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α-Synuclein Induces Unfolded Protein Response Via Distinct Signaling Pathway Independent of ER-membrane Kinases

Shin Jung Kang*, Ki Soon Shin[1] and Yunhee Kim Kwon[1]

Department of Molecular Biology, College of Natural Science, Sejong University, Seoul 143-747, Korea; [1]Department of Biology, College of Science, Kyung Hee University, Seoul 130-701, Korea

Abastract: Parkinson's disease (PD) is a neurodegenerative disease caused by selective degeneration of dopaminergic neurons in the substantia nigra. Mutations in α-synuclein have been causally linked to the pathogenesis of hereditary PD. In addition, it is a major component of Lewy body found in the brains of sporadic cases as well. In the present study, we examined whether overexpression of wild type or PD-related mutant α-synuclein induces unfolded protein response (UPR) and triggers the known signaling pathway of the resulting endoplasmic reticulum (ER) stress in SH-SY5Y cells. Overexpression of wild type, A30P, and A53T α-synuclein all induced XBP-1 mRNA splicing, one of the late stage UPR events. However, activation of ER membrane kinases and upregulation of ER or cytoplsmic chaperones were not detected when α-synuclein was overexpressed. However, basal level of cytoplsmic calcium was elevated in α-synuclein-expressing cells. Our observation suggests that overexpression of α-synuclein induces UPR independent of the known ER membrane kinase-mediated signaling pathway and induces ER stress by disturbing calcium homeostasis.

Key words: α-Synuclein, Parkinson's disease, unfolded protein response, ER stress

A hallmark of damaged neurons in many neurodegenerative diseases is the presence of abnormal aggregates of the disease-related proteins (Shastry, 2003). In Parkinson's disease (PD) characterized by selective loss of dopaminergic neurons in substantia nigra, an abnormal protein aggregate called Lewy body is observed in the disease-affected area, where α-synuclein is the major component. Overexpression of wild type α-synuclein or mutations in α-synuclein have been known to be genetic cause of PD (von Bohlen und Halbach et al., 2004).

The human α-synuclein gene encodes a small (140 amino acids) protein characterized by a remarkable conformational plasticity (Volles and Lansbury, 2003). Several independent mutations such as A30P, A53T, and E46K have been reported to be pathologically involved in PD (von Bohlen und Halbach et al., 2004). Neither wild type nor mutant α-synuclein folds into structured, globular forms in vitro (Volles and Lansbury, 2003). At high concentrations, wild type α-synuclein can self-aggregate to form fibrils and spherical assemblies and this process is accelerated in mutant α-synuclein (Conway et al., 1998). The mechanism by which abnormal α-synuclein oligomers or aggregates cause dopaminergic neuronal degeneration in substantia nigra is still unknown.

Accumulation of unfolded proteins in the ER lumen induces an adaptive program called unfolded protein response (UPR). The UPR unloads the stress by upregulating protein folding, protein degradation or slow down of protein synthesis (Kaufman, 1999). The proximal sensors of the UPR are PKR-like ER kinase (PERK), ATF6 and IRE1 (Rutkowski and Kaufman, 2004). PERK kinase phosphorylates eIF2α and thereby slows down protein translation, ATF6 is cleaved and translocated into the nucleus where functions as transcription factors for chaperones, and IRE1 induces XBP1 mRNA splicing and ultimately activates protein degradation (Rutkowski and Kaufman, 2004). When this adaptive program to reduce the level of unfolded proteins cannot unload the stress on ER, activation of Jun N-terminal kinase (JNK) and caspase-12 follows to eliminate cells with irreparable ER stress (Shen et al., 2004).

There is accumulating evidence that the UPR may play a role in the pathogenesis of both acute neurological diseases

*To whom correspondence should be addressed.
Tel: 82-2-3408-3943; Fax: 82-2-3408-3661
E-mail: sjkang@sejong.ac.kr

like brain ischemia and chronic neurodegenerative diseases like Alzheimer's disease (AD), Huntington's disease (HD) and PD (Paschen and Frandsen, 2001; Shen et al., 2004). Protein aggregates in many neurodegenerative diseases are localized to the nucleus or the cytoplasm, yet trigger the UPR and promote cell death (Shen et al., 2004). This may be due to the overload of proteosomal degradation system by the aggregates of misfolded/unfolded proteins and thereby suppressing ER-associated degradation (ERAD) of unfolded proteins.

Another aspect of ER stress is the perturbation of ER calcium homeostasis. The normal function of ER requires high concentrations of calcium within the ER lumen. Alterations in ER calcium also trigger UPR to restore the disturbed ER calcium homeostasis (Lehotsky et al., 2003).

In the present study, we examined whether overexpression of α-synuclein induces UPR and ER stress in human dopaminergic neuroblastoma, SH-SY5Y cells. XBP-1 mRNA splicing, one of the late stage UPR events and elevation of basal level of cytoplsmic calcium were detected in α-synuclein-overexpressing cells. However, activation of ER membrane kinases and upregulation of ER or cytoplsmic chaperones were not detected. Our observation suggests that overexpression of α-synuclein induces UPR independent of the known ER membrane kinase-mediated signaling pathway and induces ER stress possibly by disturbing calcium homeostasis.

MATERIALS AND METHODS

Plasmid construction

The construct of human α-synuclein (wild type) in pCDNA3.1 was a kind gift from Dr. P. Lansbury (HMS, Boston). A30P and A53T mutations were introduced using site-directed mutagenesis kit (Stratagene). The α-synuclein cDNAs were then subcloned into pEGFP-N3 or pDsRed expression vectors (Clontech). Mutations and the construction were confirmed by sequencing. ERAI construct was a kind gift from Dr. M. Miura (RIKEN, Japan).

Antibodies and other reagents

The primary antibodies used in this study are as follows: anti-eIF2-α rabbit monoclonal antibody (1 : 2000, Cell signaling), anti- eIF2-α phosphospecific (1 : 2000, Stressgen), anti-JNK rabbit monoclonal antibody (1 : 2000, Cell signaling), anti-active JNK(1 : 5000, Promega), anti-PERK (Thr980) rabbit polyclonal antibody (1 : 1000, Cell signaling), anti-phospho-PERK (Thr980) rabbit polyclonal antibody (1 : 1000, Cell signaling), anti-α-synuclein rabbit polyclonal antibody (1 : 1000, Stressgen), and anti-α-tubulin mouse monoclonal antibody (1 : 4000, Sigma). Other antibodies and chemicals were purchased from Sigma unless stated otherwise.

Cell culture and transfection

Human dopaminergic neuroblastoma cell line SH-SY5Y cells were maintained in Minimal essential medium (JBI) supplemented with 10% fetal bovine serum (JBI) in a 37°C CO_2 (5%) incubator. For the immunostaining, cells were grown on poly-L-lysine coated 12 mm coverslips in 24-well plates. Transient expression of each vector (1~2 μg of DNA/35 mm dish) in SH-SY5Y cells (2×10^5 cells/dish) was accomplished with Wellfect reagent according to the manufacturer's protocol (JBI). After 6 hr-incubation with transfection reagents, the medium was changed with fresh growth medium. Cell viability was monitored using standard dimethylthiazolyl carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay (Promega).

Immunoblot assay

SH-SY5Y cells (10^6/ml) grown on 60 mm dish were lysed by incubating in 2X SDS sample buffer and boiled at 95°C for 5 min. Total protein was subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred onto immobilon-P membrane (Millipore) and incubated with blocking solution containing 5% skim milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated with primary antibody in blocking solution at 4°C overnight. After washing three times with TBST for 10 min each, the membrane was incubated with HRP-conjugated anti-rat, anti-mouse or anti-rabbit IgG (Sigma) for 40 min at room temperature. After washing three times, the bound antibody was revealed using the ECL Western blotting reagent kit (Amersham).

Comparison of cytosolic free calcium level

Relative level of cytosolic free calcium was measured by spectrofluorometric methods (SpectraMax Gemini, Molecular Device) using calcium crimson dye (Invitrogen) as described by Lin and the colleagues (1999) with minor modifications.

Statistics

For the statistical analysis, all the experiments were repeated at least three times. The results were expressed as mean ± S.D. of at least three independent experiments, unless stated otherwise. Paired data were evaluated by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

We first examined if α-synuclein forms aggregates by itself when overexpressed in SH-SY5Y cells. As shown in Fig. 1A upper panels, neither wild type nor PD-related mutant α-synuclein fused to GFP or dsRed formed visible aggregates until 120 hr after the transfection. To examine whether GFP or dsRed tagging interfered with the aggregate

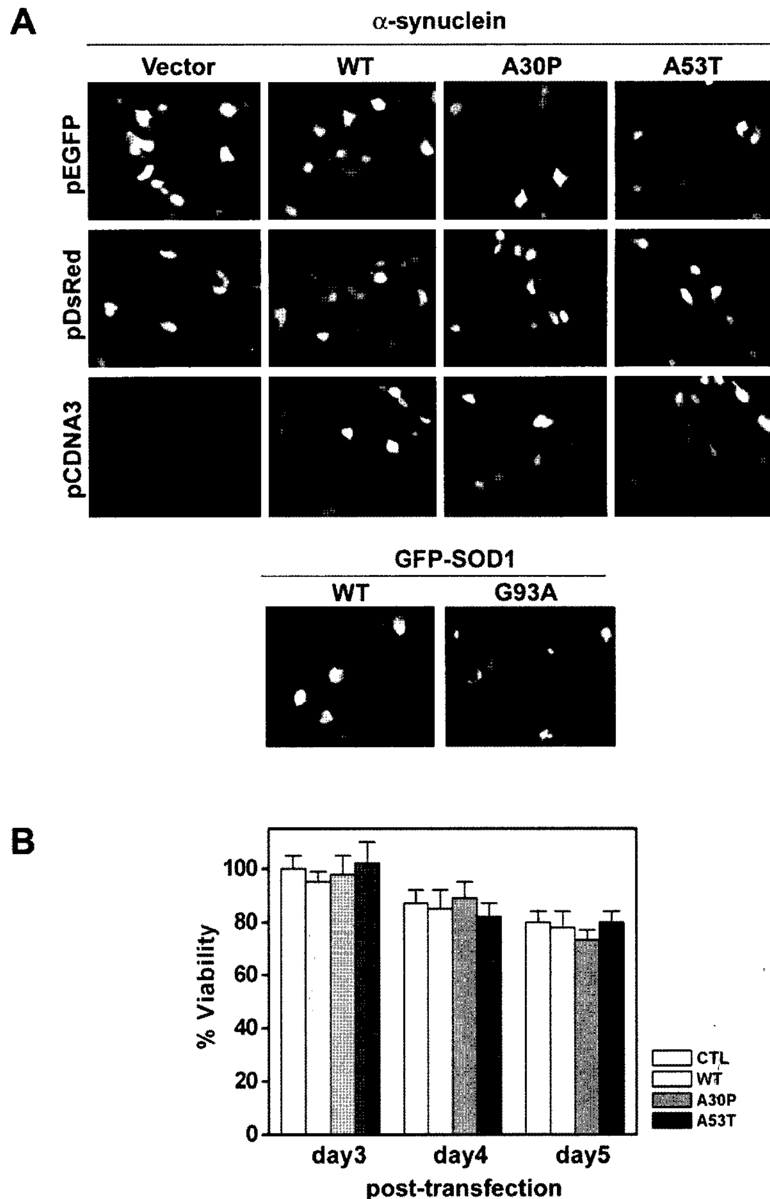

Fig. 1. Overexpression of α-synuclein alone does not induce aggregation or cell death. A. SH-SY5Y cells were transfected with α-synuclein fused to GFP or DsRed (wild type (WT), A30P or A53T mutant) or untagged α-synuclein (pCDNA3). α-synuclein did not form aggregates even at 120 hr after the transfection while G93A mutant SOD1 formed aggregates at 24 hr after the transfection. B. Viability of the α-synuclein-transfected cells were monitored by MTS assay.

formation of relatively small α-synuclein of 17 kDa, α-synuclein without any tag was overexpressed and the exogenous α-synuclein was visualized by immunostaining. As shown in Fig. 1A lower panels, α-synucleins of wild type and A30P, and A53T mutants without tags did not form aggregates, either. The level of α-synuclein expression was comparable to that of mutant SOD1 (G93A) that formed aggregates (Fig. 1A). These results suggest that α-synuclein may not form aggregates spontaneously even when its protein level is elevated.

To examine if the overexpression of α-synuclein alone induces cell death in SH-SY5Y, cell viability was measured by MTS assay. Neither wild type nor the PD-related mutant α-synuclein (A30P and A53T) induced cell death until 5 days in culture (Fig. 1B). Assessment of cell death by counting propidium iodide-positive cells among α-synuclein-expressing cells under fluorescent microscope showed the same result (data not shown). There have been conflicting observations on the death-inducing activity of α-synuclein. Mutant α-synuclein was found toxic in some reports (Oluwatosin-Chigbu et al., 2003; Volles and Lansbury, 2003; Dixon et al., 2005) or protective in others on the contrary (Albani et al., 2003; Mannig-Bog et al., 2003). These differences may be due to the different level

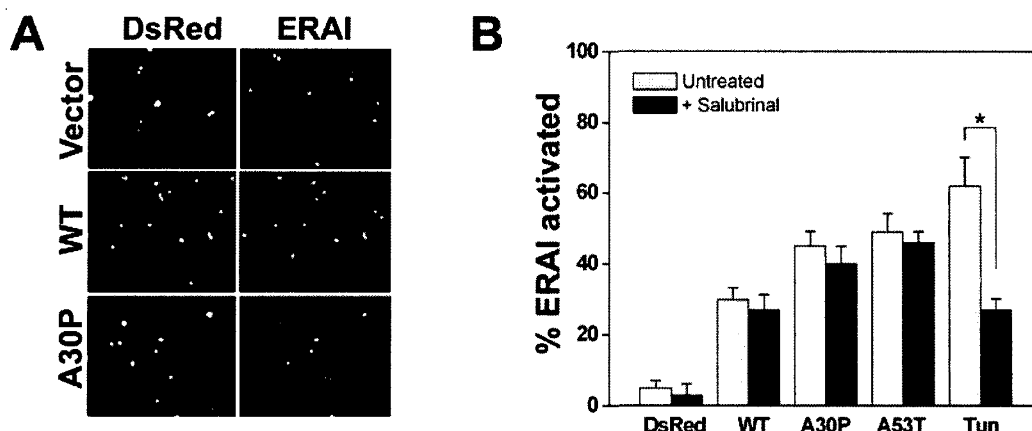

Fig. 2. Activation of ERAI, an ER stress indicator was observed by overexpression of α-synuclein. Transfection (48 hr) of DsRed-α-synuclein (wild type (WT), A30P, and A53T mutant) and tunicamycin (Tun, 1 μM, 24 hr) induced activation of ERAI. Co-treatment of eIF2α dephosphorylation inhibitor, salubrinal (20 μM) inhibited the activation of ERAI induced by tunicamycin but not by α-synuclein overexpression. Panel A shows representative micrographs of cells co-transfected with α-synuclein and ERAI construct. Percent of ERAI-positive cells in α-synuclein-positive cells were quantified in B. n = 3, *$p < 0.05$ tunicamycin vs. tunicamycin+salubrinal.

of overexpression or different cellular context. Our result implies that α-synuclein itself is not toxic whether it is mutated or not and additional cellular stress may be required to induce cell death.

A possible toxicity induced by α-synuclein has been attributed to its natively unfolded structure (Volles and Lansbury, 2003). Although it is not known whether it is synthesized in ER, functional association with ER has been observed (von Bohlen und Halbach et al., 2004). Thus it is possible that α-synuclein induces unfolded protein response (UPR) and this may be worse when α-synuclein is mutated. To examine whether α-synuclein induces UPR followed by ER stress, ER stress-activated indicator (ERAI) was monitored (Iwawaki et al., 2004). It is documented that the XBP-1 mRNA splicing occurs as a result of Ire1α activation in the late stage of the UPR and enhances the further transcription of many proteins involved in the UPR (Yoshida et al., 2001). Based on this phenomenon, Iwawaki and the colleagues (2004) developed ERAI construct composed of a part of XBP-1 cDNA fused with a GFP variant, Venus cDNA. Under normal conditions, Venus is not expressed but during ER stress when the XBP-1 mRNA undergoes splicing, a frame shift occurs in the ERAI mRNA and the Venus is expressed. Like XBP-1 protein, venus translocates into the nucleus to act as a transcription factor (Iwawaki et al., 2004). To examine whether XBP-1 mRNA splicing occurs in the α-synuclein expressing cells, ERAI and α-synuclein-pDsRed constructs were co-transfected in SH-SY5Y cells and the expression of the Venus was examined under a fluorescence microscope. As shown in Fig. 2, both wild type and mutant α-synuclein induced XBP-1 mRNA splicing as indicated by ERAI activation but the frequency of ERAI activation was significantly higher in the mutant expressing cells. Tunicamycin also activated ERAI, which was effectively suppressed by salubrinal, a recently screened ER stress inhibitor (Boyce et al., 2005). However, the activation of ERAI induced by overexpression of α-synuclein was not inhibited by salubrinal. This suggests that the UPR induced by α-synuclein overexpression may not involve activation of eIF2α-mediated signaling pathway since salubrinal suppress ER stress by inhibiting eIF2α dephosphorylation (Boyce et al., 2005).

Thus, we then examined whether overexpression of α-synuclein changes the PERK-eIF2α signaling. As can be seen in Fig. 3A, neither wild type nor mutant (A30P and A53T) synuclein induced phosphorylation of PERK or eIF2α. Overload of ER stress induces activation of JNK and caspase-12, leading to apoptosis of the stressed cells (Shen et al., 2004). As shown in Fig. 3A, overexpression of α-synuclein did not induce activation of JNK, either.

Then we examined whether chaperone proteins are upregulated by overexpression of α-synuclein. The level of Bip that is upregulated by accumulation of misfolded/ unfolded proteins in the ER lumen did not change by overexpression of wild type or mutant α-synuclein (Fig. 3B). The levels of cytoplasmic chaperones hsp25, hsp27 (αB-crystallin), and hsp70 did not change when α-synuclein of wild type or mutant was overexpressed (Fig. 3B).

Another sign of ER stress is the perturbation of ER homeostasis such as ER calcium depletion or elevation of cytoplasmic free calcium level (Shen et al., 2004). Basal levels of cytoplasmic calcium were compared between cells overexpressing wild type or mutant α-synuclein. As shown in Fig. 4, cells expressing α-synuclein showed elevated cytoplasmic calcium level compared to cells expressing empty vector when the calcium level was monitored in calcium-free media. The degree of elevated calcium level

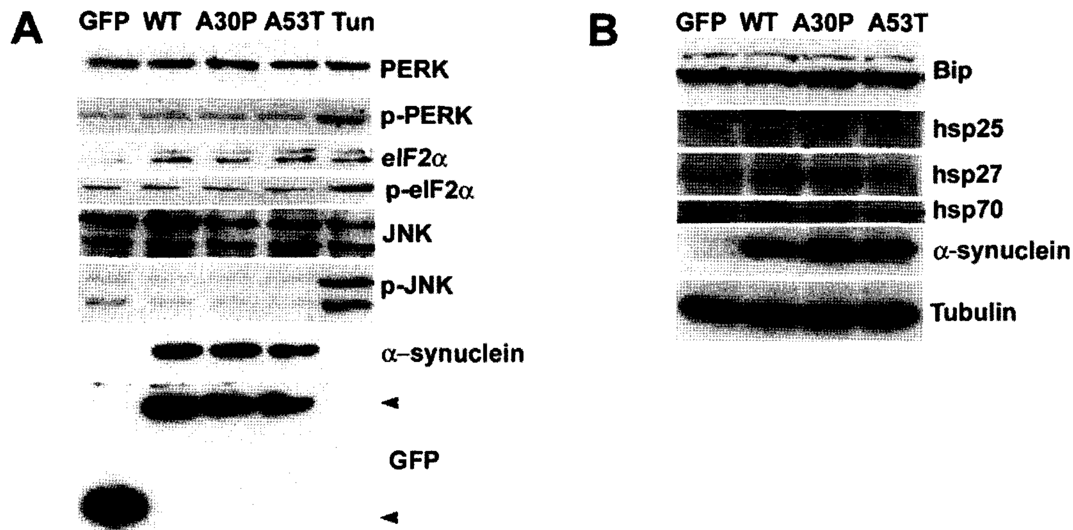

Fig. 3. Activation of ER stress kinases or upregulation of chaperones are not detected in α-synuclein overexpressing cells. A. SH-SY5Y cells were transfected with wild type (WT) or mutant (A30P, A53T) α-synuclein or treated with tunicamycin (Tun, 1 μM for 24 hr). 48 hr after the transfection, cells were harvested and analyzed by immunoblot assay to examine the activation status of PERK, eIF2α, and JNK. B. SH-SY5Y cells after 72 hr post-transfection were harvested and analyzed by immunoblot assay using anti Bip, hsp25, hsp27, and hsp70 antibodies to examine the status of ER and cytoplasmic chaperones.

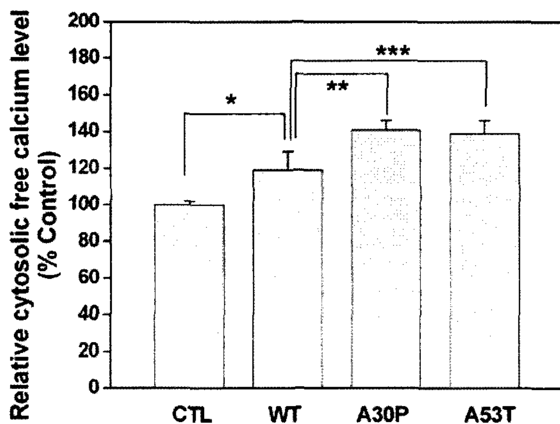

Fig. 4. Elevation of cytosolic free calcium level in cells overexpressing α-synuclein. To examine the relative level of cytosolic free calcium, α-synuclein-overexpressing cells were loaded with calcium crimson dye for 1 hr and the relative calcium level as measured by intensity of the red fluorescence were measured using spectrofluorometer (excitation at 490 nm, emission at 590 nm). n = 4, *$p < 0.05$, vector control vs. wild type; **$p < 0.05$, wild type vs. A30P; ***$p < 0.05$, wild type vs. A53T.

was slightly higher in mutant α-synuclein-expressing cells compared to wild type-expressing cells.

Our results indicate that overexpression of α-synuclein does induce UPR as evidenced by activation of ERAI but the signaling cascades may not involve activation of ER membrane kinases like PERK or ER-associated JNK. This may reflect successful management of unfolded α-synucleins by chaperone function or proteasomal degradation and thus necessity to activate translational shut down or cellular suicide mechanism being eliminated. However, basal level of cytoplasmic free calcium was slightly increased in the α-synuclein-expressing cells. This result suggests that overexpression of wild type or mutant α-synuclein induces UPR and ER stress to some degree and may cause dysregulation ER calcium homeostasis. However, it remains to be determined whether the elevated cytoplasmic calcium level in the α-synuclein-expressing cells is indeed due to release of ER calcium alone or increased net influx of free calcium from other source.

A number of neurodegenerative diseases like AD, amyotrophic lateral sclerosis, HD, and PD has a common pathological feature of protein aggregation (Shastry, 2003). There has been a controversy as to the nature of the protein aggregates or plaque found in these diseases, i.e., being toxic vs. protective. Recently, a line of evidence suggests that protein aggregation is a cellular defense mechanism by which cells can sequester toxic oligomers of the disease-related proteins (Ross and Poirier, 2005). Our observation is in agreement with this idea since we could not detect α-synuclein aggregates but still detected signs of ER stress. In the context of cultured cells, the stress imposed by unfolded protein species may be successfully managed as we observed but when it is accumulated like in human disease cases, cellular stress induced by unfolded proteins and the resulting oligomers may cause degeneration of the affected cells. Therefore, a pharmacological intervention to prevent accumulation of unfolded or misfolded proteins will be of great value to treat many neurogenerative diseases including PD.

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