Oxidants Induce α-Synuclein-Independent Toxicity in a Fission Yeast Model for Parkinson's Disease

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Abstract
Parkinson’s disease is a progressive neurodegenerative disease caused by the death of midbrain dopaminergic neurons. The misfolding and aggregation of the protein α-synuclein and accumulation of oxidants is thought to underlie the cell death, but how α-synuclein becomes toxic is unclear. Using fission yeast as a model organism, where α-synuclein expression induces aggregation but not toxicity, we tested the hypothesis that α-synuclein toxicity can result from the combination of protein aggregation and oxidative stress. Surprisingly, we found that both oxidants (hydrogen peroxide and cumene hydroperoxide) tested in our study resulted in α-synuclein-independent toxicity. Nevertheless, this work illustrates the usefulness of yeasts in evaluating genetic factors and environmental factors like oxidative stress to potentially regulate α-synuclein toxicity linked to Parkinson’s disease.

Introduction
Parkinson's disease (PD) is a progressive neurodegenerative movement disorder in which patients suffer from resting tremors, postural rigidity, bradykinesia, and poor balance. These symptoms are the result of the specific death of dopaminergic neurons in the substantia nigra pars compacta (Chua and Tang 2006). Most cases of PD are idiopathic and occur during late age, with approximately 10% of PD cases are genetically linked (Eriksen et al. 2003). PD is fatal and incurable, but over the last thirty years a variety of drugs have been developed to treat the symptoms.

Key findings in PD research point to the hypothesis that the protein alpha-synuclein plays a critical role in pathogenesis. PD is characterized by accumulation of misfolded α-synuclein in cytoplasmic inclusions called Lewy bodies within the affected dying dopaminergic neurons (Spillantini et al. 1998). There are three mutations in the α-synuclein gene that cause dominant and familial PD; A53T (Polymeropoulos et al. 1997), A30P (Kruger et al., 1998), and E46K (Zarranz et al. 2004). Transgenic mice (Masliah et al. 2000) and flies (Feany and Bender 2000) expressing the human form of α-synuclein are well established models for replicating the PD pathology (Rochet et al. 2004).

In addition to α-synuclein misfolding and aggregation, oxidants accumulate in the dying dopaminergic neurons indicating the involvement of oxidative stress in PD pathogenesis (Olanow and Tatton 1999; Dawson and Dawson 2003; Jenner 2003; Maguire-Zeiss et al. 2005). Oxidative stress occurs largely due to mitochondrial dysfunction giving rise to reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and the hydroxyl radicals (Abou-Sleiman et al. 2006). The inhibition of complex I of the mitochondria is one of the pathological features in human PD brains (Parker et al. 1989; Simon et al. 2000; Devi et al. 2008). This type of inhibition has been shown to damage dopaminergic neurons in culture (Kweon et al. 2004). It generates excess cellular ROS (Dauer and Przedborski 2003), which can cause damage to lipid membranes (Giorgio Lenaz 1998), and modulation of important metabolic and other cellular activities by oxidizing critical proteins (Alam et al. 1997). Many studies have approached modeling PD by the use of neurotoxins. Among them are methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) and rotenone which abrogate mitochondrial complex I activity, consequently inducing oxidative stress (Fallon et al. 1997; Dawson and Dawson 2003).

Whether alpha-synuclein aggregation and oxidative stress are independent inducers of cellular toxicity or they act in concert to achieve a combinatorial outcome, is still an open question. Low concentrations of oxidants (less than 5 mM) do not typically harm yeast cells (Flattery-O’Brien and Dawes 1998; Izawa et al. 1995; Jamieson et al. 1992). However, our lab recently showed in a budding yeast model that α-synuclein toxicity can be induced when coupled with oxidative stress, which was induced by exposure to low concentrations of hydrogen peroxide in yeast that lack the mitochondrial anti-oxidant enzyme SOD2 (Sharma et al. 2006). This finding supports a role for combinatorial toxicity induced by alpha-synuclein aggregation and oxidative stress.

We recently developed a fission yeast model for PD and found these yeasts are resistant to α-synuclein toxicity, despite the extensive formation of α-synuclein aggregates (Brandis et al. 2006). To better understand why fission
yeast is protective against α-synuclein toxicity, we reasoned that, just like in budding yeast, a combinatorial insult such as oxidative stress might be needed for α-synuclein to induce cellular toxicity. In this study, we treated α-synuclein expressing fission yeast with two exogenous oxidants (hydrogen peroxide and cumene hydroperoxide) at concentrations that are not toxic to yeasts, and we predicted that α-synuclein-dependent toxicity would be induced in a combinatorial manner with oxidative stress. Finally, we hypothesized that α-synuclein cellular distribution would be altered under such condition. Because oxidative stress has been linked to the initiation and progression of Parkinson’s disease, the research aim of this study is to understand an in vivo relationship between oxidative stress and the toxic property of α-synuclein aggregation. In light of these two major events in the pathology of the disease, supporting evidence will help uncover of how neurodegeneration occurs.

Materials and Methods

S. Pombe Expression Vectors

Polymerase chain reaction (PCR) was used to amplify C-terminal green fluorescence protein (GFP)-tagged wild-type α-synuclein fusion cDNA from the α-synuclein-GFP containing pYES2/TOPO S. cerevisiae vectors constructed by Sharma et al. (2006):

forward primer, 5'-GCGGCGAGCTTGCCATGGATTTATGAAAAG-3';
reverse primer, 5'-TTTGTAAGGTCATACGATGCCATAGGCAG-3'.

Similarly, PCR was used to amplify GFP cDNA from GFP-pYES/TOPO S. cerevisiae vectors constructed by Sharma et al. (2006):

forward primer, 5'-TGCGCCGACCAGCCAGAAAGGAGAGAAG-3';
reverse primer, 5'-TTTGTAAGGTCATACGATGCCATAGGCAG-3'.

These PCR products were subcloned, according to the manufacturer’s protocol (Invitrogen), into fission yeast pNMT-1 TOPO-TA expression vector. The following two vector constructs were previously created for each experiment: GFP, and wild-type α-synuclein. α-Synuclein was tagged at the C-terminus with GFP. The pNMT-1 vector and GFP in pNMT-1 vector served as expression controls.

Yeast Strains

The TCP1 strain (h-leu1-32; Invitrogen) of fission yeast was kindly provided by Judy Potashkin, Rosalind Franklin University of Medicine and Science.

Yeast Transformation

S. Pombe strains were transformed with pNMT TOPO-TA vectors (Alfa et al. 1993) using the lithium-acetate transformation method (Burke et al. 2000).

S. Pombe Expression

For selection, yeast cells were grown on synthetic-complete media lacking leucine (PDM-Leu). Presence of α-synuclein constructs was confirmed by polymerase chain reaction (PCR). The pREP vector, containing a thiamine repressible promoter, allowed for regulated α-synuclein expression. Yeast cells were first grown overnight in PDM-Leucine (10 µM thiamine) media at 30°C. Cells were washed with water and diluted to log-phase (5 × 10⁶ cells/mL) in Edinburgh minimal medium (EMM) lacking thiamine media to induce expression and grown to the time points desired for various measurements.

Fluorescence Microscopy

Yeast cells were first grown overnight at 30°C in EMM containing thiamine (10 µM). After 24 hr, cells were pelleted at 1500g for 5 minutes, washed twice in 10 ml dH₂O, resuspended in 10 ml EMM without thiamine, of which 125 mL cells were used to inoculate 25 mL EMM without thiamine (to express α-synuclein). At desired expression time points for microscopy, cells were harvested at 1500g (4°C) for 5 min and were washed in 5 mL water. Then cells were resuspended in 100-1000 µL EMM-T, of which 10 µL was pipetted onto a slide. Slides were viewed using Nikon TE-2000U fluorescence microscope at 1000X magnification. Images were deconvoluted using MetaMorph software version 4.2. Cells were first viewed under differential interference contrast (DIC) microscopy, and then viewed for GFP fluorescence. For analysis, the number of cells in the field containing α-synuclein aggregates, intracellular structure localization or plasma membrane localization was analyzed. The field was then moved three turns on the field control knob, and the process was repeated in a new field. At least 750 cells were evaluated for each sample. α-Synuclein phenotypes were scored as percent of total cells observed.
Growth Curve
Cells were grown in 5 ml PDM-Leu overnight at 30°C in the incubator which rotates at 200 rpm. Cells were harvested at 1500 x g for 5 min at 4°C, and were washed twice in 5 ml H2O. Cells were re-suspended in 5 ml H2O and were counted. Flasks with 25 ml EMM were each inoculated with 2 x 10^6 cells/ml density. At 0, 6, 12, 18, 24, 36 and 48 hours, and in duplicate measurements, 1 ml of cell was removed and placed in a cuvette to measure absorbance using a Hitachi U-2000 Spectrophotometer. Averaged absorbance readings were plotted against time points to produce a growth curve.

Spotting Analysis
For spotting, cells were grown to mid-log phase in PDM-Leu normalized to equal densities (2 x 10^7 cells/mL), serially diluted (5-fold) into 96-well microwell plates. Cell cultures either containing parent vector, GFP or wild-type α-synuclein serially diluted 5 times, were spotted onto sets of repressing (+thiamine) and inducing (-thiamine) plates, that contained 0, 0.4 or 2 mM H2O2. Photographs of the plates were scanned after 2 days of growth.

Western Analyses
Yeast cells (2.5 x 10^7 cells/mL) were washed in 50 mM Tris (pH 7.5) and 10 mM Na3 and solubilized in electrophoresis sample buffer ([ESB] Burke et al. 2000) containing 2% sodium dodecyl sulfate (SDS), 80 mM Tris (pH 6.8), 10% glycerol, 1.5% dithiothreitol, 1 mg/mL bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.7 μg/mL pepstatin A, 0.5 μg/mL leupeptin, 10 μg/mL E64, 2 μg/mL aprotinin, and 2 μg/mL chymostatin). Samples were run on precast 10–20% Trisglycine gels (Invitrogen) using SDS containing running buffer. SeeBlue (Invitrogen) was used as the molecular standard. Gels were transferred to PVDF membranes, and Western blotting was performed with different monoclonal antibodies using standard protocols and detected for alkaline phosphatase activity: anti-V5 (Invitrogen) for most blots to probe for α-synuclein, and Anti-β-Actin (Abcam) for most expression experiments, as a measure of loading control in duplicate blots.

Statistical Analyses
Statistical significance was established using a T-Test comparing the means of control cells containing no α-synuclein at 18 and 24 hours in treated versus untreated conditions.

H2O2 and Cumene Hydroperoxide Treatment
H2O2 and Cumene Hydroperoxide (CHP) were purchased from Sigma-Aldrich. Cells were grown in 10 ml PDM-Leu overnight at 30°C. Cell harvesting and calculating cell density for inoculation was performed as described above. H2O2 and CHP were added to the selected media at concentrations of 0 mM, 0.4 mM and 2 mM. Afterwards, yeast cells were inoculated into the media. Then the cells were observed at the desired time points for the following experiments: Western Analysis, Growth Curve, Spotting Analysis and Fluorescence Microscopy.

Results
H2O2 induces α-synuclein-independent toxicity in fission yeast
Our goal was to evaluate if α-synuclein dependent

A. Growth Curve

B. Spotting

Figure 1. Fission yeast cells treated with H2O2. A. Growth Curve: Fission yeast were grown in EMM-T for 48 hours expressing WT (wild-type) α-synuclein along with pNMT-1 parent plasmid and GFP as controls. Over this time course, the cells were treated with 0.4mM or 2mM H2O2 and compared to the cells that were not treated with H2O2. Each of the points on the curves represents the mean from three trials on measurement of absorbance. In addition, the standard error of the mean for a 95% confidence interval is represented by the y-bar on each of the points on the growth curves. Significant growth difference exists between pNMT-1 in 0mM H2O2 (n=3, p18hr = 0.0199 and p24hr= 0.0762) and pNMT-1 in 2mM H2O2. B. Spotting: Serially diluted cells expressing parent pYES2 plasmid, GFP, or WT-α-synuclein were spotted onto EMM-T (inducing) plates. This experiment was repeated twice.
toxicity could be induced with oxidative stress in fission yeast, an organism in which α-synuclein expression has minimal toxic effect on its own (Brandis et al., 2006). We reasoned that H$_2$O$_2$ exposure would function as an additional insult on the existing stress of α-synuclein expression (McCormacka et al., 2002; Abou-Sleiman et al., 2006). As we exposed fission yeast cells to various concentrations of H$_2$O$_2$, the growth curve shifted to the right, particularly at 2mM, indicating a toxic effect (Figure 1A). Surprisingly, this slow growth was not α-synuclein dependent, since cells that expressed GFP alone or contained the parent pNMT1 vector were also growth impaired (Figure 1A). To confirm this effect, we also evaluated fission yeast colony survival on plates by a serial dilution assay. Similar to growth curve analysis, we observed a more striking dose-dependent toxicity with H$_2$O$_2$ that was not dependent on the presence of α-synuclein (Figure 1B). With a 2 mM H$_2$O$_2$ exposure, no fission yeast growth was detected on the agar plates with or without α-synuclein (Figure 1B).

H$_2$O$_2$ induces slight alteration of α-synuclein localization without affecting expression

Next, we examined the cellular distribution of α-synuclein in fission yeast to determine whether its normal localization (cytoplasmic aggregates) changed due to oxidant exposure. Live cell GFP fluorescence images for cells expressing α-synuclein were recorded at 18 hour post-induction. As the concentration of H$_2$O$_2$ was increased to 2mM, the predominant α-synuclein localization pattern was still aggregates (A). A less common finding was the localization of α-synuclein into as-yet-unidentified internal cellular structures (Figure 2A). In fact, some H$_2$O$_2$-exposed cells had both of these α-synuclein localization patterns (Figure 2A). Quantification of cellular distribution indicated that there was no statistical significance in the number of α-synuclein aggregates between H$_2$O$_2$-exposed cells versus untreated cells (Figure 2B). Meanwhile, the expression of α-synuclein remained the same with or without H$_2$O$_2$ treatment, as determined by Western blot (Figure 2C).

CHP induces α-synuclein independent toxicity in fission yeast

Lastly, we examined whether the H$_2$O$_2$ toxicity on yeast cells had a broader effect representative of more than one oxidant. As such, we induced oxidative stress in fission yeast by exposing them to cumene hydroperoxide. Though the growth curve analysis was conducted only once, the cellular toxicity under CHP treatment was more dramatic than under H$_2$O$_2$ treatment (Figure 3). The toxicity was not α-synuclein dependent, since cells that expressed GFP alone or contained the parent vector were also growth impaired (Figure 3).

Discussion

A connection between α-synuclein misfolding, aggregation, and oxidative stress is not fully understood. As examined initially by Brandis et al. (2006), S. pombe does not exhibit toxicity when α-synuclein is overexpressed. We inquired whether an additional stress (e.g. oxidant exposure) on fission yeast is critical for toxicity to occur. Our
major finding is that oxidants (H$_2$O$_2$ and CHP) exerted unexpected toxicity on fission yeast at low concentrations, independent of $\alpha$-synuclein expression and also altered $\alpha$-synuclein's cellular localization.

**Toxicity of oxidants in fission yeast**

The slow growth of fission yeast in media containing 2mM H$_2$O$_2$ was a surprising finding. At this same concentration, H$_2$O$_2$ does not impair growth of budding yeast (Sharma et al., 2006). More surprisingly, CHP exerted stronger toxicity on fission yeast than H$_2$O$_2$ at low of a concentration as 0.4mM. According to Mutoh et al. (2005), fission yeast has close to 100% survival rate at such a low concentration, but they are certainly growth impaired. In their study, they show 10-fold reduction in yeast colony forming ability within four hours after 0.1mM CHP treatment. One possibility for this conundrum is that budding yeast and fission yeast might have varying sensitivity to oxidants. Though fission yeast produce antioxidants and enzymes to break down reactive oxygen species generated by H$_2$O$_2$ and CHP, these might not be adequate to maintain cellular redox homeostasis as oxidant concentrations rise (Chen et al., 2003). One study points out that a 2mM H$_2$O$_2$ concentration inhibits the fission yeast cell growth by 58% (Pekmez et al., 2008). Also, the increased H$_2$O$_2$ concentrations lower the glutathione (GSH) levels; 2.0 mM H$_2$O$_2$ treatment showed significant decrease in GSH level in fission yeast (Pekmez et al., 2008). GSH is an abundant thiol that has been shown to protect against the deleterious effects of reactive oxygen species in budding yeast cells (Grant et al., 1998; Izawa et al., 1995). GSH expression in budding yeast and fission yeast (or within various fission yeast strains) may perhaps be different hence leading to difference in tolerance for H$_2$O$_2$ and perhaps also CHP. Along with GSH, there might be group of other regulatory genes in response to oxidative stress that may be responsible for why fission yeast is sensitive to the oxidants.

Contrary to our hypothesis, $\alpha$-synuclein-dependent toxicity was not induced in a combinatorial manner with oxidative stress. Both the $\alpha$-synuclein expressing cells and those that did not express the protein experienced the same magnitude of growth defect along the time course with exposure to the two oxidants. We did not observe any changes in $\alpha$-synuclein expression level in treatment with H$_2$O$_2$, but contrast that to a study done in neuronal cells, acute exposure to H$_2$O$_2$ increases the level of $\alpha$-synuclein expression where the protein protected neurons from apoptosis (Quilty et al., 2006). Whether $\alpha$-synuclein is cytotoxic or cytoprotective along with oxidative stress remains a contentious subject.

**Figure 3. Fission yeast cells treated with CHP.**

Growth Curve: Fission yeast was grown in EMM-T for 48 hours expressing WT (wild-type) $\alpha$-synuclein along with pNMT-1 parent plasmid and GFP as controls. Over this time course, the cells were treated with 0.4mM or 2mM CHP and compared to the cells that were not treated with CHP. Each of the points on the curves represents measurement of absorbance from one trial.
H₂O₂ and CHP are powerful oxidizing agents, but their interaction with α-synuclein inside the cell may not have been enough to cause α-synuclein dependent cell death, partly because α-synuclein rapidly forms aggregates in this organism and may prevent such interactions (Brandis et al. 2006). Secondly, because the oxidants produced significant toxicity on their own in fission yeast, this may mask any additional toxic contribution that would have come out of a combined effect of both oxidant and α-synuclein expression. Lastly, additional cellular dysfunction maybe required before α-synuclein dependent toxicity can be elevated. We presume that protective measures exist in fission yeast to suppress both α-synuclein toxicity and oxidative stress. For example, the anti-oxidant gene sod2 was shown to protect budding yeast cells from α-synuclein toxicity (Willingham et al., 2003). When the sod2 gene is deleted, α-synuclein toxicity was exhibited and further enhanced with H₂O₂ treatment (Sharma et al., 2006). An important future study should be to repeat our current experiments in fission yeasts knocked down for key anti-oxidant genes. In S. Pombe, oxidative stress activates transcription factors that induce the expression of ROS defensive genes. Transcription factors like Atf1, Pap1 and Prr1 (Wilkinson et al., 1996; Vivancos et al., 2004; Ohiya et al., 1999) which are known to respond to oxidative stress in fission yeast can be targeted for deletion.

In conclusion, it is not clear how oxidative stress is a critical factor to the etiology of Parkinson’s disease. On the other hand, the abnormal aggregation of alpha-synuclein into Lewy bodies has acquired much of the focus in the research field. Both of these events could be connected because several in vitro studies suggest that increased cellular oxidative stress increases the propensity for alpha-synuclein to aggregate within cells (Hashimoto et al., 1999; Souza et al., 2000). Our current in vivo study in fission yeast, where α-synuclein aggregation is prominent but it exhibits no cytotoxicity, was an attempt to dissect how oxidative stress could potentially induce α-synuclein dependent toxicity. Ongoing research that examines α-synucleinopathy with oxidative stress will be helpful for looking at potential therapeutic targets in the oxidative stress pathway to combat this debilitating brain disease.

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