autophagic flux. Because we observed enlarged autophagosomes in the cytoplasm, we attempted to determine whether the autophagic process was activated or interrupted. First, we examined the level of the autophagosomal markers LC3-II and p62 by Western blotting. MCF-7 breast cancer cells were treated with various concentrations of reserpine (0, 1, 2.5, 5, 10, and 20 μM) and the expression of LC3 and p62 was examined. The levels of both LC3-II and p62 increased in a dose-dependent manner (Fig S1B). p62 is a useful marker for autophagy and the p62 protein level is generally decreased by autophagy induction, suggesting that autophagic flux was interrupted by reserpine. We examined the level of LC3-II in a time-dependent manner, and found that the level of LC3-II increased after 24 h of reserpine treatment and was maintained at 48 h and 72 h (Fig S1C).

3.2. Reserpine inhibits autophagic flux

Because reserpine increased the level of p62, we hypothesized that reserpine inhibits the autophagic flux. To test our hypothesis, we used a mRFP-GFP-LC3 reporter construct (tfLC3) for autophagic flux whereby autophagosomes appear yellow and autolysosomes appear red [19]. HEK293 cells were transfected with plasmid encoding mRFP-GFP-LC3 and then incubated with reserpine (0, 1, 2.5, 5, 10, and 20 μM). Consistent with previous results, reserpine induced the formation of enlarged vesicles that appeared yellow, indicative of autophagosomes (Fig 1A). In contrast, starvation induced vesicles that were small and red, indicating the formation

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