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Research Paper

Trehalose does not improve neuronal survival on exposure to alpha-synuclein pre-formed fibrils

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ABSTRACT

Parkinson's disease is a debilitating neurodegenerative disorder that is pathologically characterized by intracellular inclusions comprised primarily of alpha-synuclein (αSyn) that can also be transmitted from neuron to neuron. Several lines of evidence suggest that these inclusions cause neurodegeneration. Thus exploring strategies to improve neuronal survival in neurons with αSyn aggregates is critical. Previously, exposure to αSyn pre-formed fibrils (PFFs) has been shown to induce aggregation of endogenous αSyn resulting in cell death that is exacerbated by either starvation or inhibition of mTOR by rapamycin, both of which are able to induce autophagy, an intracellular protein degradation pathway. Since mTOR inhibition may also inhibit protein synthesis and starvation itself can be detrimental to neuronal survival, we investigated the effects of autophagy induction on neurons with αSyn inclusions by a starvation and mTOR-independent autophagy induction mechanism. We exposed mouse primary cortical neurons to PFFs to induce inclusion formation in the presence and absence of the disaccharide trehalose, which has been proposed to induce autophagy and stimulate lysosomal biogenesis. As expected, we observed that on exposure to PFFs, there was increased abundance of pS129-αSyn aggregates and cell death. Trehalose alone increased LC3-II levels, consistent with increased autophagosome levels that remained elevated with PFF exposure. Interestingly, trehalose alone increased cell viability over a 14-d time course. Trehalose was also able to restore cell viability to control levels, but PFFs still exhibited toxic effects on the cells. These data provide essential information regarding effects of trehalose on αSyn accumulation and neuronal survival on exposure to PFF.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative condition that clinically manifests with abnormalities in movement, olfactory senses, gastrointestinal function and an overall decline in cognitive abilities as the disease progresses. These symptoms are associated with the progressive loss of dopaminergic neurons in the substantia nigra [1]. Sharing with other neuropathologies, including dementia with Lewy bodies and multiple systems atrophy, the majority of PD patient postmortem brains exhibit accumulation of α-synuclein (αSyn) protein in highly phosphorylated, ubiquitinated and insoluble aggregates observed as Lewy bodies and Lewy neurites [2–4]. Recent studies indicate that αSyn may be released from neurons and seed aggregations in adjacent neurons thus propagating disease progression [5].

Abbreviations: AraC, cytosine arabinoside; αSyn, α-synuclein; BSA, bovine serum albumin; Con, control; CQ, chloroquine; DIV, days *in vitro*; DMSO, dimethyl sulfoxide; *E. coli*, *Escherichia coli*; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HMW, high molecular weight; HSC70, heat shock cognate 70; HSP104, heat shock protein 104; ICC, immunocytochemistry; KO, knockout; LAMP1, lysosomal-associated membrane protein 1; LAMP2A, lysosome-associated membrane protein 2A; LC3, microtubule-associated proteins 1 light chain 3; MAP2, microtubule-associated protein 2; mTOR, mammalian target of rapamycin; MTT, , 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide); p-αSyn, phosphorylated-α-synuclein; PBS, phosphate buffered saline; PD, Parkinson's disease; Penn/Strep, Penicillin/Streptomycin; PFFs, pre-formed fibrils; PLL, poly-l-lysine; Pon S, ponceau S; p-S129, phosphorylated serine 129; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFEB, transcription factor EB; Tre, trehalose; TX-100, triton X-100; US FDA, United States Food and Drug Administration; WT, wildtype

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Recently studies have started to investigate how αSyn intracellular inclusion formation impacts recipient neuronal survival using a model in which αSyn pre-formed fibrils (PFFs) can enter the cell and recruit endogenous αSyn in the neuron to form insoluble, ubiquitinated and phosphorylated high molecular weight αSyn, characteristic of those in human Lewy bodies and Lewy neurites [5–7]. Since one of the strategies to help maintain intracellular protein homeostasis is through macroautophagy [8], determining the impact of macroautophagy regulators on accumulation of αSyn aggregates and neuronal survival in response to PFF exposure is thus essential to understanding PD.

Autophagy can be activated by the inhibition of the mammalian target of rapamycin (mTOR) by rapamycin. Many *in vitro* and *in vivo* studies have reported positive results using rapamycin to mitigate the effects of αSyn toxicity [9]. However, clinical use of rapamycin carries with it disadvantages that make its prolonged use in humans undesirable [10]. In addition, previous studies have demonstrated that autophagy induction by starvation or rapamycin post PFF transduction did not decrease PFF-induced intracellular αSyn aggregations and further exacerbated cell death [6]. Given that targeting of mTOR has limited therapeutic applications in humans in the realm of neurodegenerative treatments, compounds that activate autophagy independently of mTOR have been tested. One compound that has been found to be effective in activating autophagy in cell lines is the disaccharide trehalose [11], which appears to mediate its effects through both initiation of autophagy and activation of TFEB, turning on genes for increased lysosomal biogenesis [12–14]. Important for our study, neurons lack the enzyme trehalase that breaks down trehalose, thus the level of trehalose should be persistent over long periods of time in culture [15]. In humans, a single bolus of 50 g or less has been deemed safe, and in 2000 the US FDA gave notice that trehalose is generally regarded as safe for human consumption [16]. Although its administration has not been studied in the context of humans and PD, trehalose decreases α-synuclein in PC12 cells [11], decreases Tau accumulation in mice [17], and reduces neurodegeneration in amyotrophic lateral sclerosis [18], Huntington's [19], and Alzheimer's [20] disease models. In this study we specifically test the effects of trehalose on decreasing αSyn aggregation and cell death upon exposure to PFFs, thus helping to evaluate the therapeutic potential of trehalose in PD.

2. Methods

2.1. Cell culture

Primary wildtype (C57BL/6 strain bred in house from mice ordered from Charles River and WT mice bred from a cathepsin D knockout colony) [21–23], or αSyn knockout (The Jackson Labs C57BL/6N-Snca^{tm1Mjff}/J) [24] cortical mouse neurons were derived from p0 pups. All mouse experiments were done in compliance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee guidelines (IACUC09019 and IACUC20354). Briefly, cells were seeded on plates coated with Poly-L-Lysine solution containing 0.1 mg/mL Poly-L-Lysine, 50 mM Boric acid, and 10 mM Borax for a minimum of 1 h. Cells were grown and maintained in Neural Basal A medium supplemented with Glutamax, Penn/Strep, and B27 neuronal supplement. Treatments were performed by removing half the media and adding back the same volume of media with the treatment media. Tissue culture medium and reagents were obtained from Life Technologies. Trehalose (T0167–25G), chloroquine (C6628–25G) sucrose (S-5016) and AraC (C1768-500MG) were obtained from Sigma.

2.2. Generation of αSyn PFFs

A human wildtype αSyn gene was cloned into pRK172 and expressed in *E. coli* as previously reported [25]. Bacteria grown under antibiotic selection were harvested, homogenized and dialyzed before purification through size exclusion and ionic exchange columns. Five mg/mL of protein was incubated at 37 °C for 1 week to produce fibrils. Before applying to the cells, fibrils were sonicated 60 times over 40 s [6]. Using the Pierce LAL chromogenic endotoxin quantification kit, we have determined that ≤0.004 ng/mL of endotoxin was present in the PFF samples.

2.3. Cell viability

Cell viability was measured in two ways. First, viability was measured utilizing the trypan blue exclusion method. Cells were trypsinized and then trypan blue was added to the cells. Cells excluding the dye were counted. Second, viability was measured using the MTT cell death/proliferation assay. Briefly, a media and tetrazolium salt mixture was added to the cells where it is reduced to insoluble formazan crystals. These crystals were dissolved in DMSO and measured at 550 nm using a plate reader. Cells were plated on 96-well plates at 80,000 cells per well for both assays.

2.4. Immunocytochemistry

Cells were seeded at 240,000 cells per well on autoclaved glass coverslips that were placed in 24-well plates. After treatment, cells were fixed with a mixture of 4% paraformaldehyde and 4% sucrose. The addition of 1% Triton X-100 to the fixative was used to determine the soluble from insoluble protein in the cell. After fixing, cells were permeabilized with 0.1% Triton X-100 and then blocked with 3% BSA in PBS. Cells were probed with antibodies for p-Ser-129 using either Affinity Bioreagents (PA1-4686 1:2000) for Fig. 1G or Covance-81A (MMS-5091 1:5000) for the remaining figures. For non-αSyn staining, cells were plated and fixed as above but were blocked with 10% horse serum and 5% FBS in PBS. Cells were probed with either LC3 (L8918 Sigma 1:500), MAP2 (Sigma M4403 1:1000), or GFAP (Dako Z0334 1:500). Alexa Fluor 488 (Invitrogen A11001, A11008 1:500) or 568 (Invitrogen A11004 1:500) secondary antibodies were subsequently added to the wells. Cells were then counter stained with nuclear dye Hoechst 33342 (Sigma 861405) and mounted with Fluoromount-G (Southern Biotechnology). All images were acquired using a Leica TCS SP5 V confocal laser scanning microscope.

2.5. Western blot analysis

For immunoblot analysis, cells were plated at 240,000 cells per well on 48 well plates or 480,000 per well on 24 well plates and then washed with ice cold PBS and then lysed with RIPA buffer (50 mM Tris pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100%, and 0.1% SDS) in the presence of protease (Roche) and phosphatase inhibitors (Sigma) after treatments. After 20 min on ice, cells were scraped from the wells and placed in 1.5 mL Eppendorf tubes for centrifugation at 16,800×*g* for 20 min at 4 °C. Protein content in supernatant was determined by DC Protein assay (Bio-Rad). Equal amounts of protein for each sample were loaded and separated by SDS-PAGE using 12% or 15% gels. Protein was wet-box transferred to PVDF membranes and probed with the following antibodies: LC3 (Sigma L8918 1:2000), p62 (Abnova H00008878-M01 1:2000), α-synuclein (Santa Cruz sc-7011-R 1:2500), and β-actin (Sigma A5441 1:2500). Blots were visualized by film or Amersham™ Imager 600 (GE Healthcare Biosciences; Pittsburg PA).

2.6. Statistical analysis

All data are reported as the mean ± SEM, p values of less than 0.05 were deemed statistically significant after being analyzed by Student's *t*-test or ANOVA.