

Impairment of redox state and dopamine level induced by α -synuclein aggregation and the preventive effect of hsp70

FuRong Yu^a, HuaTai Xu^c, Ming Zhuo^a, LanYing Sun^a, AiWen Dong^a,
XinYuan Liu^{a,b,*}

^a *Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, People's Republic of China*

^b *Xin Yuan Institute of Medicine and Biotechnology, School of Life Science, Zhejiang University of Technology and Science, Zhejiang, Hangzhou, Xia Sha, High Educational Park, West Part, Hangzhou 310018, China*

^c *Institute of neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, People's Republic of China*

Received 24 February 2005
Available online 2 April 2005

Abstract

One hypothesis for the etiology of Parkinson's disease (PD) is that the formation of proteinaceous inclusion, which is mainly composed of α -synuclein, may contribute to the selective loss of dopaminergic neurons. To further explore the role of α -synuclein in neurodegeneration of PD, we examined the possible effects of aggregated α -synuclein on the intracellular redox state, dopamine level, and cell death of SK-N-SH cells. Our present studies show that α -synuclein aggregation gives rise to both elevated intracellular oxidative state and dopamine level in SK-N-SH cells. Moreover, α -synuclein aggregation results in a higher ratio of apoptosis population ($55.8\% \pm \text{SEM}$) in cells overexpressing α -synuclein aggregation, compared to their normal control groups ($8.0\% \pm \text{SEM}$). In contrast, coexpression of hsp70 with α -synuclein suppresses the oxidative state shift, restores the normal dopamine levels and blocks neuron cell loss. Therefore, our data provided one possible mechanism by which the α -synuclein aggregation may lead to the neurodegeneration in PD via regulating the level of cytoplasmic dopamine and then disturbing the intracellular redox homeostasis. On the other hand, hsp70 can mitigate the degenerative effect conferred by α -synuclein, acting as a protective factor in treatment of PD. © 2005 Elsevier Inc. All rights reserved.

Keywords: Dopamine; Lewy body; Neurodegeneration; Oxidative stress; Parkinson's disease; SK-N-SH cell line

Parkinson's disease (PD), the second most common neurodegenerative disease after Alzheimer's disease (AD), is a chronic progressive disorder [5]. PD is characterized by the presence of cytoplasmic protein aggregates called Lewy bodies in affected neurons. Although the exact mechanism underlying the pathogenesis of PD still remains unknown, several lines of evidence indicate that the α -synuclein may play a pivotal role in the etiology of PD. First, α -synuclein protein is the major component

of proteinaceous lewy bodies, which are found in the remaining dopaminergic neurons of PD patients [8]. Second, transgenic mice and fly models expressing α -synuclein recapitulated some characters of PD [7,4]; and finally, the formation of α -synuclein aggregation affects the oxidative stress in cultured cells suggesting the association of α -synuclein with the redox status. Therefore, we speculated that aggregated α -synuclein might exert its degenerative effect through affecting the cytoplasmic dopamine level and thereby, impairing the redox state of dopaminergic neurons. The present studies demonstrated increasing cytoplasmic dopamine level, shifting redox state as well as cell apoptosis occur

* Corresponding author. Fax: +86 21 54921126.
E-mail address: xyliu@sibs.ac.cn (X. Liu).

resulting from overexpression of α -synuclein in dopaminergic SK-N-SH cells.

It was reported that molecular chaperone hsp70 colocalized with α -synuclein in the Lewy bodies in *Drosophila* model of PD and mitigate the α -synuclein-inducing toxicity [1]. To further explore the possible role of hsp70 in protecting neurons from degeneration, hsp70 was coexpressed with α -synuclein in SK-N-SH. Our data show that hsp70 overexpression can rejuvenate the normal physical dopamine level and the normal intracellular oxidative state. Co-immunoprecipitation assay indicated the direct interaction between α -synuclein and molecular chaperone hsp70. Here we provide further evidence for the possibility of chaperone therapeutics in neurodegenerative diseases.

Materials and methods

All chemicals, unless otherwise noted, were purchased from Sigma Chemical (Sigma–Aldrich China). The SK-N-SH cell line was purchased from the Cell Storage of Institute of Biochemistry and Cell Biology, Shanghai, China. The expression vectors pcDNA3- α -synuclein (α -synuclein) and pcDNA3-hsp70 were generous gifts from Dr. Kenny K.K. Chung and Dr. J.H. Wang, respectively. The mouse hsp70 antibody was bought from Boster (Wuhan, China) and goat α -synuclein antibody was from Santa Cruz Biotechnology (Shanghai, China).

Cell cultures. SK-N-SH cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum (FBS). Cells (10^6) were transfected with hsp70 (2 μ g) and/or α -synuclein (2 μ g) expressing vectors.

Co-immunoprecipitation analysis and Western blot. For co-immunoprecipitation, all steps were performed at 4 °C according to the manufacturer's protocols (Santa Cruz Biotechnology). Briefly, cells (1×10^7) were harvested and total proteins were extracted using NP-40 lysis buffer (1% NP-40, 150 mM NaCl, and 50 mM Tris, pH 8.0). Lysates were precleared by adding 1.0 μ g control IgG, together with 20 μ l protein A-agarose. After centrifugation and transferring the supernatant to new tubes, 1 μ g anti- α -synuclein antibody and 20 μ l protein A-agarose were added and incubated at 4 °C on a rocker platform for overnight. Samples were separated on 8% of SDS-PAGE gels, then transferred to nitrocellulose membrane, incubated with primary mouse anti-hsp70 monoclonal antibody (at a 1/500 dilution), HRP-coupled rabbit anti-mouse secondary antibody (at a 1/10,000 dilution), and visualized by chemiluminescence.

Th T fluorescence staining. SK-N-SH cells were fixed in 4% paraformaldehyde/PBS solution for 30 min, then incubated for 8 min with 0.05% Thioflavin T solution, and washed three times with PBS before mounting [14]. Images were analyzed using Zeiss fluorescence microscope coupled with a numeric Nikon camera photo apparatus.

Estimation of intracellular redox state using DCFH. The intracellular redox state was estimated by the level of intracellular peroxides, which was monitored by green fluorescence analysis with DCFH-DA. DCFH-DA is a non-polar compound that readily diffuses into cells, where it is converted into non-fluorescent polar derivative, 2',7'-dichlorofluorescein (DCFH) by cellular esterase. DCFH is membrane impermeable and rapidly oxidizes to a highly fluorescent compound 2',7'-dichlorofluorescein (DCF), in the presence of cellular H_2O_2 /other peroxides [10]. Thus, intracellular fluorescence intensity is directly proportional to the level of intracellular peroxides. At 48 h after transfection, the culture medium was replaced with freshly prepared medium containing 5 μ M DCFH-DA and incubation for 30 min, then the fluorescence intensity was observed with fluorescence microscope. The intracellular redox state was analyzed basing on the

intensity fluorescence at different time point 8, 16, 24, and 48 h, respectively.

Assay for dopamine level. The dopamine level in SK-N-SH cells was examined using the SPG (sucrose-potassium phosphate-glyoxylic acid) histochemistry method, as previously described [3]. Cells coated in 24-well plates were first washed thoroughly with PBS, then collected on a coverslip, dipped in SPG solution, and air-dried for several minutes. Finally, cells were examined under the fluorescence microscope and images were photographed using coupled numeric Nikon camera apparatus (Zeiss). The dopamine levels were analyzed at different time points: 0, 8, 16, 24, and 48 h, respectively.

Acridine orange/ethidium bromide staining. Cells (10^6) were centrifuged at 200g for 10 min at 4 °C. The cell pellets were resuspended in 1 ml PBS with 2 μ l each 100 μ g/ml acridine orange and ethidium bromide (AO/EB) diluted in PBS [13]. After staining for 8 min at room temperature, cells were immediately observed under fluorescence microscope with blue channel.

Results

The overexpression of hsp70 and α -synuclein in SK-N-SH cells

The overexpression of hsp70 and α -synuclein in SK-N-SH cells was confirmed by Western blot analysis. As indicated in Figs. 1 and 2, the expression levels of hsp70 (Fig. 1A) and α -synuclein (Fig. 2A) in transfected cells were markedly increased compared to that of the control group. Quantitative analysis revealed that the relative

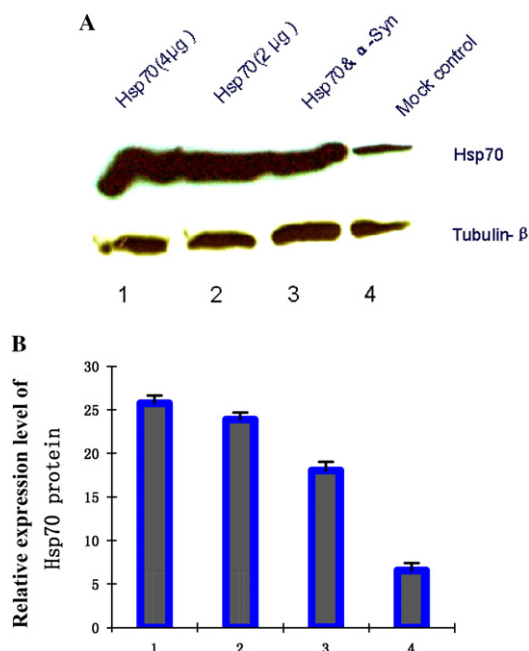


Fig. 1. Overexpression of hsp70 in SK-N-SH cells. (A) Western blot analysis of the expression of hsp70. Lanes 1 and 2, cells were transfected with 4, 2 hsp70 expressing vectors, respectively; lane 3, Cells were cotransfected with hsp70 and α -synuclein expressing vectors; and lane 4, mock control sample. (B) Quantitative analysis of hsp70 expression level—after the normalization to tubulin- β levels in SK-N-SH cells—was performed using laser densitometry. Each point, the mean of triplicate analyses with SD ($P < 0.05$).

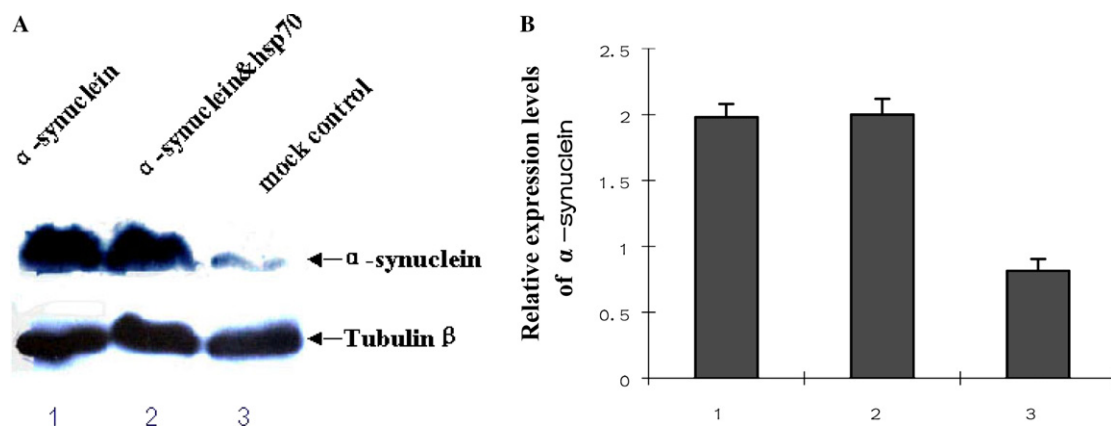


Fig. 2. Overexpression of α -synuclein in SK-N-SH cells. (A) Western blot analysis of α -synuclein expression. Lane 1, cells were transfected with α -synuclein expressing vector; lane 2, cells were cotransfected with α -synuclein and hsp70 expressing vectors; lane 3, mock control sample (tubulin- β as for internal references). (B) Quantitative analysis of α -synuclein expression level—after the normalization to tubulin- β levels in SK-N-SH cells—was performed using laser densitometry. Each point, the mean of triplicate analyses with SD ($P < 0.05$).

expression level of hsp70 (Fig. 1B) was increased to 5-fold of that of the mock control. The expression of α -synuclein (Fig. 2B) was increased to 3-fold of that of the control (Figs. 1A and B; tubulin- β as internal reference).

The formation of aggregation induced by α -synuclein overexpressing

To find out whether overexpression of α -synuclein would affect the formation of α -synuclein accumula-

tion, we adopted the Th T labeling method to monitor the information of aggregates. As shown in Fig. 3B, a lot of granules appeared in the α -synuclein overexpressing cells (indicated with arrows). The mean number of the aggregate complexes were $120 \pm 10/100$ cells (means \pm SD, Fig. 3D, $n = 600$). In contrast, no or very few aggregate complex was found in the mock control cells (Fig. 3A) and in cells coexpressing hsp70 and α -synuclein cells (Fig. 3C). Accordingly, the mean number of the aggregates complex was 10 ± 1.5

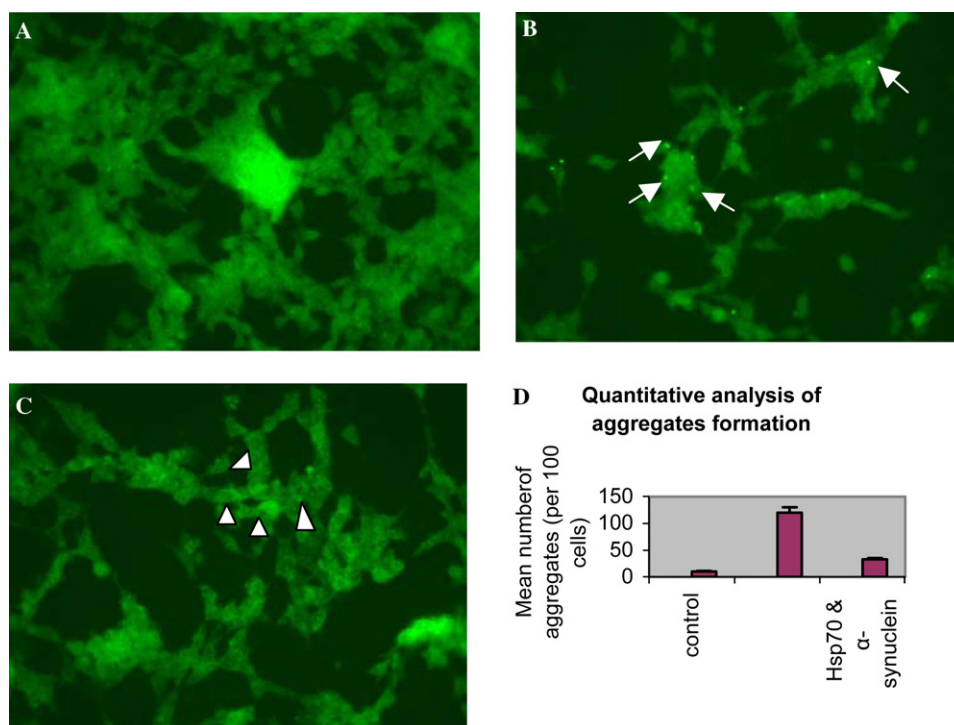


Fig. 3. The formation of intracellular α -synuclein aggregations indicated by Th T fluorescence (20 \times). (A) Mock control cells. (B) Cells were transfected with α -synuclein-expressing vector (the aggregations are indicated by arrows). (C) Cells were transfected with hsp70 and α -synuclein expressing vectors (small cavities are indicated by arrowheads). (D) Quantitative analysis of the formation of aggregates in control cells, α -synuclein expressing and hsp70 and α -synuclein coexpressing cells. Shown is the mean of aggregated complex number per 100 cells ($n = 600$).

(mean \pm SD, Fig. 3D, $n = 600$) in control cells and 33 ± 2.2 (mean \pm SD, Fig. 3D, $n = 600$) in cells coexpressing hsp70 and α -synuclein. The most interesting aspect was that small cavities appeared after 2-day coexpression of hsp70 and α -synuclein, which were suspected to be the remains of aggregates complexes shown in Fig. 3B (Fig. 3C, indicated by arrowheads). These results suggested that overexpression of α -synuclein may result in the formation of aggregated complexes and the coexpression of molecular chaperone hsp70 rescues the proteins from aggregating and therefore can mitigate the damage caused by dissoluble complexes loading against cells.

The intracellular redox state shift in cells overexpressing α -synuclein

The change in the intracellular redox state was estimated through the intracellular oxidative state, which was monitored by the green fluorescence intensity and flow cytometric analysis of DCFH (data not shown). In α -synuclein overexpressing cells, the intracellular oxidative state began to increase at 24 h after transfection (data not shown) and reached the highest level at 48 h after transfection (Figs. 4A and B). However, co-expres-

sion of hsp70 recovered the intracellular oxidative state to the level similar to that of control samples (Figs. 4A and C). These results suggest overexpression of α -synuclein might induce the shift of intracellular redox state by elevating peroxide level. On the other hand, molecular chaperone hsp70 may suppress the redox shift through rescuing the misfolded protein from aggregating. To confirm this speculation, we further examined the changes in intracellular dopamine level after overexpression of α -synuclein.

The intracellular dopamine level increased in cells overexpressing α -synuclein

Dopamine is one of the most vulnerable neurotransmitters to oxidation and its oxidative derivatives is one of the sources resulting in the increase in oxidative state. To find out whether the shift in oxidative state is associated with changes in dopamine level, we measured the intracellular dopamine level using SPG method, which was developed for the visualization of catecholamines and serotonin in tissue or culture cells. As shown in Fig. 5, the dopamine level was significantly increased in cells overexpressing α -synuclein (Fig. 5B, as arrows indicated) compared to the

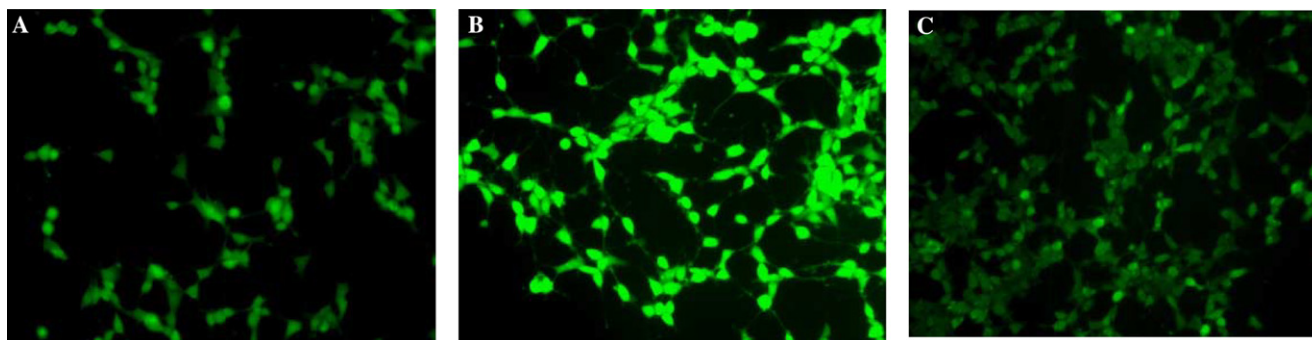


Fig. 4. The intracellular peroxide state was elevated resulting from overexpression of α -synuclein (20 \times). (A) Mock control cells. (B) Cells were transfected with α -synuclein expressing vector. (C) Cells were transfected with hsp70 and α -synuclein expressing vectors.

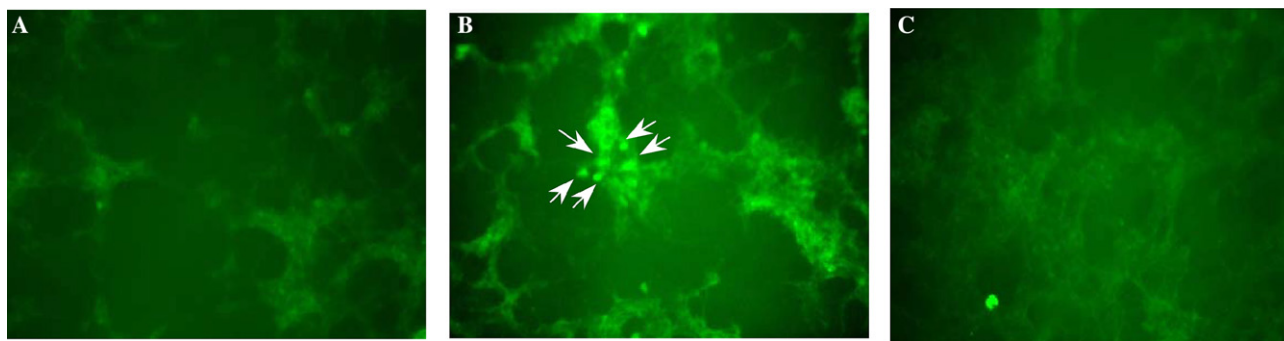


Fig. 5. The dopamine level was elevated by α -synuclein overexpression (20 \times). (A) Mock control cells. (B) Cells were transfected with α -synuclein expressing vector. (C) Cells were cotransfected with hsp70 and α -synuclein expressing vectors.

control cells (Fig. 5A) and cells coexpressing hsp70 and α -synuclein (Fig. 5C). The changes in fluorescence intensity showed that dopamine level began to increase at 12 h and reached the most significant value at about 30 h after transfection (data not shown). These data indicate that the overexpression of α -synuclein may lead to the increase in intracellular dopamine level and the coexpression of hsp70 blocked this elevation in dopamine.

The interaction between hsp70 and α -synuclein in the SK-N-SH cell

To evaluate the potential binding interaction between hsp70 and α -synuclein, we performed co-immunoprecipitation (Co-IP) assay. The whole cell extract was immunoprecipitated with antibody to α -synuclein or preabsorbed antibody coupled to protein G beads. Co-IP with α -synuclein antibody precipitated the expected band at 70 kDa which was verified by the immunoblot using hsp70 antibody. This result indicates the intracellular interaction between α -synuclein and hsp70 (Fig. 6).

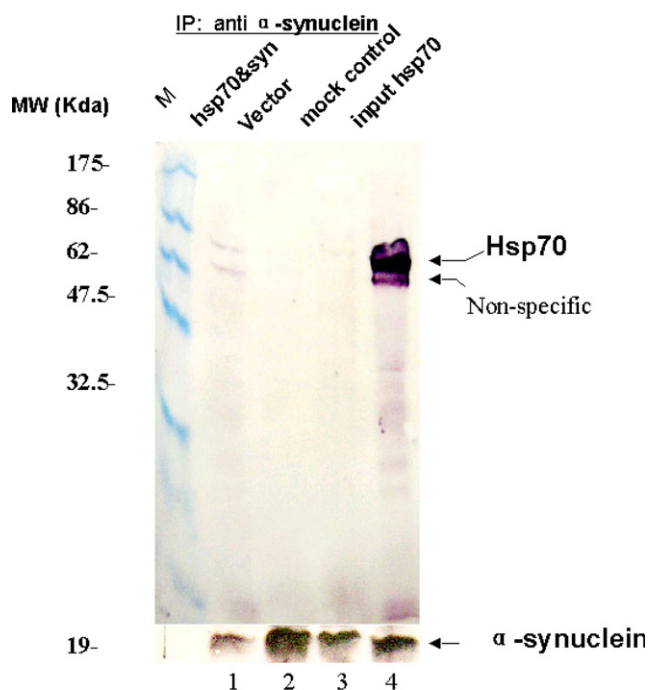


Fig. 6. Co-immunoprecipitation (Co-IP) assay for the interaction between hsp70 and α -synuclein. Data from a representative experiment showing Western blot (WB) reacted with the hsp70 antibody. Whole cell extracts were immunoprecipitated by anti- α -synuclein antibody (α -synuclein-IP). The precipitated complexes were run on 8% SDS-PAGE gel and probed for hsp70 using mouse anti-hsp70 antibody. Lane 1, WB of α -synuclein-IP sample from coexpression of α -synuclein and hsp70 cells; lane 2, cells were transfected with pcDNA3 vector; lane 3, mock control cells; and lane 4, inputting hsp70 protein (1/3 of whole cell extract used for IP) as positive protein control.

Neuron apoptosis in cells overexpressing α -synuclein

To investigate the possible degenerative effects of α -synuclein on the dopaminergic neurons, we adopted the AO/EB staining method to examine the apoptosis phenomenon. As shown in Fig. 7, the apoptosis/necrosis cells showed yellow or yellowish-green color (Fig. 7B), while the live cells (or control cells) were stained with green color fluorescence (Figs. 7A and C). The result indicates the break down of DNA double strands and mitochondria damages occurred in these cells.

Discussion

The exact mechanism of neurodegeneration in PD is not fully known. The discovery of two missense mutants in the gene encoding α -synuclein indicates the inheritance factor involved in the pathogenesis of PD [5,8]. Several lines of evidence from cells culture and animal models have shown that the pathogenesis of PD may be associated with the aggregation of insoluble complexes which mainly contains α -synuclein. What is the exact role of α -synuclein in the dopaminergic neuron degeneration? One report said that α -synuclein acting like a molecular chaperone could regulate the dopamine biosynthesis through binding to the dephosphorylated-tyrosine hydrolase (TH) form [9,11]. However, there is little knowledge about how aggregated α -synuclein led to neuron cell loss. As previous experiments indicated, oxidative stress plays a key role in neurodegeneration which is exemplified by intrastriatal injections of 6-hydroxydopamine (6-OHDA) causing the degeneration of dopaminergic neurons in a manner that can mimic the early stage in PD [6]. In our studies, we investigated the role of α -synuclein aggregation in the induction of oxidative stress in dopaminergic neurons. We found that overexpression of α -synuclein, which mainly formed aggregates in the cells, can induce the redox state shift and cell apoptosis. This result is out of our expectation because the other group reported that oxidative factors such as ferric ion, H_2O_2 can induce the aggregation of α -synuclein [15]. To examine the mechanism by which oxidative stress was elevated, we further explored the possibility of changes in cytoplasmic dopamine level. Experimental results showed that there is significant increase in dopamine level in cells overexpressing α -synuclein compared to the control.

Heat shock protein 70 (hsp70) is another component found in the Lewy body in experiments in transgenic *Drosophila* model of overexpression of hsp70 which showed that hsp70 can prevent the TH-immunopositive neuron loss under the oxidative stress conditions [2]. In our studies, hsp70 can protect the neuron cells from apoptosis as well as suppress the increase in dopamine level and oxidative state shift after coexpression with

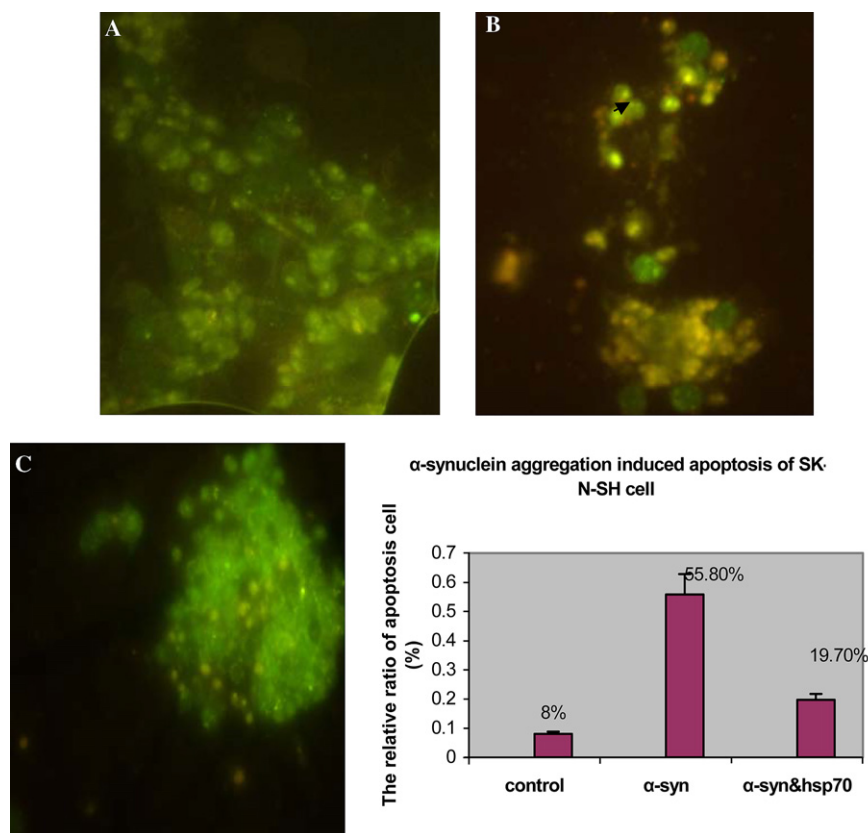


Fig. 7. Measurement of cell apoptosis by AO/EB staining. Acridine orange/ethidium bromide staining showed the apoptosis of cells expressing α -synuclein. (A) Mock control cells; (B) cells overexpressing α -synuclein; (C) cells coexpressing hsp70 and α -synuclein.

α -synuclein in dopaminergic neurons. The possible mechanism by which hsp70 can inhibit the formation of α -synuclein aggregates is through direct interaction with α -synuclein. Previous experiments also indicate that hsp70 can mitigate the neurotoxicity caused by misfolding proteins such as polyQ-expanded proteins [12]. Our studies, together with previous results, provide the promise for treatment of neurodegenerative diseases through up-regulation of chaperone pathways.

Acknowledgments

This work was supported by a Grant-in-Aid for Great Projects from the Chinese Academy of Sciences (No. KJ951-B1-608). We thank Dr. X.L. Fan for the review for manuscript, and Dr. Ruth G. Perez and Prof. Yongjian Liu (University of Pittsburgh School of Medicine) for providing us with pcDNA3- α -synuclein plasmid.

References

- [1] P.K. Auluck, H. Chan, J.Q. Trojanowski, V. Lee, N.M. Bonini, Chaperone suppression of α -synuclein toxicity in a *Drosophila* model for Parkinson's Disease, *Science* 295 (2002) 865–868.
- [2] N.M. Bonini, Chaperoning brain degeneration, *Proc. Natl. Acad. Sci. USA* 99 (Suppl. 4) (2002) 16407–16411.
- [3] J.C. De la Torre, An improved approach to histochemistry using the SPG method for tissue monoamines, *J. Neurosci. Methods* 3 (1980) 1–5.
- [4] M.B. Feany, W.W. Bender, A *Drosophila* model of Parkinson's disease, *Nature* 404 (2000) 394–398.
- [5] M. Goedert, Alpha-synuclein and neurodegenerative disease, *Nat. Rev. Neurosci.* 2 (7) (2001) 492–501.
- [6] C.S. Lee, H. Sauer, A. Bjorklund, A dopaminergic neuronal degeneration and motor impairments following axon terminal lesion by intrastratial 6-hydroxydopamine in the rat, *Neuroscience* 72 (1996) 641–653.
- [7] E. Masliah, E. Rockenstein, I. Veibergs, M. Mallory, M. Hashimoto, A. Takeda, Y. Sagara, A. Sisk, L. Mucke, Dopaminergic loss and inclusion body formation in α -synuclein mice: implications for neurodegenerative disorders, *Science* 287 (2000) 1265–1268.
- [8] L. Narhi, S.J. Wood, S. Steavenson, Y. Jiang, G.M. Wu, D. Anafi, S.A. Kaufman, F. Martin, K. Sitney, P. Denis, J.C. Louis, J. Wypych, A.L. Biere, M. Citron, Both familial Parkinson's disease mutations accelerate α -synuclein aggregation, *J. Biol. Chem.* 274 (14) (1999) 9843–9846.
- [9] R.G. Perez, J.C. Waymire, E. Lin, J.J. Liu, F. Guo, M.J. Zigmond, A role for the α -synuclein in the regulation of dopamine biosynthesis, *J. Neurosci.* 22 (8) (2002) 3090–3099.
- [10] T. Sasada, S. Iwata, N. Sato, Y. Kitaoka, K. Hirota, K. Nakamura, A. Nishiyama, Y. Taniguchi, A. Takabayashi, J. Yodoi, Redox control of resistance to *cis*-diamminedichloroplatinum(II) (CDDP): protective effect of human thioredoxin against ADDP-induced cytotoxicity, *J. Clin. Invest.* 97 (10) (1996) 2268–2276.

- [11] H. Sauer, W.H. Oertel, Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat, *Neuroscience* 59 (1994) 401–415.
- [12] J.M. Warrick, H.Y. Chan, G.L. Gray-Board, Y. Chai, H.L. Paulson, N.M. Bonini, Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone hsp70, *Nat. Genet.* 23 (4) (1999) 425–428.
- [13] A.B. Zubrow, M. Delivoria-Papadopoulos, Q.M. Ashraf, J.R. Ballesteros, K.I. Fritz, O.P. Mishra, Nitric oxide-mediated expression of Bax protein and DNA fragmentation during hypoxia in neuronal nuclei from newborn piglets, *Brain Res.* 954 (2002) 60–67.
- [14] C. Alves Da Costa, E. Paitel, B. Vincent, F. Checler, Alpha-synuclein lowers p53 dependent apoptotic response of neuronal cells, *J. Biol. Chem.* 277 (52) (2002) 50980–50984.
- [15] M. Hashimoto, L.J. Hsu, Y. Xia, A. Takeda, A. Sisk, M. Sundsmo, E. Masliah, Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro, *Neuroreport* 10 (4) (1999) 717–721.