Enhancement of autophagy and neuroprotection of rapamycin in lactacystin-induced injury of dopaminergic neurons *in vitro*

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BACKGROUND

In Parkinson's disease (PD), death of dopaminergic neurons in the substantia nigra is associated with accumulation of aggregated protein, such as α-synuclein, within inclusions called Lewy bodies. Thus, preventing aggregation or disaggregating misfolded proteins might provide potential therapeutic benefit by favorably modifying the progression of PD.

The traditional view is that such proteins are cleared by the ubiquitin–proteasome pathway. Several lines of evidence have converged to suggest that failure of the ubiquitin-proteasome system (UPS) to degrade misfolded proteins plays a significant role in the etiopathogenesis of familial PD as well as sporadic PD. Besides proteosomes, lysosomes, often considered as non-specific systems for protein degradation, have also been shown to play an important role in degradating intracellular proteins (1,2). The process of bulk degradation of cytoplasmic proteins or organelles in the lytic compartment is termed autophagy (3-5). Autophagy is particularly crucial in the aging nervous system for protecting cells from cumulative oxidative damage to proteins and membranes, from synthesis of defective proteins, and from other genetic and environmental insults (6,7). Now, autophagy dysfunction is emerging as a theme in neurodegenerative diseases in which mis-aggregated proteins accumulate, including Alzheimer's disease, PD and the polyglutamine expansion diseases (8). Genetic ablation of autophagy in mice has been recently shown to induce neurodegeneration and accumulations of ubiquitinated proteins (9,10). The decline in lysosome function and efficiency of autophagy during aging (11-13) suggests the increased need for autophagy in the aging brain, and explains in part the increased risk that aging confers for neurodegenerative disorders that involve accumulation of abnormal proteins. Features of autophagy have also been observed in neurons of the substantia nigra of PD patients (14).

Effective neuroprotective strategies are needed in the management of PD. The autophagy pathway has not been targeted pharmacologically in PD. Considering the role of the protein aggregation in PD and the autophagy effect on the aggregated proteins, we propose to investigate the ability of autophagy enhancement on the removal of misfolded protein aggregates induced by environmental insults, such as lactacystin, one of the specific proteasome inhibitors, and the neuroprotective effect on lactacystin-induced cell injury.

Rapamycin is an FDA-approved antibiotic and immunosuppressant. It inhibits the activity of a protein called mTOR (mammalian target of rapamycin) which normally serves as an inhibitor of autophagy, and thereby, promotes autophagy (15). In this study, we use rapamycin to induce autophagy and to correlate the neuroprotection of rapamycin on lactacystin-induced cell injury to the induction of autophagy.

METHODS

Cell culture PC12 cells, a dopaminergic cell line that assumes a neuronal phenotype following exposure to the neurotrophin nerve growth factor (NGF), and has been extensively studied as a model for neuronal degeneration, were grown in 5% CO2 at 37oC. The growth medium consisted of DMEM supplemented with 5% heat-inactivated fetal bovine serum and 5% heat-inactivated horse serum, and penicillin/streptomycin. The medium was changed every 2 days and cells were passaged once a week. Cells were plated in growth medium at 5×104 cells per well in poly D-lysine-coated 6-well plates and allowed to attach overnight. The next day, cells were washed and incubated in DMEM supplemented with 1% heat-inactivated fetal bovine serum, 1% heat-inactivated horse serum, 100 ng/ml mouse 2.5S NGF to PC12 cells for 5 days followed by various doses of experimental compounds. Controls for each drug condition consisted of sister cultures treated with the vehicle used to dissolve that drug.

Application of reagents The pharmacological inhibitor of the 26S proteasome lactacystin (A.G. Scientific, Inc., San Diego, CA) was prepared in sterile dH2O at a stock concentration of 1 mM. Rapamycin (Sigma, St. Louis, MO), an inducer of autophagy through its inhibitory activity on mTOR proteins, was prepared in DMSO at a stock of 1 mm. They were diluted in serum-free medium prior to addition to the cultures for the desired final concentration for the indicated times. 3-methyladenine (3-MA, Sigma), which inhibits autophagy at the sequestration stage, where a double-membrane structure forms around a portion of the cytosol and sequesters it from the rest of cytoplasm to form the autophagosome, was prepared as a stock of 100 mM by heating in dH2O, and was added concurrently with rapamycin to the cultures at a final concentration of 10 mM.

Autophagy detection The induction of autophagy was detected by evaluation the development of acidic vesicular organelles (AVOs), which is characteristic of autophagy (16), using the FACScan flow cytometer and CellQuest software as described previously (16,17) by staining the cells with acridine orange (1μg/ml) for 15 min after rapamycin treatment. The induction of autophagy was also assessed by detecting an increase of the autophagosomal membrane form of microtubule-associated protein light chain 3 (LC3-II), which is a specific marker of autophagy, both in mRNA level and protein level (18).

Inhibition of autophagy To pharmacologically inhibit autophagy, 3-MA was added to the culture medium in the presence of rapamycin. To specifically inhibit autophagy, siRNA directed against autophagy-related gene *Beclin 1* (19) (accession number: NM_001034117; NM_053739; Ambio, the RNA company) were transfected to PC12 for 24 to 72 h using lipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Non-targeting siRNA was used as a negative control.

RNA extraction and quantitative RT-PCR Total RNA fractions were isolated from the culture cells by using SV total RNA isolation system (Promega, Madison, WI). One microgram of total RNA was used for reverse transcription using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) in a volume of 20 μl. All PCRs were performed by using HotStarTaq Master® Mix Kit (QIGEN Inc., Valencia, CA) with the specific primers targeting LC3 (F, 5'- CGTCCTGGACAAGACCAAGT-3';R,5'-CCATTCACCAGGAGGAAGAA-3'), β-Actin was used as internal control for equal loading of cDNA. RT-PCR products were separated on 1.5% agarose gel, and quantified by densitometric analysis with quantity one system (Bio-Rad, Richmond, CA).

Cell viability assay After a specific period of treatment, cell viability was assayed by adding 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide (MTT) to each well of 96-well plate with one-tenth of the total volume in the well, followed by incubation at 37oC for 3h. Then the culture medium was replaced by dimethylsulfoxide (DMSO) and read in duplicate at wavelengths of 570 nm.

Immunocytochemistry The primary mensencephalic cells were fixed in 4% paraformaldehyde for 20 min after being treated with lactacystin with or without rapamycin pretreatment. Then the cells were washed with PBS and incubated with 0.5% H2O2 for 10 min at room temperature followed by overnight incubation with 1:1000 diluted primary antibody to tyrosine hydroxylase (TH) (Protos Biotech Corporation, New York, NY) at 4 ° C. The cells were then washed and incubated with anti-Rabbit IgG 1:300 (Vector Laboratories Inc. Burlingame, CA) for 1 hour at room temperature, followed by avidin-biotin complex reaction (anti-rabbit ABC, Vector Laboratories, Inc. Burlingame, CA) and 3,3'-diaminobenzidine colorization (Sigma, St. Louis, MO).

Immunoblotting assay After specific treatment, the cells were harvested and the protein was isolated from the cell pellets. For the expression levels of various proteins, total protein was isolated with mammalian tissue lysis/extraction reagent (Sigma-aldrich, St. Louis, MO, USA) according to the manufacture's protocol. Equal amounts of lysate protein were loaded onto a 7.5%, 4 – 20% SDS−polyacrylamide gel electrophoresis (PAGE) gel, and separated by Laemmli method. Proteins were electrophoretically transferred to a nitrocellulose membrane and the nonspecific sites were blocked in 6% nonfat dry milk in Tris-buffered saline containing 0.3% Tween-20 for 45 min. Membranes were then incubated in the presence of respective primary antibodies p-p70 S6K, LC3 II, beclin 1, cytochrome c, anti-p-MAPK41/42, or antibody against β-actin (1:5,000; Sigma). Antibody binding and chemilluminescence enhancement were performed using the corresponding secondary antibody (1:2000) and signals were detected using ECL (Amersham, Piscataway, NJ, USA). To determine the changes in ubiquitination both the detergent soluble and insoluble fraction were subjected to immunoblotting assay and visualized by probing the membranes with a rabbit anti-ubiquitin antibody (1: 2000).

RESULTS

➢Rapamycin treatment enhanced the development of AVOs in differentiated PC12 cells (Fig. 1A) and increased in the autophagosomal membrane form of microtubule-associated protein LC3 both in mRNA (Fig. 1 B, C) and protein (Fig. 1 D,E) levels. The increased protein level of LC3 by rapamycin was inhibited by 3-MA (Fig. 1 F). Transfection of cells with siRNA of *Beclin 1* significnatly inhibited the expression of beclin 1 in dose- and time-dependent manner (Figure 1 G, H).

➢Rapamycin treatment did not change the cell viability significantly (Fig. 2 A). The cell viability was significant decreased by the treatment with lactacystin, which was dose-dependently (Fig. 2 B). Pretreatment of cells with rapamycin reduced the lactacystin-induced decrease of cell viability in PC12 cells (Fig. 2 C), which was blocked by autophagy inhibitor 3-MA (Fig. 2 C). Pretreatment of cells with rapamycin protected against lactacystin-induced neuron death in cultured rat primary embryonic mesencephalic cells as determined by counting TH-positive neurons (Fig. 2 D). Rapamycin pretreatment decreased the lactacystin-induced ubiquitinated-protein aggregation as determined by immunoblotting assay in the insoluble fraction of cells (Figure 2 E).

➢Immunoblotting assay revealed that both the protein levels of p-mTOR and the phosphorylation of 70-kDA ribosomal protein S6 kinase (p70-S6K), a downstream protein of mTOR, were decreased by rapamycin treatment, demonstrating the partial inactivation of mTOR kinase pathway, which is consistent with the induction of autophagy (Fig. 3 A-C). In addition to the inhibition of p-mTOR and p-P70 S6K, rapamycin treatment increased the protein levels of LC3 and p-MAPK41/42 as determined by immunoblotting assay (Fig. 3 C). Rapamycin pretreatment reduced lactacystin-incuced release of cytochrome c from mitochondria as assessed by immunoblotting assay (Fig. 3 D).

FIGURES

Figure 1

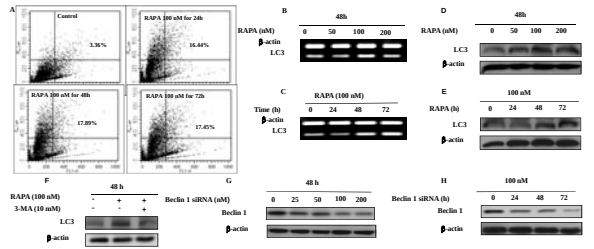


Figure 2

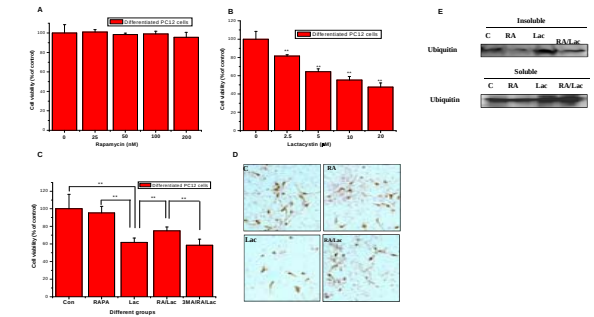
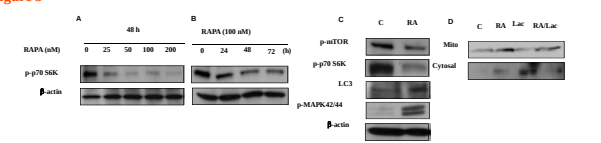


Figure 3



LEGENDS

Figure 1 Induction and inhibition of autophagy in differentiated PC12 cells. Differentiated PC12 cells pretreated with rapamycin (100 nM) for different time duration were stained with acridine orange (1 μg/ml) and then subjected to FACScan flow cytometric analysis (A). FL1-H, green color intensity; FL3-H, red color intensity. Top of the grid was considered as AVO (A). The cells were treated with rapamycin at various concentrations for specific time period. The mRNA levels were detected by RT-PCR using primers specific for *LC3* (B, C), and the protein levels of LC3-B were detected by immunoblotting assay using an antibody against LC3 (D,E). The cells pretreated with rapamycin with or without 3-MA，and the protein level of LC3 was assessed by immunoblotting assay (F). SiRNA of *beclin 1* at various concentrations were transfected into PC12 cells, and the protein level of beclin 1 was measured by immunoblotting assay (G, H).

Figure 2 The cells were treated with rapamycin ranging from 0 to 200 nM for 48 h (A) or treated with lactacystin ranging from 0 to10 μM for 24 h (B) and the cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were treated with lactacystin (10 μM) with or without rapamycin (100 nM) or 3-MA (10 mM) pretreatment followed by the MTT assay (C). The cultured rat primary embryonic mesencephalic cells were treated with lactacystin with or without rapamycin followed by the immunocytochemistry assay with anti-TH antibody to detect the TH-positive neurons (D). Differentiated PC12 cells treated with lactacystin with or without rapamycin pretreatment were harvested and proteins in soluble and insoluble fraction were subjected to immunoblotting assay with anti-ubiquitin antibody (E). C = control; RA = rapamycin; Lac = lactacystin; **: $p < 0.01$.

Figure 3 Effect of rapamycin on mTOR activity in differentiated PC12 cells. The cells were treated with rapamycin at various concentrations for 48 h (A) or at 100 nM for different time duration (B). Protein levels of p70-S6K were determined by imunoblotting assay using an antibody against phosphor-Thr389-specific p70-S6K (A, B). Protein levels of p-mTOR, p-MAPK41/42 were also detected by immunoblotting assay in differentiated PC12 cells treated with rapamycin (100 nM) for 48 h (Fig. 3 C). The release of cytochrome *c* from mitochondria induced by lactacystin was also detected in mitochondrial fraction of cells treated with lactacystin with or without rapamycin treatment by immunoblotting assay (Fig. 3 D).

CONCLUSIONS

- Our results showed that autophagy can be enhanced by rapamycin treatment in differentiated PC12 cells.
- Rapamycin treatment may partially prevent lactacystin-induced dopaminergic neuronal injury and protein aggregation, which may be abolished by inhibition of autophagy, indicating that neuroprotective effect of rapamycin may be mediated through the induction of autophagy. The increase in p-MAPK 41/42 indicated that the MAPK (Erk1/2) signaling pathway may also contribute to the neuroprotection of rapamycin against lactacystin-induced injury in PC12 cells.
- The findings we obtained may provide evidence that induction of autophagy is a potential neuroprotective strategy for the management of PD. Further studies are needed to investigate the mechanisms involved in the neuroprotection and evaluate the neuroprotective effect of rapamycin in *in vivo* models of PD.

REFERENCES

1. Ravikumar et al. Hum Mol Genet. 2002; 11(9):1107-17.
2. Hideshima T et al. Clin Cancer Res. 2005;11(24 Pt 1):8530-3.
3. Shintani T et al. Science 2004; 306(5698):990-5.
4. Klionsky DJ et al. Science 2000; 290:1717–21.
5. Levine B et al. Dev. Cell. 2004; 6:463–77.
6. Brunk UT et al. Eur. J Biochem. 2002; 269:1996–2002.
7. Sohal RS et al. Free Radic. Biol. Med. 2002; 33: 575–586.
8. Nixon RA.Trends in Neurosciences2006; 29:528-535.
9. Hara T et al. Nature 441 (2006), pp. 885–889.
10. Komatsu M et al. Nature 2006; 441:880–884.
11. Lynch G et al. Neurochem. Res. 2003;28:1725–1734.
12. Bahr BA et al. J Neurochem. 2002;83:481–489.
13. Martinez-Vicente M et al. Exp. Gerontol. 2005;40: 622–633.
14.Anglade P et al. Histol Histopathol. 1997;12(1):25-31.
15. Berger Z et al. Hum Mol Genet. 2006;15(3):433-42.
16.Takeuchi H et al. Cancer Res. 2005; 65(8):3336-46.
17. Daido S et al. Cancer Res, 2004; 64: 4286-4293.
18.Aoki H, Iwamaru A, Kondo Y, Yamamoto A, Aldape K, Kobayashi R, Sawaya R, Kondo S. Detection of autophagy in malignant glioma cells and tissues. In preparation.
19.Scarlatti F et al. J Biol Chem. 2004;279(18):18384-91.

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