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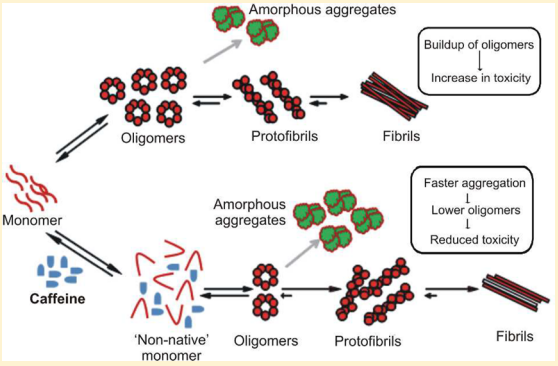
Understanding Caffeine's Role in Attenuating the Toxicity of α-Synuclein Aggregates: Implications for Risk of Parkinson's Disease

Jay Kardani and Ipsita Roy*

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, Punjab 160 062, India

Supporting Information

ABSTRACT: Epidemiological studies report a beneficial relationship between drinking coffee and the risk of developing Parkinson's disease (PD). This is likely due to caffeine, a constituent of coffee, acting as an adenosine A_{2A} receptor antagonist. This study was planned to investigate whether caffeine has any effect on the aggregation of α-synuclein, present in Lewy bodies, the pathological hallmark of PD, which may account for this positive association. Aggregation of recombinant α-synuclein was followed *in vitro* and in a well-validated yeast proteotoxicity model of PD. Caffeine was found to have twin effects: it accelerated the process of aggregation and also altered the nature of mature aggregates. Aggregates formed in the presence of caffeine displayed amorphous as well as fibrillar morphology. In the presence of caffeine, the toxicity of oligomers and aggregates was diminished, with concomitant reduction in intracellular oxidative stress, decreased oxidative proteome damage, and increased cell survival. Caffeine-treated samples showed improved binding to phospholipids, a property likely to be important in cellular functioning of α-synuclein. Far-UV CD spectroscopy and fluorescence quenching analysis revealed that caffeine induced transient changes in this intrinsically disordered protein, forming a non-native species that enhanced the rate of aggregation of α-synuclein and modified the population of mature aggregates, introducing a higher fraction of amorphous, less toxic species. Increasingly, it is felt that the process of fibrillation itself, along with the nature of mature aggregates, dictates the cytotoxicity of the process. Our results provide a rationale for the observed epidemiological link between drinking coffee and developing PD.



KEYWORDS: *Amorphous aggregates, caffeine, fibrillation, quenching, α-synuclein*

Parkinson's disease (PD) is the second most common neurodegenerative disorder, preceded only by Alzheimer's disease (AD). The twin hallmarks of the disorder are selective degeneration of dopaminergic neurons in the substantia nigra of the midbrain and the appearance of cytoplasmic inclusions referred to as Lewy bodies (LBs), which appear as dense clumps of protein aggregates. The major component of LBs is the natively disordered protein, α-synuclein.[1,2] This presynaptic protein follows a biphasic aggregation pattern, which is characteristic of many protein deposition disorders such as AD, Huntington's disease, prion diseases, amyotrophic lateral sclerosis, and others. Pharmacological intervention strategies in PD include molecules like L-DOPA (L-3,4-dihydroxyphenylalanine), dopamine agonists, and inhibitors of enzymes like COMT (catechol-O-methyl transferase) and MAO-B (monoamine oxidase-B).[2−4] These approaches provide temporary symptomatic relief but do not modify the course of the disease. As the presence of cytosolic inclusions seems to have adverse effects on cell survival, efforts are underway to develop strategies that can slow down or inhibit the aggregation of α-synuclein.[5−8] Although the function of α-synuclein in the cell is not well understood, it is known to interact with a number of binding partners within the cell. Its aggregation is likely to result in reduced cell viability due to loss of function, which is in addition to a probable gain-of-function mechanism of the proteotoxic aggregates themselves.[1,2]

PD is increasingly classified as an environmental disorder.[9,10] As such, lifestyle habits like consumption of tea, coffee, and alcohol as well as cigarette smoking have been shown to correlate with the occurrence of the disease.[11−13] Several epidemiological studies have reported an inverse relationship between drinking coffee and the risk of developing PD.[11,14−17] Caffeine, the major component in coffee responsible for this beneficial effect in PD,[18] is an adenosine analogue and acts as a nonselective adenosine A_{2A} receptor antagonist, offering protection to dopaminergic neurons.[19,20] A recent study has attributed the therapeutic effect of caffeine in experimental PD models to its function as an adenosine A_{2A} receptor inverse agonist.[21] Caffeine has multiple cellular targets, with the most important one probably being target of rapamycin complex 1 (TORC1).[22] Caffeine resulted in a longer life span in budding and fission yeast and in the nematode, *Caenorhabditis*

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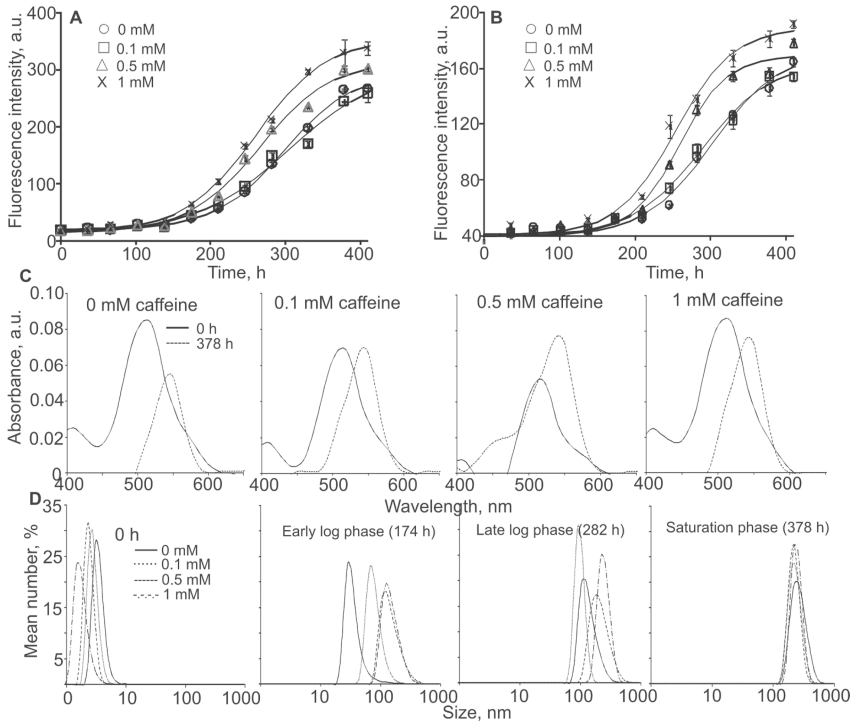

Figure 1. Kinetics of aggregation of α-synuclein. (A) Thioflavin T fluorescence assay was used to monitor aggregation of α-synuclein in the absence and presence of caffeine. Data represent the mean ± SEM of three independent experiments. (B) ANS fluorescence assay was used to monitor aggregation of α-synuclein. Data represent the mean ± SEM of three independent experiments. (C) Congo Red difference spectroscopy was carried out before and after completion (378 h) of α-synuclein aggregation in the presence of caffeine. (D) Measurement of mean particle size of α-synuclein by dynamic light scattering in the absence and presence of caffeine at different time intervals. Size represents the diameter of particles.

elegans.[22−24] Administration of caffeine also slowed the decline in cognitive ability[25] and recognition memory[26] in aged subjects. Caffeine has been reported to protect against degeneration of the blood−brain barrier associated with AD and PD.[27] Preliminary studies[28] have implicated the inhibition of fibrillation of human islet amyloid polypeptide by caffeine *in vitro* to explain the negative correlation observed between drinking coffee and developing type 2 diabetes mellitus. Administration of caffeine was successful in reducing Aβ levels in brain interstitial fluid and plasma in mice in an age-independent manner,[29] presumably explaining the lower incidences of AD upon habitual coffee drinking. No correlation, however, was observed between plasma caffeine level and cognitive behavior. In this work, we investigated whether the beneficial effect of caffeine is exerted only through its function as a modulator of the adenosine A_{2A} receptor or whether caffeine has an additional role as a modulator of α-synuclein aggregation. We employed the *Saccharomyces cerevisiae* proteotoxicity model of Parkinson's disease to explore the role of caffeine in the aggregation of α-synuclein. Phenotypic screening using this PD model revealed a small molecule acting on a druggable network that was able to antagonize α-synuclein toxicity in yeast cells.[30] This molecule was further able to attenuate toxicity in mammalian cell and animal models of PD,[30] validating the use of *S. cerevisiae* as a model organism to study aggregation of heterologous α-synuclein. In fact, the yeast model was successful in discovering early pathogenic PD phenotypes in cortical neurons derived from induced pluripotent stem cells.[31] We report here that caffeine is indeed able to alter the aggregation pathway of α-synuclein by introducing species with reduced proteotoxicity. This has beneficial effects on the proteomic integrity of the cell and its survival.

RESULTS AND DISCUSSION

Effect of Caffeine on Aggregation Kinetics of α-Synuclein *in Vitro*. α-Synuclein has been reported to form higher-order aggregates *in vitro*. These aggregates exhibit characteristic fibrillar structures similar to the fibrils found in the brains of PD patients.[32,33] In order to follow the kinetics of aggregation in the presence of caffeine, α-synuclein was purified as per a reported protocol (Figure S1).[34,35] During the initial phase of incubation (lag phase), natively disordered α-synuclein did not show any change in the fluorescence intensity of Th T (Figure 1A). This was followed by a sudden increase in the fluorescence intensity of Th T (log phase), which indicated the formation of fibrillar α-synuclein aggregates. Finally, the fluorescence intensity of the dye achieved a plateau (saturation phase), indicating that fibrillation reached stationary phase (Figure 1A). Protein fibrillation kinetics follows mostly a nucleation-dependent process.[35−37] A characteristic sigmoidal curve, with distinct nucleation, growth (fibrillation), and equilibrium (saturation) stages, was observed during the aggregation process. The presence of caffeine was found to increase the extent of aggregation of α-synuclein in a concentration-dependent manner (Figure 1A). The lag time of nucleation (τ, the time required for seed formation) was seen to decrease with increasing concentration of caffeine. In the absence of caffeine, the lag time was 207.6 h, which decreased to 180.7, 170.9, and 167.2 h in the presence of 0.1, 0.5, and 1 mM caffeine, respectively. The apparent rate constant of fibrillation (k_{app}) was found to increase marginally in the presence of caffeine. With k_{app} starting at 0.0217 h^{-1} in the

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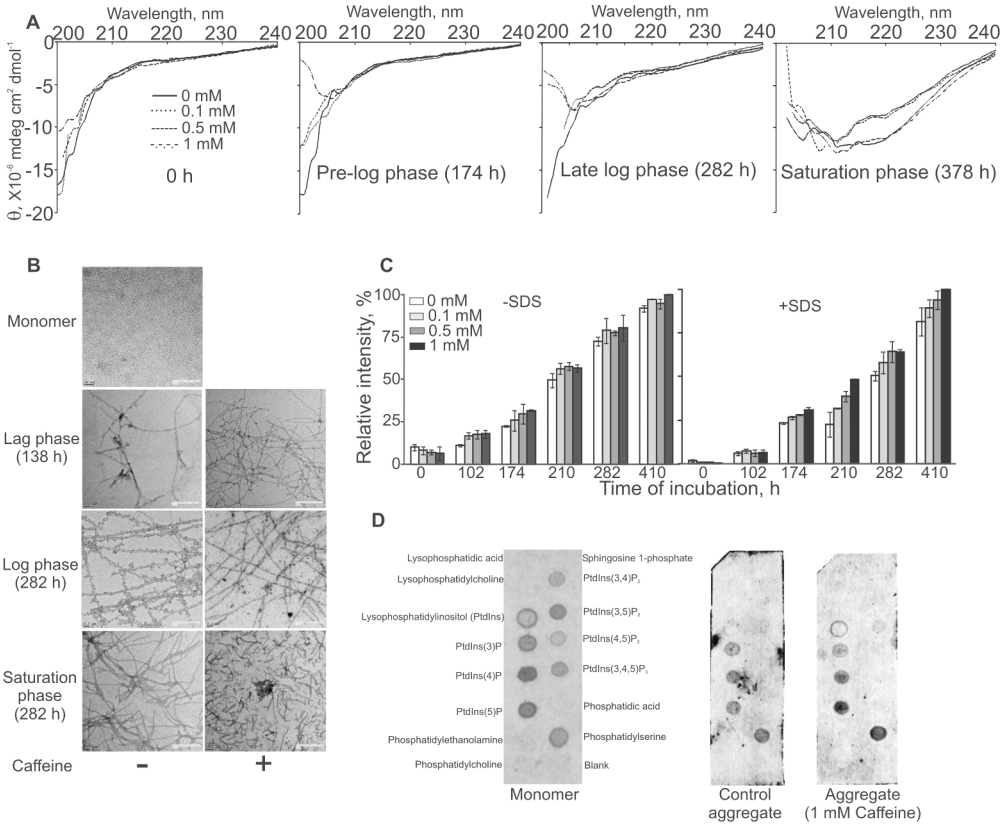

Figure 2. Changes in the conformation of α-synuclein upon incubation. (A) Far-UV CD spectra of α-synuclein were recorded in the absence and presence of caffeine at different time intervals. (B) TEM images of α-synuclein were recorded in the presence of 1 mM caffeine at different time intervals. Bar = 0.2 μm in all cases except monomer, where the bar = 0.02 μm (11 500× magnification). (C) Filter retardation assay of α-synuclein in the absence and presence of caffeine. Densitometry (Figure S2) was carried out using ImageQuant TL software (GE Healthcare). Relative intensity was calculated by assigning the intensity of the dot obtained in the presence of 1 mM caffeine at 410 h in each case (absence and presence of SDS) as 100%. Data represent the mean ± SEM of three independent experiments. (D) Protein−lipid overlay assay to determine the lipid specificity of α-synuclein. The membrane was probed with an anti-α-synuclein antibody as the primary antibody and a FITC-conjugated goat anti-mouse antibody as the secondary antibody.

absence of caffeine, it increased to 0.0220, 0.0226, and 0.0249 h^{-1} in the presence of 0.1, 0.5, and 1 mM caffeine, respectively. Lower lag times and higher k_{app} values in caffeine-treated samples suggested that both the formation of oligomers and the conversion from oligomers to fibrils took place rapidly in the presence of the alkaloid. Thus, caffeine appeared to accelerate the nucleation step as well as to hasten the process of fibrillation, reducing the time required for the process to be completed. Rapid conversion of oligomers to fibrils reduces the population of oligomers, which are generally considered to be the more toxic species in the aggregation pathway.[1,38−42] The hydrophobic dye ANS was used to monitor changes in the conformation around hydrophobic residues during α-synuclein aggregation. The fluorescence intensity of the dye was found to increase as the incubation period increased (Figure 1B). Aggregation was seen to follow sigmoidal kinetics similar to that in the Th T assay. Thus, the formation of hydrophobic surfaces and mature aggregates occurred simultaneously during incubation. Similar to the results of the Th T assay, a concentration-dependent enhancement in ANS fluorescence kinetics was observed in the caffeine-treated samples.

To confirm the presence of fibrillar aggregates in the samples, a Congo Red (CR) spectral shift assay was carried out (Figure 1C). CR is a diazo dye that is highly specific to amyloids.[43] When CR binds to fibrillar aggregates, it exhibits a red shift in its absorbance spectrum, from 490 to 540 nm. There was no significant difference in the absorption spectra of monomeric α-synuclein (0 h sample) in the absence and presence of caffeine (Figure 1C). From the Th T aggregation assay, it was observed that aggregation achieved saturation at around 400 h (Figure 1A). In the aggregated samples (378 h), the absorption maxima of CR exhibited a bathochromic shift to around 550 nm (Figure 1C). A red shift in the CR spectrum, characteristic of amyloid fibrils, was also observed in caffeine-treated samples (Figure 1C). The difference in CR absorbance values in caffeine-treated and untreated samples suggested that individual samples may vary in the amount of fibrils finally formed, with a relatively higher amount of aggregates being formed in the presence of caffeine, similar to observations using Th T (Figure 1A).

Protein fibrillation increases the hydrodynamic radius of the molecule by assembling monomers into ordered macrostructures.[1,44] Aggregation of α-synuclein was followed by dynamic light scattering (DLS) to monitor changes in the hydrodynamic radius of the protein with time. In the monomeric state (0 h), the hydrodynamic radius of the protein was in the range of 2−4 nm (Figure 1D), which matched with the reported size of α-synuclein monomer (approximately 3 nm).[44] As per the kinetics of aggregation followed using the Th T assay (Figure 1A), 282 h was selected to represent the mid log phase of aggregation. An increase in the average size of the protein (between 100 and 200 nm) was observed for all of the samples after 282 h when compared with that of the

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monomeric protein (Figure 1D). In mid log phase, caffeine-treated samples showed larger aggregates, in terms of size, when compared with that of the untreated sample. Increasing the concentration of caffeine led to the formation of larger sized intermediates in mid log phase as compared to that of the control sample (Figure 1D). From Th T assay, it was observed that the presence of caffeine increased the rate of formation of the nucleus and also accelerated the conversion of oligomers to fibrils. A probable reason for the observed higher size range of particles during mid log phase in caffeine-treated samples could be the acceleration of fibrillation from oligomers in the presence of caffeine (Figure 1A). Size measurements were also done for samples in the stationary phase of aggregation (378 h). After completing the fibrillation process (378 h), the size of the protein was more than 200 nm in all cases (Figure 1D). A gradation in intensity was observed, with a relatively higher concentration of aggregates seen with increasing concentrations of caffeine. Thus, at the stationary phase, the aggregates' size was in a similar range in all cases, as indicated by the almost superimposable peaks, although the extent of aggregation was different. Taken together, the increase in the fluorescence intensity of Th T with increasing concentrations of caffeine at this time point (Figure 1A) and the results of DLS (Figure 1D) point to a higher amount of mature aggregates being formed in the presence of caffeine.

Effect of Caffeine on Conformation of α-Synuclein. Changes in the conformation of α-synuclein during aggregation were studied by far-UV CD spectroscopy. Far-UV CD spectra of α-synuclein incubated in the presence of caffeine were recorded at different time intervals (Figure 2A). α-Synuclein is an intrinsically disordered protein, and its CD spectrum reflects the disordered nature of the protein.[44] At 0 h, the spectrum of α-synuclein was typical of a misfolded polypeptide, with the absence of minima in the range of 210−230 nm (Figure 2A). Caffeine treatment did not lead to any visible change in the conformation of the monomeric protein. As the incubation time increased, changes in the spectra were observed. At 174 h (pre log phase), marginal ordering of α-synuclein was observed in the presence of 1 mM caffeine, with an increase in β-sheet content. After 282 h of incubation (mid log phase), the CD spectrum of α-synuclein alone showed enhanced negative ellipticity, which suggested a change in its secondary structure (Figure 2A). Similar changes in ellipticity were observed in samples treated with 0.1, 0.5, and 1 mM caffeine after 282 h of incubation. At the stationary phase (378 h), the CD spectrum of α-synuclein alone showed minima at around 218 nm (Figure 2A). A sharp minima at 218 nm, which is characteristic of β-sheet-rich structures, was not observed, indicating the possibility of the presence of amorphous aggregates in the samples. Enhanced negative ellipticity in the CD spectra of α-synuclein treated with 0.1, 0.5, and 1 mM caffeine was observed after 378 h of incubation, with the sample treated with 1 mM caffeine showing the maximum decline (Figure 2A).

Transmission electron microscopy (TEM) was used to detect changes in the morphology of α-synuclein aggregates, if any, formed in the presence of caffeine. Monomeric α-synuclein (0 h) showed a dotted appearance in the TEM image (Figure 2B), which confirmed the absence of aggregates. Formation of oligomers gives rise to the nucleus, which forms protofilaments.[1] In nucleation-dependent aggregation, formation of protofilaments is followed by actively growing forms called protofibrils, and protein aggregation achieves saturation with the formation of mature fibrils. Electron microscopic analysis revealed that with time α-synuclein formed aggregates and that the formation of protofilaments was observed after incubation for 138 h (Figure 2B). Protofilaments are smaller structures with diameters in the range of a few nanometers. Very few protofilaments, with a long and thin stick-like appearance, were seen at the completion of lag phase (Figure 2B). In TEM analysis, α-synuclein alone (282 h, mid log phase) showed long and thick protofibrillar aggregates. These protofibrils displayed the appearance of beads on the growing fiber axis. At the saturation phase (410 h), mature fibrils were observed in electron micrographs. These fibrils were short and dense in the TEM micrograph. α-Synuclein treated with 1 mM caffeine was also analyzed by TEM. Initially, in the lag phase (138 h), the caffeine-treated sample showed the presence of numerous protofilaments (Figure 2B). These protofilaments appeared to be like complexes of several branched and/or unbranched protofilaments. Thick and elongated protofibrils were seen in caffeine-treated samples in the mid log phase (282 h) of aggregation. Protofibrils formed in the presence of caffeine exhibited a different morphology than that of untreated α-synuclein (Figure 2B). Compared with the untreated sample, protofibrils in the caffeine-treated sample appeared to be thicker, longer, and unbranched in nature. During the stationary phase (410 h), the treated sample showed the presence of a mixed population, with mature fibrils as well as nonfibrillar aggregates, which was different from the morphology observed for the untreated α-synuclein sample (Figure 2B). Although the end point of aggregation remained the same, the composition of aggregates formed was different in the presence of caffeine, similar to what was observed with DLS studies. A more heterogeneous population was seen in this case.

The fibrillar nature of aggregates can also be confirmed by their (in)solubility in denaturing detergents. The filter retardation assay is based on the fact that protein aggregates are selectively retained on a cellulose acetate membrane, whereas soluble protein, under the same conditions, is not.[1] Incubated α-synuclein samples were removed at predetermined time intervals and were filtered through a cellulose acetate membrane to analyze the extent of aggregation. The membrane was probed with an α-synuclein-specific antibody (Figure S2). Initially, aggregation was low in samples incubated for 174 h (prelog phase), as observed by the lower intensity of the dots. Aggregation was found to increase with incubation time. Maximum aggregation was observed in samples incubated for 410 h (stationary phase), as indicated by the intensity of the dots (Figure 2C). Caffeine-treated samples were also analyzed by the filter retardation assay. Aggregation was found to increase with incubation time in treated samples (Figure S2). Aggregation of α-synuclein alone was observed to be around 25% in the pre log phase (174 h), which increased to around 75% during mid log phase (282 h) (Figure 2C).

α-Synuclein aggregates comprise a mixture of species, such as oligomers, fibrils, and amorphous.[1,40,45] Among these, fibrils and certain amorphous types are considered to be detergent-insoluble, whereas oligomers remain soluble. As the function of α-synuclein in the cell is not well understood, the implications of oligomerization and fibrillation in cell survival are a matter of debate. Some reports indicate that the soluble oligomers form pore-like structures in the membrane, increasing its conductance, which is accompanied by higher Ca^{2+} influx and results in neuronal degeneration, likely by activating the apoptosis pathway.[2,45−48] Treatment with the anionic detergent, SDS, serves as a good approach to distinguish fibrils from

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soluble oligomers.[49] Detergent treatment helped to solubilize other aggregates, and only detergent-insoluble aggregates were retained on the membrane after filtration. The initial appearance of detergent-insoluble aggregates was observed in samples incubated for 174 h (Figure S2). This amount was found to increase with incubation time. Note that the concentration of SDS used in these experiments was significantly higher than the critical micellar concentration (CMC) of the detergent, which may impact the fibrillogenesis of α-synuclein. The sample obtained at the stationary phase (410 h) showed the maximum retention of insoluble aggregates on the membrane. A greater amount of insoluble aggregates was observed in caffeine-treated samples incubated for 174 h (prelog phase) compared to that for α-synuclein alone (Figure 2C). Again, the amount of insoluble aggregates in the samples was found to increase with the concentration of caffeine, and a higher fraction of detergent-insoluble aggregates was seen in caffeine-treated samples (Figure 2C), with the maximum aggregation seen in the sample treated with 1 mM caffeine. Formation of detergent-insoluble aggregates was found to be 25% at the nucleation phase (210 h) in the absence of caffeine, whereas it was 50% in the presence of caffeine under the same conditions. Thus, caffeine accelerated the conversion of detergent-soluble oligomers to detergent-insoluble aggregates, which is in accordance with the results observed earlier with the Th T fluorimetric assay (Figure 1A).

Effect of Caffeine on α-Synuclein−Phospholipid Interaction. Natively disordered α-synuclein acquires an α-helical conformation after binding with various physiologically important phospholipids. This orderliness is important for it to perform its biological functions.[50] α-Synuclein shows affinity toward phospholipid membranes, mainly via its N-terminal region.[51] Any change in its lipid binding property can also lead to a change in the normal physiological functions of α-synuclein. To analyze the lipid interacting property of aggregates, samples were incubated with a nitrocellulose membrane prespotted with different types of phospholipids (PIP Strip membrane). The protein−lipid complexes were detected by immunoblotting with an α-synuclein-specific antibody. α-Synuclein binds with acidic phospholipids like phosphatidylserine (PS) and phosphatidyl inositol (PtdIns) but not with neutral phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE).[52] Monomeric α-synuclein was seen to bind to some physiologically important phospholipids like phosphatidyl inositol and phosphatidylserine (Figure 2D). Intense dots were observed with PS and PtdIns, whereas no signal was observed with PC and PE (Figure 2D). The monomer also showed affinity toward phosphate, bisphosphate, and trisphosphate derivatives of PtdIns. PtdIns and phosphatidylserine have important roles in critical cellular processes like cell signaling, membrane trafficking, and apoptosis. α-Synuclein aggregates showed no interaction with PtdIns and also exhibited reduced or negligible binding toward mono-, bis-, and tris-phosphate derivatives of PtdIns (Figure 2D). The change in the binding properties of aggregates toward lipids may explain the observed loss of α-synuclein function[53,54] upon formation of Lewy bodies. α-Synuclein aggregates formed in the presence of caffeine were analyzed for lipid specificity. Aggregates formed in the presence of caffeine were seen to retain higher lipid binding ability, similar to that of the monomeric protein (Figure 2D). In contrast to interactions observed for aggregates formed by α-synuclein alone, intense dots were observed with PtdIns and derivatives of PtdIns in the presence of caffeine, whereas interaction with PS remained unaffected. Stronger binding was seen toward PtdIns and its derivatives, which was not observed in the case of aggregates formed in the absence of caffeine. Thus, the presence of caffeine helps α-synuclein to retain its lipid-binding properties even after the formation of aggregates, and this may partially account for the correlation with a lower risk of disease onset, as reported in epidemiological studies.[11,14−17]

Effect of Caffeine on α-Synuclein Aggregate-Mediated Oxidative Stress Generation in Mammalian Cells. Soluble protein oligomers are known to induce cytotoxicity by forming transient pores in the plasma membrane.[2,45−48] This damage to the membrane leads to an increase in intracellular ROS levels in the cell. The cell-permeable dye, DCFH-DA, is cleaved by esterases present in the cell to form DCFH, which gets oxidized by ROS present in the cell to form the fluorescent derivative, DCF.[55] Rat RBCs were used to estimate the oxidative stress induced by various types of aggregates formed by purified recombinant human α-synuclein. A strong signal was observed in RBCs treated with α-synuclein monomer, suggesting that ROS were generated (Figure 3A), which may be

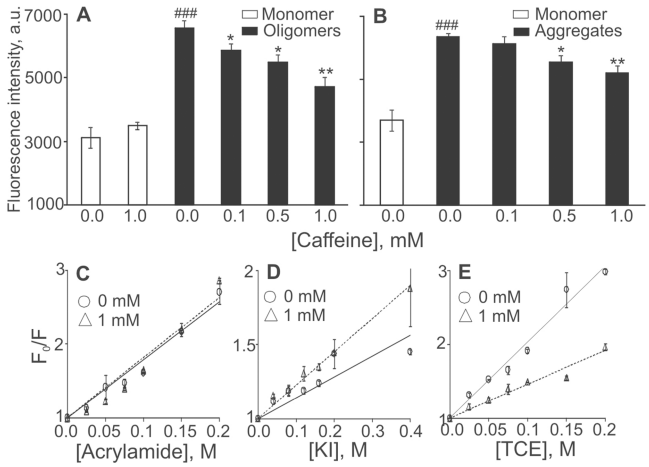

Figure 3. Estimation of the level of reactive oxygen species in RBCs pretreated with aggregates formed after (A) 210 h (early log phase) and (B) 410 h (saturation phase). DCFH-DA assay was used to determine the amount of ROS produced in RBCs after incubating the cells with monomeric (0 h) or oligomeric/aggregated α-synuclein for 2 h at 37 °C. Data represent the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ against ROS produced by oligomeric/aggregated α-synuclein in the presence of 0 mM caffeine; ###, $p < 0.001$ against monomeric α-synuclein (in the absence of caffeine). Quenching of intrinsic tyrosine fluorescence in α-synuclein was measured in the absence and presence of (C) acrylamide, (D) potassium iodide (KI), and (E) 2,2,2-trichloroethanol (TCE). In the case of KI, the varying ionic strengths were adjusted with NaCl. Stern−Volmer plots are shown for monomeric α-synuclein in the absence and presence of 1 mM caffeine. F_0/F describes the ratio of fluorescence intensities in the absence and presence of quencher. Data represent the mean ± SEM of three independent experiments.

because of the cellular stress pathway being disrupted in mammalian cells by membrane-bound α-synuclein at higher concentrations. Overexpression of wild-type or A53T α-synuclein caused the release of cytochrome c from mitochondria.[56] Expression of A53T α-synuclein alone led to elevated intracellular ROS.[57] In the presence of caffeine, α-synuclein monomer showed a similar level of ROS as compared to that with the untreated monomer, suggesting that caffeine does not

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play any role in ROS generation in RBCs (Figure 3A). α-Synuclein aggregates formed at the end of the nucleation phase (210 h) were selected as the fraction with maximum soluble oligomers, whereas samples in the stationary phase (410 h) were considered to be the fraction with the maximum amount of mature aggregates (Figure 1A). Samples formed at the end of the nucleation phase (210 h), which are mostly oligomers, showed a significant increase in ROS levels when compared with those from the monomer. The level of ROS generated due to oligomers formed in the presence of caffeine was significantly lower than that with oligomers formed in its absence (Figure 3A). The reduction in ROS levels in treated samples was found to depend on the concentration of caffeine. The maximum reduction in the level of ROS was observed with 1 mM caffeine (Figure 3A). Mature aggregates (410 h) also showed an increased level of ROS as compared to that with the monomer (Figure 3B). Treatment of RBCs with aggregates formed in the presence of caffeine showed a significant decrease in the production of intracellular ROS. This reduction was dependent on the concentration of caffeine. Mature aggregates formed in the presence of 1 mM caffeine showed the lowest amount of intracellular ROS when compared with that from aggregates formed in the absence of caffeine (Figure 3B). This indicated that caffeine induced a change in the nature of the aggregates, which exerted an ameliorative effect on intracellular oxidative stress.

Interaction of Caffeine with α-Synuclein. α-Synuclein forms a mixture of aggregates with fibrillar and amorphous species. Results obtained from studies on aggregation kinetics, conformation, morphology, and detergent sensitivity of aggregates suggested that caffeine induced conformational changes in α-synuclein. The presence of caffeine favored the enrichment of an aggregate population with amorphous forms, with reduced toxicity. Although they are still SDS-insoluble, they were less toxic than the aggregates that were mainly fibrillar, which were formed in the absence of caffeine. The secondary structure of α-synuclein was monitored in the presence of caffeine to measure any major conformational change in the protein. Far-UV CD spectra suggested that caffeine did not induce any significant conformational change in the secondary structure of α-synuclein (Figure S3A). To explore whether any direct interaction between caffeine and α-synuclein occurred, the binding of caffeine with α-synuclein was analyzed by RP-HPLC. The amount of caffeine in the unbound fraction was analyzed after the completion of α-synuclein aggregation in the presence of 1 mM caffeine (410 h). The amount of caffeine in the supernatant at the start of the incubation (0 h) was found to be 181.06 ± 1.89 μg mL^{-1}, whereas it was 184.69 ± 0.81 μg mL^{-1} after 410 h of incubation, which is similar (p = 0.22) to that in the starting concentration (Figure S3B). This binding analysis indicated that caffeine remained largely unbound, possibly ruling out any direct binding and/or complexation with α-synuclein. However, the possibility of transient interaction cannot be ignored. One reason to consider transient-type interactions is because of the mechanism of binding of intrinsically disordered proteins (IDPs) with their target partners.[58] IDPs show very rapid association with their target molecules and also dissociate very rapidly upon completion of their task.

α-Synuclein contains four tyrosine residues at positions 39, 125, 133, and 136 and does not contain any tryptophan. The intrinsic fluorescence of tyrosine could be an ideal tool to assess the interaction of caffeine with α-synuclein. Quenching of tyrosine fluorescence has been used to measure conformational changes in the protein[59] and can provide information about the relative compactness of a protein's structure. Addition of the neutral quencher, acrylamide (AM), to α-synuclein samples showed a significant reduction in tyrosine's fluorescence intensity. A Stern−Volmer plot showed a linear relationship between the concentration of quencher and the quenching effect (Figure 3C), which suggested that the quenching process is dynamic in nature.[59] In the presence of caffeine, α-synuclein showed a linear Stern−Volmer plot, similar to that of α-synuclein alone. Stern−Volmer constants (K_{SV}) were calculated to be 7.83 M^{-1} for α-synuclein alone and 8.13 M^{-1} for α-synuclein in the presence of 1 mM caffeine. The low but increased value of K_{SV} for the latter indicated that tyrosine residues were more accessible to the quencher in the presence of caffeine than they are in α-synuclein alone. A significant reduction in fluorescence intensity was also observed in the presence of the ionic quencher, potassium iodide (KI). A linear relationship was observed in the Stern−Volmer plot (Figure 3D), which indicated a collisional process of quenching in the presence of KI. Values of K_{SV} were 1.40 M^{-1} for α-synuclein alone and 2.24 M^{-1} for α-synuclein treated with 1 mM caffeine. Thus, tyrosine molecules remained easily accessible to the ionic quencher. As iodide is negatively charged and is a hydrated molecule, it interacts mainly with the surface residues of a folded protein.[59] The stronger quenching of fluorescence intensity in caffeine-treated α-synuclein by KI suggested that a conformational change in α-synuclein due to the presence of caffeine allowed the tyrosine residues to be more easily accessible to the quencher.

To study the hydrophobic regions in the vicinity of tyrosine residues in α-synuclein, 2,2,2-trichloroethanol (TCE) was used as the quencher. A linear relationship in the Stern−Volmer plot was also observed in the presence of caffeine (Figure 3E). K_{SV} values for α-synuclein alone and in the presence of caffeine were 10.23 and 4.57 M^{-1}, respectively. The higher K_{SV} value for α-synuclein alone indicated that tyrosine residues near the hydrophobic pockets remained easily accessible to TCE. The lower amount of quenching in the presence of caffeine suggested that the three-dimensional structure of α-synuclein underwent a change such that the tyrosine residues were shielded from the hydrophobic quencher and were not accessible to it, resulting in an increased retention of fluorescence intensity of the protein, especially at lower concentrations of the quencher. The higher K_{SV} values for α-synuclein treated with caffeine in quenching experiments with AM and KI indicated that caffeine induced conformational changes in α-synuclein, which allowed tyrosine residues to remain easily accessible to polar (AM) and ionic (KI) quenchers. Similarly, the reduction in quenching observed in the presence of caffeine confirmed that tyrosine remained inaccessible to the nonpolar quencher, TCE. A comparison of the data from the three quenchers indicated minor but significant rearrangement of the three-dimensional structure of α-synuclein, especially its hydrophobic residues surrounding tyrosines, in the presence of caffeine. It is interesting to note that none of the tyrosine residues, whose proximal residues undergo conformational changes, is present in the nonamyloid component (NAC) region (residues 60−95) of α-synuclein, which is important in the fibrillation of the protein.[60,61] Thus, the flanking sequences are also important in the aggregation of the protein.

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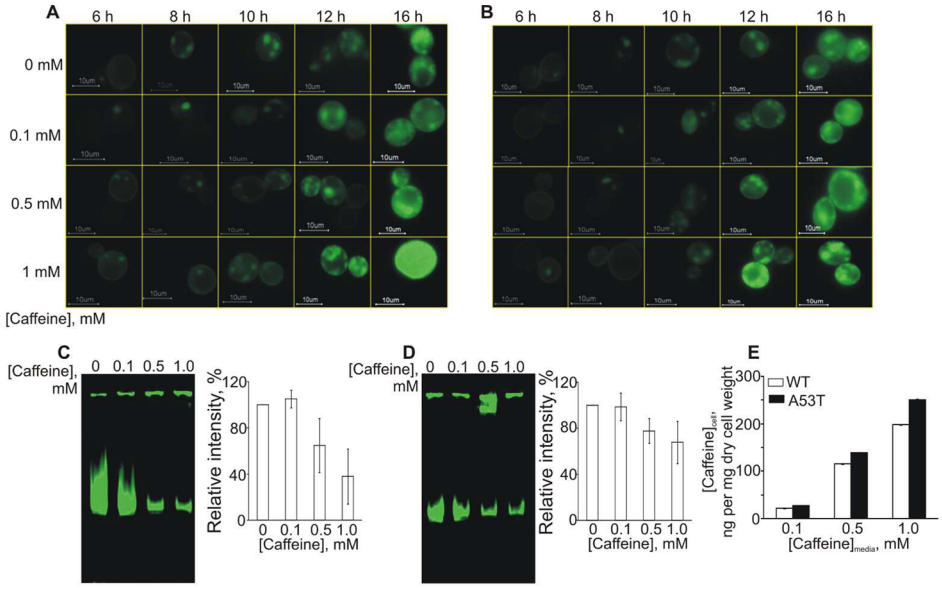

Figure 4. Expression of (A) WT and (B) A53T α-synuclein monitored in *S. cerevisiae* cells. Fluorescence micrographs of cells were recorded after different periods of induction in the presence of varying concentrations of caffeine. Bar = 10 μm. Solubility of (C) WT and (D) A53T α-synuclein expressed in cells grown for 16 h at 30 °C in the absence and presence of caffeine monitored by native PAGE analysis. Soluble fractions of cell lysates were loaded on the gels. Densitometric analysis of the band for the soluble protein was carried out using ImageQuant TL software (GE Healthcare). Relative intensity was calculated by assigning the intensity of the band for the protein expressed in the absence of caffeine after 16 h in each case as 100%. Data represent the mean ± SEM of three independent experiments. (E) Determination of the amount of caffeine taken up by yeast cells expressing WT or A53T α-synuclein. Cells were grown in the presence of different concentrations of caffeine, as indicated. Data represent the mean ± SEM of three independent experiments.

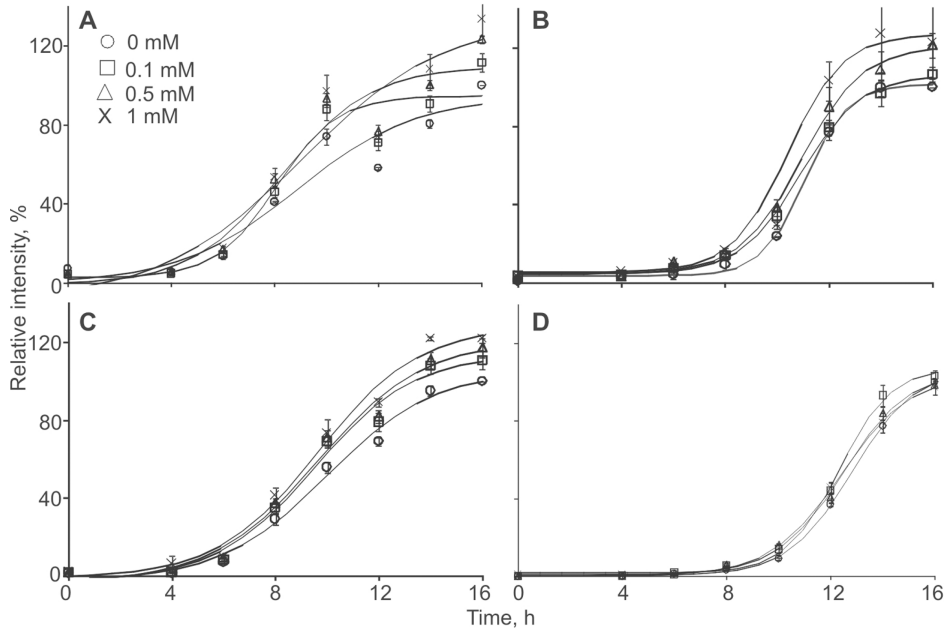

Figure 5. Determination of detergent solubility of aggregates formed by (A, B) WT and (C, D) A53T α-synuclein in yeast cells grown in the absence and presence of caffeine. Cell lysis was carried out in the presence of lysis buffer without (A,C) and with (B, D) 0.2% SDS, and a filter retardation assay was performed. Densitometry of the resulting dots (Figure S5) was carried out using ImageQuant TL software (GE Healthcare). Relative intensity was calculated by assigning the intensity of the dot obtained in the presence of 1 mM caffeine at 16 h in each case (absence and presence of SDS, WT, and A53T α-synuclein) as 100%. Data represent the mean ± SEM of three independent experiments.

Effect of Caffeine on Preformed Aggregates. Remodelling preformed aggregates to less toxic conformations has been reported in the presence of certain molecules and is considered to be an effective mechanism to combat protein aggregation.[62,63] To determine if caffeine can reverse the aggregation process, preformed mature α-synuclein aggregates (410 h) were incubated with caffeine for 48 h at 37 °C, and the resulting change in Th T fluorescence intensity was measured. No change was observed in treated samples as compared to aggregates formed without caffeine (Figure S4). This confirmed that caffeine was not able to dissociate preformed aggregates of α-synuclein.

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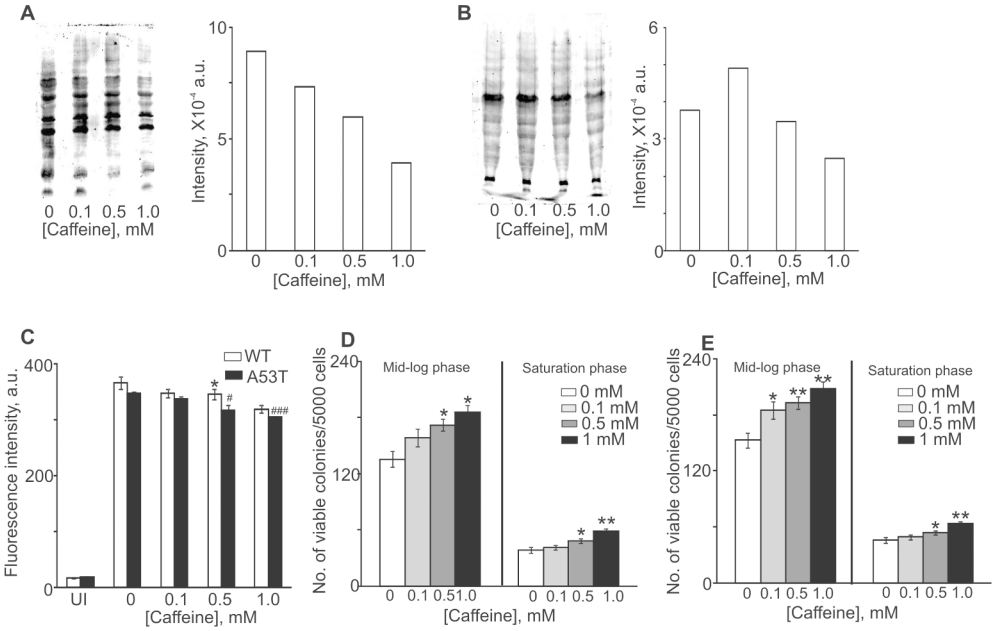

Figure 6. Extent of oxidative damage to cellular proteins in cells expressing WT α-synuclein after (A) 10 h (mid log phase) and (B) 16 h (saturation phase) of aggregation in the presence of different concentrations of caffeine monitored using an anti-DNP antibody as the primary antibody. Densitometric analysis of whole lanes was carried out using ImageQuant TL software (GE Healthcare). (C) Sytox Orange assay to determine toxicity in yeast cells expressing WT or A53T α-synuclein in the presence of different concentrations of caffeine. Toxicity in uninduced (UI) cells is shown for comparison. Data represent the mean ± SEM of three independent experiments. *, $p < 0.05$ against cells expressing WT α-synuclein in the absence of caffeine. ###, $p < 0.001$; #, $p < 0.05$ against cells expressing A53T α-synuclein in the absence of caffeine. Viability of yeast cells expressing (D) WT or (E) A53T α-synuclein in the absence and presence of caffeine. Cells were withdrawn after 10 h (mid log phase) or 16 h (saturation phase) and plated on SC-URA + 2% dextrose plates. Data represent the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ against corresponding yeast cells expressing α-synuclein in the absence of caffeine.

Expression of WT and A53T α-Synuclein in *S. cerevisiae* Cells. Time-dependent expression of WT and A53T α-synuclein in yeast cells was studied in the presence of caffeine by fluorescence microscopy. Intracellular α-synuclein aggregates were observed as fluorescent foci in the cytoplasm after 8 h of induction. In all cases (treated and untreated), WT α-synuclein expression followed a similar pattern of initial localization to the membrane followed by intracellular inclusion formation after 8 h of induction (Figure 4A). Expression of WT α-synuclein was found to be similar between caffeine-treated and untreated cells, i.e., caffeine did not alter the aggregation pattern of α-synuclein. A53T α-synuclein followed a similar pattern of expression to that of WT α-synuclein in caffeine-treated and untreated samples (Figure 4B).

Native PAGE was performed to monitor changes in the aggregation of α-synuclein. The presence of caffeine decreased the amount of soluble WT α-synuclein after 16 h of induction (Figure 4C). Caffeine-treated samples showed a decrease in the amount of soluble monomeric WT α-synuclein in a concentration-dependent manner. Quantification by densitometry revealed that the lowest amount of soluble protein was found in the presence of 1 mM caffeine (Figure 4C). A53T α-synuclein showed a similar trend in the presence of caffeine, which was confirmed by densitometry (Figure 4D).

Uptake of Caffeine by Yeast Cells. Before studying any effect of caffeine on the aggregation of α-synuclein (WT and A53T) in yeast cells, the ability of *S. cerevisiae* cells to take up caffeine from the media was determined. Yeast cells expressing WT and A53T α-synuclein were incubated with 0.1, 0.5, and 1 mM caffeine for up to 16 h of induction. The amount of caffeine in the samples was determined by RP-HPLC. The availability of caffeine inside the cells was found to increase in a concentration-dependent manner (Figure 4E). The amount of caffeine taken up by yeast cells was found to be 22.27, 115.50, and 197.61 ng per mg of dry cell weight, respectively, for 0.1, 0.5, and 1 mM caffeine (Figure 4E). The amount of caffeine taken up by yeast cells expressing A53T α-synuclein was 27.05, 138.84, and 250.55 ng per mg of dry cell weight, respectively, for 0.1, 0.5, and 1 mM caffeine treatment (Figure 4E).

Effect of Caffeine on Aggregation of α-Synuclein in Yeast Cells. The aggregation of α-synuclein was followed by a filter retardation assay for up to 16 h of induction. Initially, no aggregation was observed (Figure S5). Beyond 6 h, aggregation was found to increase with induction time (Figure 5A). Caffeine-treated and untreated samples showed a time-dependent pattern of aggregation, with the maximum aggregation occurring at 16 h of induction. Treatment with caffeine increased the aggregation of WT α-synuclein (Figure S5). Densitometric analysis showed three distinct stages of amyloid-specific aggregation, viz., nucleation, growth, and saturation phases (Figure 5A). In the initial lag phase, no aggregates were observed (up to 6 h); a subsequent exponential growth phase was seen with increased retention of aggregates, followed by a final equilibrium phase, indicating the saturation phase of fibrillation (16 h). To differentiate between detergent-insoluble aggregates and soluble oligomers, samples were treated with the anionic detergent, SDS, and filtered on a cellulose acetate membrane. Detergent-insoluble aggregates were observed after 10 h of induction. The extent of aggregation was found to increase with time, with maximum aggregation occurring at 16 h (Figure S5). Similar to results obtained with *in vitro* aggregation (Figure 2C), formation of

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detergent-insoluble aggregates was higher in caffeine-treated samples (Figure 5B), suggesting that caffeine increased the rate of conversion of oligomers to mature aggregates. Densitometry data were fitted into a sigmoidal curve to analyze the kinetics of aggregation and showed a longer lag phase for the formation of SDS-insoluble aggregates as compared to that for the formation of aggregates in the absence of detergent (Figure 5B).

Similar to WT α-synuclein, aggregation of A53T α-synuclein was studied by a filter retardation assay. Aggregation of A53T α-synuclein in caffeine-treated and untreated samples was found to increase with the time of induction, and maximum aggregation was observed at 16 h of induction (Figure S5). Aggregation of A53T α-synuclein was found to be higher in caffeine-treated samples when compared with that of the untreated sample at the end point (Figure 5C). The total aggregates formed increased as the concentration of caffeine increased from 0.1 to 1 mM. Densitometric analysis showed that caffeine did not alter the lag time of nucleation for A53T α-synuclein in a significant manner but accelerated the formation of mature aggregates (Figure 5C).

The detergent solubility pattern of aggregates formed by A53T α-synuclein was similar to that of WT α-synuclein. A higher fraction of detergent-insoluble aggregates was formed in the presence of caffeine (Figure 5D), although the effect was not as pronounced in this case as it was with WT α-synuclein. We have observed a similar increase in the rate of formation of mature aggregates of α-synuclein in the presence of caffeine metabolites theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) (Patel, Kardani, and Roy, unpublished results), indicating that the xanthine scaffold may be important in modulating the conformation of α-synuclein and in altering its aggregation properties.

Effect of Caffeine on α-Synuclein Aggregation-Induced Cellular Protein Oxidation. Protein aggregation can lead to severe deleterious effects in the cell, such as damage to organelles, oxidative stress, improper functioning of the proteasome system, and impairments in normal cellular homeostasis and functions.[1,2] Oxidative stress initiates damage to the cellular proteome by formation of protein carbonyls, and this has been used as a cellular biomarker of damage due to oxidative stress.[64] Formation of carbonyls in yeast cells expressing WT α-synuclein was monitored after 10 h of induction. Densitometric analysis showed reduction in the overall protein carbonylation level in yeast cells expressing WT α-synuclein in the presence of caffeine (Figure 6A). The lowest protein carbonyl level was found in yeast cells expressing WT α-synuclein in the presence of 1 mM caffeine. Similar to the carbonyl level in yeast cells expressing WT α-synuclein, carbonyl groups were found to be lower in cells expressing A53T α-synuclein treated with caffeine (data not shown).

To study the formation of protein carbonyls in the presence of mature aggregates, oxidative damage to the proteome in yeast cells was also analyzed after 16 h of induction. The signal was negligible in uninduced yeast cells, whereas significant oxidative damage was observed in yeast cells expressing WT α-synuclein (Figure 6B). The presence of a very low level of protein carbonyls in uninduced yeast cells suggested that the observed protein carbonyls in induced cells were mainly because of aggregation of α-synuclein, which induced oxidative stress in the cells. Densitometric analysis of bands obtained in caffeine-treated cells revealed reduction in the level of protein carbonylation (Figure 6B). Yeast cells expressing A53T α-synuclein also showed the formation of protein carbonyls after 16 h of induction (data not shown). This again confirmed the ameliorative effect of caffeine toward the formation of the less toxic protein species with beneficial effects toward cell survival.

Effect of Caffeine on Viability of Yeast Cells Expressing α-Synuclein. Expression of α-synuclein in yeast cells is reported to affect their normal growth and reduce the viability of these cells.[65] The viability of yeast cells transformed with the empty vector (*α-SYN*-deleted) and grown for 16 h was found to remain unaltered in the presence of 1 mM caffeine (Figure S6). Thus, the presence of caffeine had no effect on the viability of the yeast cells up to 1 mM concentration. The viability of cells expressing WT (Figure 6C) and A53T (Figure 6D) α-synuclein was studied at mid log phase (10 h) and stationary phase (16 h). As the time of induction increased from 10 to 16 h, the viability of the cells was found to decrease significantly in all cases. This may be due to the fact that overexpressed α-synuclein is itself toxic to the cell,[56,57] as was observed in the case of increased oxidative stress in RBCs (Figure 3A). The cell is unable to recover from the toxic challenge of oligomers formed at the initial stages of aggregation. An increase in viability was observed at both time points in cells treated with caffeine as compared to that for untreated cells (Figures 6C,D), and this effect was dependent on the concentration used. As caffeine did not have any effect on the viability of yeast cells *per se* (Figure S6), the increased viability was likely due to the less toxic nature of α-synuclein aggregates formed in the presence of caffeine. As the concentration of caffeine increased from 0.1 to 1 mM, a significant improvement in viability was observed in cells at mid log phase (10 h) as well as at stationary phase (16 h) of aggregation. The higher viability of yeast cells expressing A53T α-synuclein (Figure 6D), as compared to that of cells expressing WT α-synuclein (Figure 6C), could be correlated with the observed higher uptake of caffeine by cells expressing A53T than by those expressing WT α-synuclein (Figure 4E). This observation suggested that the availability of caffeine in the yeast cytoplasm was a key determinant of the kinetics of α-synuclein aggregation and its consequent effect on cellular survival. We are unable to understand why caffeine uptake should be higher in cells expressing A53T α-synuclein. Although A53T α-synuclein is reported to aggregate faster than WT α-synuclein,[66] this has not be correlated conclusively with any difference in toxicity of the two protein variants to date. Our studies showed that caffeine was able to attenuate the toxicity of WT and A53T α-synuclein with equal efficiency. For analysis of the dead cell population, yeast cells were treated with the fluorescent dye, Sytox Orange. The dye selectively stains dead cells by being incorporated into their DNA, whereas live cells are relatively impermeable to the dye.[67] The population of dead cells was significantly lower in caffeine-treated cells expressing WT α-synuclein for 16 h as compared to that of the untreated control (Figure 6E). Taken together with the increased viability of cells expressing α-synuclein in the presence of caffeine, this result showed that the faster conversion of the more toxic oligomers [possibly by generating higher oxidative stress (Figure 3A)] to the less toxic mature aggregates [via decreased oxidative stress (Figure 3B)] in the presence of caffeine might partially explain the beneficial role of caffeine in PD-induced pathology.[11,14−17]

Effect of Caffeine on Morphology of α-Synuclein Aggregates Formed in Yeast Cells. As observed in *in vitro* and yeast cell studies, caffeine likely altered the nature of aggregates formed by α-synuclein and reduced the concen-

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tration of the more toxic oligomers. Immunogold staining was carried out to study the morphology of WT α-synuclein aggregates formed in the presence of caffeine. No gold particles were seen when the cells were transformed with the empty vector (α-*SYN*-deleted) (Figure 7). Fibrillar structures were

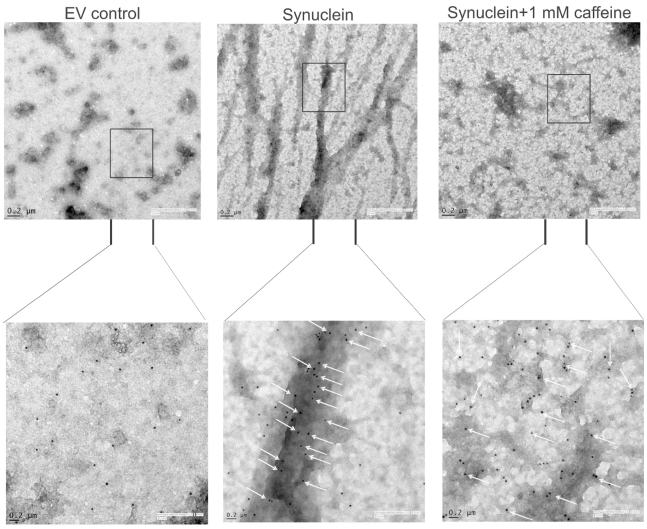

Figure 7. Electron micrographs of WT α-synuclein aggregates formed in yeast cells in the presence of 1 mM caffeine following immunogold staining. The first panel shows the electron micrograph of the empty vector (EV) control. Lower panels show an expanded view (11 500× magnification) of the area (indicated by a square) in the corresponding upper panels (3500× magnification). Arrows indicate the presence of gold-conjugated secondary antibodies. Bar = 0.2 μm.

clearly seen in the case of aggregates formed by WT α-synuclein expressed in the absence of caffeine. In caffeine-treated cells, on the other hand, a mixture of amorphous and fibrillar aggregates was observed (Figure 7). Thus, caffeine increased the rate of aggregation and altered its course to form a mixture of detergent-insoluble fibrillar and amorphous aggregates that were less toxic to the cell than the mainly fibrillar aggregates formed in the absence of caffeine. Similar aggregates were seen when yeast cells expressed A53T α-synuclein (data not shown).

CONCLUSIONS

Some recent reports indicate that the process of fibrillation, rather than the formation of fibrils, is toxic to the cell.[1,73] In support of this, we find that once aggregation has commenced in yeast cells the cell is unable to overcome the resulting toxicity due to intermediate oligomers even as the pathway moves toward completion, i.e., formation of mature aggregates. The presence of caffeine has an ameliorative effect in that it attenuates the toxicity at similar time points. Any molecule that can facilitate the completion of aggregation will have a dampening effect on cytotoxicity. As caffeine reduces the lag time and accelerates the apparent rate of fibrillation of α-synuclein, the action of caffeine seems to favor this hypothesis. More than any other protein that is misfolded in neurodegenerative disorders, α-synuclein has the ability to assume alternate aggregation pathways due to its natively disordered structure. This seems to be heightened by the presence of caffeine. Our results offer a plausible explanation for the observations from epidemiological studies that coffee consumption is inversely related to the risk of onset of PD.

METHODS

Materials. Mouse anti-α-synuclein monoclonal, FITC-conjugated goat anti-mouse, goat anti-mouse IgG-gold, and FITC-conjugated goat anti-rabbit antibodies were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., Bengaluru, India. Rabbit anti-2,4-dinitrophenol antibody was obtained from Molecular Probes, Invitrogen, USA. 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was a product of Cayman Chemical Company and was purchased from Genetix Biotech Asia Pvt. Ltd., New Delhi, India. Caffeine was purchased from Himedia, Mumbai, India. PIP Strip membrane and Sytox Orange were purchased from Invitrogen Bioservices India Pvt. Ltd., Bengaluru, India. Cellulose acetate membrane (0.2 μm) was purchased from Sartorius AG, Goettingen, Germany. *S. cerevisiae* strain W303-1B (MATα *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11*,15) was a product of Open Biosystems and was purchased from SafLabs, Mumbai, India. All other reagents and chemicals used were of analytical grade or better.

Aggregation of Recombinant α-Synuclein. Recombinant human α-synuclein was purified as per a reported protocol.[34,35] The aggregation assay was set up as described previously.[35] Samples of α-synuclein (7 mg mL^{-1}) were incubated at 37 °C in the presence of different concentrations of caffeine (0.1, 0.5, and 1 mM). At predefined time intervals, aliquots were withdrawn and biophysical characterization of the sample was carried out by different methods.

Thioflavin T Assay. Final concentrations of protein and Thioflavin T (Th T) were 10 and 20 μM, respectively. Fluorescence emission intensity of the dye was recorded at 485 nm with excitation at 440 nm, with slit widths of 5 and 10 nm for excitation and emission, respectively. The emission intensity of the Th T solution in the presence of buffer (with or without caffeine) was subtracted as blank. Aggregation kinetics was followed by fitting the data using the following equation[66]

$$y = y_i + mx_i + \frac{y_f + mx_f}{1\left|e^{\frac{x - x_0}{\tau}}\right.}$$

where $(y_i + mx_i)$ is the initial line, $(y_f + mx_f)$ is the final line, and x_0 is the midpoint of maximum signal. The apparent rate constant (k_{app}) is $1/\tau$, and lag time is calculated to be $x_0 - 2\tau$.

1-Anilinonaphthalene-8-sulfonic Acid Assay. Aliquots were withdrawn at defined time intervals and added to a 1-anilinonaphthalene-8-sulfonic acid (ANS) solution (5 mM) such that the molar ratio of protein to dye was 1:10. The increase in fluorescence emission intensity of the dye at 460 nm was recorded at an excitation wavelength of 360 nm, with excitation and emission bandwidths of 5 and 10 nm, respectively. The emission spectrum of the dye alone was subtracted from each spectrum.

Congo Red Spectral Shift Assay. The method used was described previously.[43] Aliquots of α-synuclein were incubated with Congo Red (CR) at a molar ratio of 1:5 for 30 min. Absorption spectra were recorded in the range 400−700 nm. The shift in the spectrum was recorded by subtracting the spectrum of CR alone from the spectrum of CR with α-synuclein samples.

Far-UV Circular Dichroism Spectroscopy. Far-UV circular dichroism (CD) spectra of incubated α-synuclein samples (5 μM) were collected on a spectropolarimeter (J-815, Jasco). Spectra were recorded in the wavelength range 200−240 nm in a cuvette with a 0.1 cm path length. The average of three scans was recorded for each spectrum, and the spectrum of buffer was subtracted from that of the protein.

Dynamic Light Scattering Studies. Particle size distribution of incubated α-synuclein samples (5 μM) was measured by a Zetasizer (Malvern Instruments). Peaks below 0.1 nm and above 1000 nm were excluded from analysis.

Filter Retardation Assay. Protein samples were incubated without and with 0.5% SDS and filtered through a cellulose acetate membrane (0.2 μm) using a dot blot apparatus (Whatman Schleicher & Schuell) followed by overnight blocking with 10% skim milk. The membrane was incubated with a mouse α-synuclein monoclonal

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antibody (1:5000) followed by a goat anti-mouse FITC-conjugated antibody (1:2000) and scanned on an image scanner (Typhoon TRIO, GE Healthcare) at excitation and emission wavelengths of 488 and 526 nm, respectively.

Transmission Electron Microscopy. Aliquots were withdrawn at different time intervals and centrifuged at 15 000*g* for 30 min, and the pellet was resuspended in deionized water. Samples (10 μL) were placed on carbon-coated copper grids, allowed to dry for 15 min, negatively stained with 2% uranyl acetate, and viewed under an electron microscope (TF20, FEI).

Protein−Lipid Interaction Assay. Protein samples were centrifuged at 1 00 000*g* for 1 h, and the resulting pellets were resuspended in 20 mM Tris HCl, pH 7.8. The overlay assay was performed as per the manufacturer's instructions. Briefly, the aggregate suspension was incubated with a preblocked (3% BSA) PIP Strip membrane for 4 h at room temperature, followed by sequential incubation with a mouse α-synuclein antibody (1:5000) and a goat anti-mouse FITC-conjugated antibody (1:2000). The bound protein was detected by scanning the membrane on a gel scanner as described above.

Measurement of Reactive Oxygen Species. All experiments were performed with prior approval from the Institutional Animal Ethics Committee (IAEC/13/21). Rat red blood cells (RBCs) were washed thrice with isotonic phosphate buffered saline (PBS), pH 7.4, and counted using a hemocytometer. RBCs (7×10^6) were incubated with α-synuclein aliquots (2.4 μM), withdrawn after different periods of incubation, at 37 °C and 15 rpm for 2 h. After the incubation was complete, cells were washed with PBS and intracellular ROS was measured using DCFH-DA assay.[55] Cells were incubated with 10 μM DCFH-DA and 50 μM H_2O_2 for 30 min at room temperature, and the fluorescence intensity was measured at 519 nm following excitation of the samples at 504 nm.

RP-HPLC Analysis. Caffeine content determination in the samples was done by RP-HPLC analysis. Aliquots were centrifuged at 1 00 000*g* for 30 min, and supernatants were injected into a Zorbax C-18 analysis column (Agilent Technologies). The eluate was monitored at 254 nm using a photodiode array (PDA) detector. The mobile phase was a mixture of acetonitrile and 0.1% formic acid (aqueous) at a ratio of 10:90 (v/v) and a flow rate of 1 mL min^{-1}.[68] For determination of caffeine uptake by yeast cells, cells were lysed as described below, and clarified cell lysates were injected into the column.

Fluorescence Quenching Studies. α-Synuclein (7 mg mL^{-1}) was incubated with 1 mM caffeine at 37 °C for 1 h. A final concentration of 10 μM for the protein and different concentrations of quenchers were used. Fluorescence emission intensity of tyrosine was recorded at 305 nm following excitation at 285 nm, at slit widths of 5 nm for both excitation and emission. Fluorescence quenching data were analyzed using the Stern−Volmer equation[59] $[F_0/F = 1 + K_{SV}[Q]]$, where F_0 is the fluorescence intensity in the absence of the quencher, F represents fluorescence intensity in the presence of the quencher, K_{SV} is the Stern−Volmer constant, and [Q] is the concentration of the quencher.

Expression of WT and A53T α-Synuclein in Yeast Cells. *S. cerevisiae* cells were transformed as per a standard protocol.[69] Transformed cells were grown in SC-URA + 2% dextrose at 30 °C and 200 rpm. Expression of proteins was induced by the addition of 2% galactose[70] in the presence of different concentrations of caffeine. Expression of WT and A53T α-synuclein was monitored up to 16 h by fluorescence microscopy (Eclipse E600, Nikon) and native PAGE analysis. For the latter, cells were lysed by the glass beads method,[71] and the soluble fraction was loaded on a nondenaturing (12% cross-linked) polyacrylamide gel. The gel was scanned using an image scanner (Typhoon Trio, GE Healthcare) at excitation and emission wavelengths of 488 and 526 nm, respectively.

Yeast Cell Toxicity Assay. Induced cells were washed twice with 50 mM MES buffer, pH 5.5, and resuspended in 1 mL of MES buffer. The A_{600} was adjusted to 1.0, and samples were incubated with 1 μM Sytox Orange at 30 °C and 200 rpm for 30 min.[67] Fluorescence intensity of Sytox Orange was recorded at 570 nm with an excitation wavelength of 547 nm.

Cell Viability Assay. Induced cells (5×10^3) were withdrawn at different time periods, plated on SC-URA + 2% dextrose agar plates, and grown at 30 °C for 2 to 3 days. The number of viable cells was calculated by considering one colony forming unit as a single cell.

Immunoelectron Microscopy. Yeast cell lysates were centrifuged at 800*g* for 10 min, and the supernatants obtained were re-centrifuged at 1 00 000*g* for 1 h. The pelleted aggregates were applied on carbon-coated copper grids (200 mesh), dried for 10 min, and blocked with 10% BSA. The grid was incubated with a mouse α-synuclein antibody (1:300) for 2 h followed by incubation with a goat anti-mouse gold-conjugated antibody (1:50) (10 nm gold particles) for 2 h. The grid was negatively stained with 2% uranyl acetate, and samples were viewed under an electron microscope (TF20, FEI).

Estimation of Oxidative Damage. Protein samples from induced yeast cell lysates were separated by 12% SDS-PAGE followed by electroblotting on PVDF membrane (0.45 μm). Carbonyl groups in proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH).[72] The immunoblot was probed with a rabbit DNP antibody (1:2000) as the primary and a FITC-conjugated goat anti-rabbit antibody (1:2000) as the secondary antibody.

Statistical Analysis. All data are expressed as the mean ± standard error of mean (SEM) of three independent experiments and analyzed by Student's *t* test. Statistically significant difference was determined by a probability (p) value of less than 0.05.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1: Purification of recombinant human α-synuclein. Figure S2: Filter retardation assay to determine the detergent solubility of aggregates. Figure S3: Interaction of monomeric α-synuclein with caffeine. Figure S4: Effect of caffeine on dissociation of preformed aggregates. Figure S5: Filter retardation assay to determine the detergent solubility of WT or A53T α-synuclein aggregates formed in yeast cells. Figure S6: Effect of caffeine on viability of *S. cerevisiae* cells. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00158.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 91-172-229 2061. Fax: 91-172-221 4692. E-mail: ipsita@niper.ac.in.

Notes

The authors declare no competing financial interest.

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