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Oxidative stress induces nuclear translocation of C-terminus of α-synuclein in dopaminergic cells

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Abstract

Growing evidence suggests that oxidative stress is involved in the neuronal degeneration and can promote the aggregation of α-synuclein. However, the role of α-synuclein under physiological and pathological conditions remains poorly understood. In the present study, we examined the possible interaction between the α-synuclein and oxidative stress. In a dopaminergic cell line MES23.5, we have found that the 200 μM H_2O_2 treatment induced the translocation of α-synuclein from cytoplasm to nuclei at 30 min post-treatment. The immunoactivity of α-synuclein became highly intensive in the nuclei after 2 h treatment. The protein translocated to nucleus was a 10 kDa fragment of C-terminus region of α-synuclein, while full-length α-synuclein remained in cytoplasm. Thioflavine-S staining suggested that the C-terminal fragment in the nuclei has no β-sheet structures. Our present results indicated that 200 μM H_2O_2 treatment induces the intranuclear accumulation of the C-terminal fragment of α-synuclein in dopaminergic neurons, whose role remains to be investigated.

Keywords: α-Synuclein; Nuclear translocation; Oxidative stress; Parkinson's disease; Dopaminergic neuron

Parkinson's disease (PD) is one of the most common movement disorders of the elderly. Its pathological characteristics include loss of dopaminergic neurons and the presence of intracellular inclusion of Lewy bodies (LBs). Wild-type α-synuclein is a major component of LBs in sporadic PD, dementia with LBs, a subtype of Alzheimer's disease known as the LB variant of AD, as well as of glial cytoplasmic inclusions in multiple system atrophy (MSA) [1]. Wilt-type α-synuclein as well as aggregated and truncated α-synuclein have been discovered from purified LBs, and α-synuclein solubility is reduced in affected regions of LB disease and multiple system atrophy brains [2]. So far, the mechanisms underlying neurodegeneration in PD are unknown, but the findings that α-synuclein accumulates in LBs and the mutations of α-synuclein cause familial PD suggest that α-synuclein critically participates in the pathophysiology of PD [3].

Growing evidence has been showing that oxidative stress is involved in the neurodegeneration. Oxidative modifications of α-synuclein and generation of stable oligomers may play an important role in the neurotoxicity of oxidative stress [4]. Recent studies indicated that oxidative stress could induce up-regulation of the expression of α-synuclein and promote the fibrillization and aggregation of α-synuclein. Conversely, the overexpression, fibrillization, and aggregation of α-synuclein result in the high level of reactive oxygen species (ROS) and neurotoxicity [5–7]. Moreover, oxidative stress could disrupt nuclear membrane and translocated α-synuclein from perinuclear region into the disrupted nucleus [8]. It was reported that a dramatic

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increase in α-synuclein immunoreactivity in both the cytosol and nuclei of nigral cells after administration of herbicide paraquat to mice [9]. Because the mechanisms underlying the interaction between α-synuclein and oxidative stress have not been explored, we set out to further investigate the role of oxidative stress in regulation and modification of α-synuclein in dopaminergic neurons.

Materials and methods

Cell culture. A hybrid cell line MES23.5, produced by fusing rat embryonic mesencephalic dopaminergic neurons with mouse neuroblastoma cell line N18TG2, was obtained by courtesy of Dr. Wei-Dong Le [10]. MES23.5 cells were cultured in DMEM/F12 medium containing 5% (vol/vol) FBS (Hyclon), 100 U penicillin/streptomycin, 2 mM L-glutamine (Sigma), and Sato's (50 × Sato's: insulin 25 mg; transferring 25 mg; pyruvic acid, 243 mg; putrescine 20 mg; 1 mg/ml sodium selenate, 25μl; 0.315 mg/ml progesterone, 100 μl in 100 ml DMEM/F12 medium) at 37 °C with 5% (vol/vol) CO_2.

MTT assay. The MTT assay was used to measure the cell viability. Cells were seeded in 96-well plates cultured for 2 days and treated with 200, 400, and 800 μM H_2O_2, respectively, for 24, 48, and 72 h at 37 °C. At the end of the incubation period, MTT was diluted to a final concentration of 0.5 mg/ml in the culture media. Cells were incubated for 2 h at 37 °C, and the media were carefully removed. The formazan crystals were dissolved in acidified isopropanol (40 mM HCl in isopropanol) and absorbance was determined at 590 nm. The data were obtained from a representative of at least three experiments shown as percentage of control SD and were measured in quadruplicate wells.

Immunofluorescence. Cells were seeded in Chamber-slides (Falcon) coated with 0.01% (w/vol) poly-lysine (Sigma) and cultured for 4 days. Cells were treated with H_2O_2 (200 μM H_2O_2 was used in all the following experiments) and harvested at 30 min, 1, 2, 4, and 8 h. Cells were fixed with 4% (w/vol) paraformaldehyde (Sigma) for 30 min at 4 °C and then washed 3 times with PBS, permeabilized with 0.3% (vol/vol) Triton X-100 (Sigma) for 30 min at room temperature (RT). Cells were blocked in 5% (vol/vol) normal horse serum for 30 min at RT. Cells were incubated with primary antibodies of rabbit polyclonal anti-tyrosine hydroxylase (TH, Santa Cruz, at a dilution of 1:2000) or anti-human α-synuclein MDV2 (at a dilution of 1:4000) that recognizes the N-terminal region [2]; and mouse monoclonal anti-α-synuclein antibody 3D5 (1:5000) that recognizes the C-terminal region [11] (amino acids 114–120), overnight at 4 °C. Cells were washed with 0.3% (w/vol) BSA in PBS and then incubated for 2 h with two secondary antibodies, i.e., biotin-conjugated horse anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG, and washed again and then incubated for 2 h with a tertiary FITC-conjugated donkey anti-goat IgG and Streptavidin Cy3 conjugate (Sigma). Cells were visualized using a Bio-Rad MRC-1024 confocal microscope.

Thioflavine-S histochemistry. After immunofluorescence staining of α-synuclein with 3D5 antibody, the cells were washed twice with PBS and incubated with 0.5% (w/vol) thioflavine-S (Sigma) for 8 min at RT, washed 3 times with 80% (vol/vol) ethanol, washed once with H_2O, and then mounted and observed using the confocal microscope.

Preparation of subcellular extracts. The Nuclear Extract Kit (Active-motif, USA) was used for preparation of subcellular extracts according to the protocol provided. In brief, cultured cells (ca. 1×10^7) were washed with 5 ml ice-cold PBS/phosphatase inhibitors. After removing solution, 3 ml ice-cold PBS/phosphatase inhibitors were added. Cells were collected from flask by scraping gently with a cell scraper. Cells were transferred to a pre-chilled 15 ml conical tube and centrifuged for 5 min at 200*g* in a centrifuge at 4 °C. After discarding supernatant, cell pellet was kept on ice. Cells were resuspended gently in 0.5 ml of 1× hypotonic buffer, transferred to a pre-chilled microcentrifuge tube, and incubated for 15 min on ice. Twenty-five microliter of detergent was added and vortexed 10 s at the highest setting. The suspension was centrifuged for 30 s at 14,000*g* in a microcentrifuge at 4 °C. Supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube. The pellet was used for collecting the nuclear fraction. Nuclear pellet was resuspended in 50 μl complete lysis buffer by pipetting, vortexed 10 s at the highest setting, and incubated for 30 min on ice on a rocking platform at 150 rpm. The lysate was vortexed for 30 s at the highest setting and centrifuged for 10 min at 14,000*g* in a microcentrifuge at 4 °C. Supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube.

Western blot analysis. Samples were separated by 15% (w/vol) SDS–PAGE. Proteins were transferred onto a PVDF membrane as described previously [12]. α-Synuclein was detected using primary anti-α-synuclein antibodies, MDV2 (1:10,000) or 3D5 (1:500) with incubation overnight at 4 °C. HRP-conjugated horse anti-mouse IgG at 1:2000 dilution was applied with 2 h incubation at 4 °C. ECL blotting reagents (Amersham Biosciences) were used for protein visualization.

Results

The effects of different concentrations of H_2O_2 treatment on cellular viability

To determine the effects of different concentrations of H_2O_2 on the viability of MES dopaminergic neuronal cells, we monitored the cell viability using MTT assay. After 24 h exposure with 800 μM H_2O_2, the viability decreased to 23.2%; and at 48 h, the viability decreased to 0.5%; at 72 h post-treatment, no vital cell was detected (Fig. 1). This study showed the neurotoxicity produced by 800 μM H_2O_2 treatment was too strong to investigate the response of MES23.5 to H_2O_2 treatment. However, 200–400 μM H_2O_2 exposure induced a dose- and time-dependent reduction of cell viability (Fig. 1). Therefore, 200 μM H_2O_2 was used in the following experiments.

α-Synuclein translocate from cytoplasm to nucleus

Although most studies reported that the location of α-synuclein was in presypaptic membrane and perinuclear region, some investigators found the location in nucleus in normal neurons [13,14]. The immunostaining pattern of α-synuclein may be different, depending on the primary antibodies. Therefore, the location of α-synuclein was

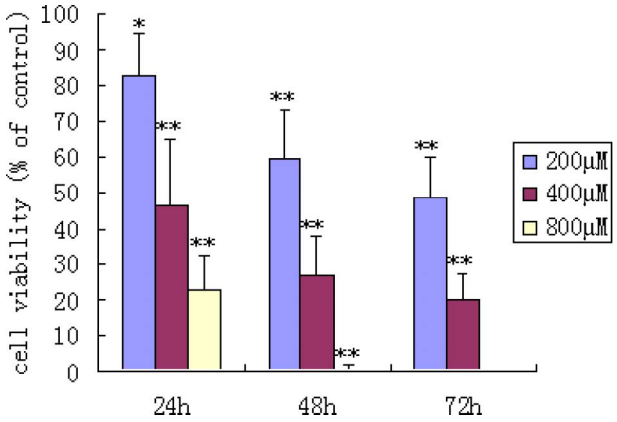

Fig. 1. H_2O_2 induced dose- and time-dependent toxicity. MES23.5 cells are treated with 200, 400, and 800 μM H_2O_2 for 24, 48, and 72 h. Cell viability is measured by MTT assay. $^*P < 0.05$; $^{**}P < 0.01$.

investigated in MES23.5 cells with two primary antibodies, MDV2 and 3D5. As shown in Figs. 2A and B, the MDV2 and 3D5 immunoactivities were in cytosol in untreated MES23.5 cells. Since MES23.5 cells express tyrosine hydroxylase (TH), a key enzyme for dopamine synthesis, the cells were also double stained with antibodies against α-synuclein (3D5) and TH, a cytosolic marker for dopaminergic neurons. Confocal imaging revealed that in untreated MES23.5 cells α-synuclein and TH were co-localized within the cytosol (Figs. 3A–C). After 30 min treatment with H_2O_2, the α-synuclein immunoreactivity appeared rapidly in nuclei and the intensity of staining was similar to that of the cytosol (Figs. 3D–F). After 1 h exposure with H_2O_2, the intensity of immunostaining in nuclei was increased (Figs. 3G–I), and after the treatment for 2 h, α-synuclein apparently accumulated highly in nuclei and the staining in nuclei became much stronger than in the cytosol (Figs. 3J–L), while TH remained in the cytosol.

The intensity and pattern of α-synuclein immunoreactivity (Fig. 3O) were different at 8 h post-treatment in different cells. α-Synuclein accumulated significantly within nucleus in some cells (arrowheads); in other cells, α-synuclein staining was weak or absent in nucleus and only presented in cytosol (arrows). The levels of the expression of TH were also different in different cells (Fig. 3P). The levels were significantly higher in some cells (arrowheads) than others (arrows). The merged images (Fig. 3Q) showed that the levels of the expression of TH were much higher (arrowheads) in those cells where α-synuclein was accumulated significantly in nucleus. Conversely, the expression of TH was very low, or even disappeared (arrows) in those cells where α-synuclein was not accumulated significantly in nucleus.

To investigate the status of α-synuclein in the nucleus, thioflavine-S was used to stain the cells with the histochemistry method. It is demonstrated that the staining of β-sheet structures was apparently not coincident with α-synuclein at 8 h post-treatment, suggesting that α-synuclein accumulated in nuclei was not aggregated or fibrillated (Fig. 4).

The protein translocated into nucleus was C-terminal fragment of α-synuclein

To assess whether the protein translocated into nucleus after H_2O_2 treatment was full-length α-synuclein or a fragment of it, we employed immunofluorescent double labeling and Western blot analysis using N-terminal region (MDV2) and C-terminal region (3D5) specific anti-α-synuclein antibodies. The immunofluorescent double labeling indicated that the N-terminus staining appeared only in cytosol and the C-terminal immunoreactivity was both in cytosol and in nuclei (Figs. 5A–C). Western blot analysis using C-terminus antibody showed that a ca. 10 kDa band appearing in the nuclear fraction of H_2O_2-treated cells and the full-length α-synuclein was in the cytoplasmic fraction of treated cells (Fig. 6A). However, when N-terminus antibody was used, there was no positive band in the nuclear fraction of treated cells, whereas the size of the bands appeared in the cytoplasmic fraction of both untreated and treated cells was about 18 kDa (Fig. 6B), the size of full-length α-synuclein. Altogether, these results suggest that the protein in the cytoplasmic fraction is a full-length α-synuclein, and that a 10 kDa C-terminal fragment of α-synuclein accumulated in the nuclei after the H_2O_2 treatment.

Discussion

Our current study revealed that H_2O_2 treatment induces the translocation of α-synuclein from the cytoplasm to nucleus in dopaminergic MES23.5 neuronal cells. Immunofluorescent double labeling and Western blot analysis identified that the protein translocated to nucleus is the C-terminal fragment of α-synuclein and the size of the fragment is about 10 kDa. Thioflavine-S histochemistry showed that the C-terminal fragment of α-synuclein translocated to nucleus is apparently not aggregated or fibrillated. Furthermore, at 8 h post-treatment, the expression of TH is down-regulated in those cells where α-synuclein was not significantly accumulated in nucleus.

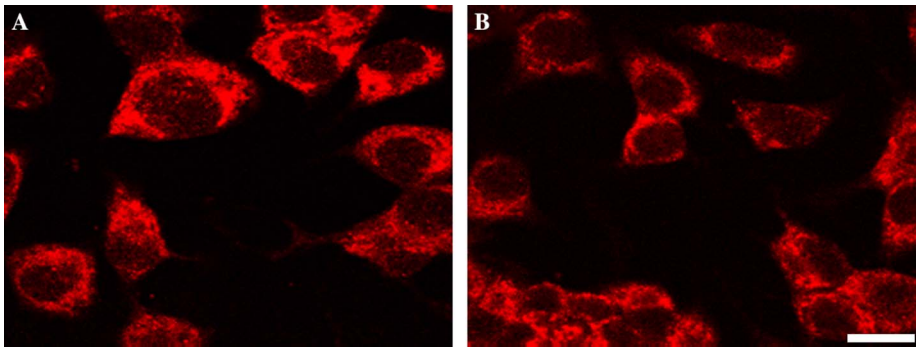

Fig. 2. Immunostaining of untreated cells is performed with MDV2 (A) and 3D5 (B). The staining is visualized by fluorescence (red). The α-synuclein immunoreactivity is present only in the cytoplasm in untreated cells with both two antibodies. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

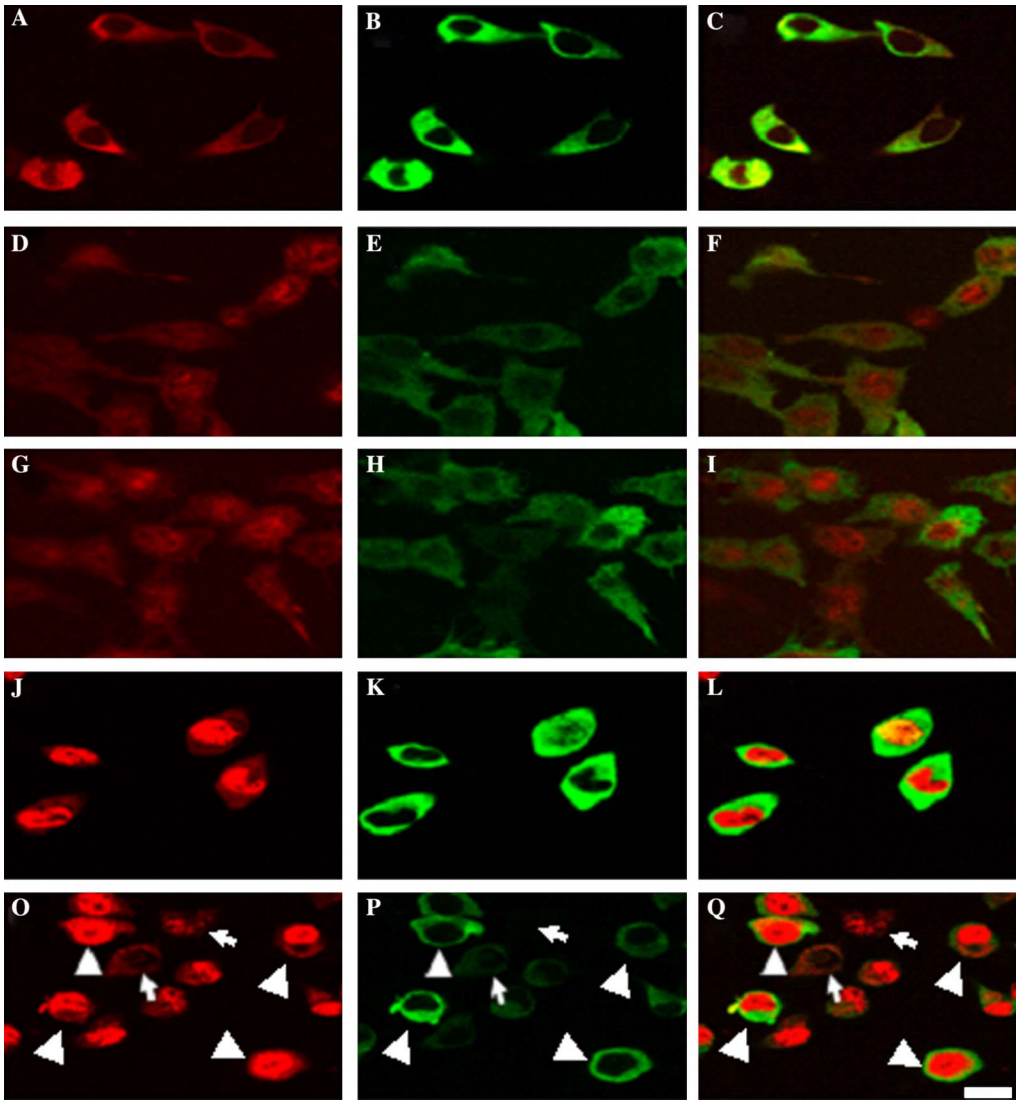

Fig. 3. H_2O_2 treatment induces the translocation of α-synuclein from cytoplasm to nuclei and visualized using fluorescent microscopy. MES23.5 cells are double stained for α-synuclein shown in red (3D5) (A, D, G, J, and O) and TH shown in green (B, E, H, K, and P) immunoreactivity. The merged image (C) shows that α-synuclein and TH are co-localized in the cytosol in untreated cells as shown in yellow, and the merged images (F, I, L, and Q) show that only α-synuclein immunoreactivity is present in the nuclei in H_2O_2-treated cells. At 8 h post-treatment (O–Q), the α-synuclein is accumulated significantly within nuclei in some cells (O, arrowheads), meanwhile α-synuclein staining in nucleus is weaker or even totally absent in nuclei (O, arrows). Note that in O, P, Q, the levels of the expression of TH are higher in those cells where α-synuclein is significantly accumulated in nuclei than those where less α-synuclein is accumulated (P,Q). Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Recent reports have shown that the oxidative stress disrupted nuclear membrane and translocated α-synuclein from perinuclear region into the disrupted nucleus in cultured SK-N-SH cell line [8]. It was also reported that a dramatic increase in α-synuclein immunoreactivity in both the cytosol and nuclei of nigral cells after intraperitoneal administration of herbicide paraquat to mice [9]. In our study, the H_2O_2 treatment induced rapid accumulation of the C-terminal fragment of α-synuclein in the nuclei, while the nuclei of untreated cells lacked α-synuclein immunoreactivity. From 1 h post-treatment, the intensity of α-synuclein positive staining became much stronger in nuclei than in cytosol. It is likely that the C-terminal fragment of α-synuclein was transported into nuclei positively because nuclear membrane was apparently intact in our observation. A specific transporter for the C-terminal fragment of α-synuclein in nuclear membrane might play a pivotal role in this process. Future studies will be needed to address these issues.

Although it was suggested that the proteasomal pathway is involved in α-synuclein degradation [15], some in vitro studies failed to show any relationship between α-synuclein and the proteasome [16]. Some proteases have been identified to degrade α-synuclein in vitro [17,18]. One in vitro study reported that the calcium-activated neutral protease, Calpain I predominantly cleaved wild-type

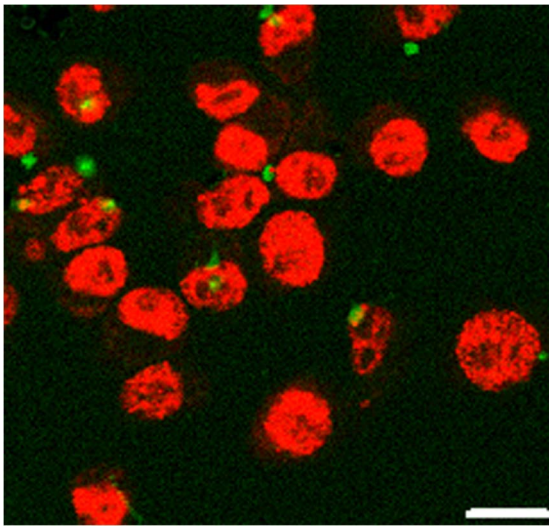

Fig. 4. α-Synuclein translocated into nucleus has apparently no β-sheet structures. Thioflavine-S histochemistry (green) and α-synuclein immunofluorescence double staining show that the two staining patterns are not coincident, suggesting that α-synuclein in nuclei is not aggregated, or fibrillated. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

α-synuclein in the N-terminal segment after amino acid 57, and to a lesser degree within the NAC region [19] after amino acids 73, 75, and 83, while fibrillized wild-type and A53T α-synuclein were cleaved predominantly in the C-terminus after amino acids 114 and 122 [18]. Recently, it was found that parkin induced the degradation of α-synuclein via the activation of Calpain in vivo [20]. However, the cleavage pattern of Calpain I to α-synuclein in vivo is still unclear. The present results indicated that the size of the C-terminal fragment of α-synuclein translocated into nuclei is about 10 kDa. Oxidative stress should trigger the up-regulation of the concentration of calcium that should activate Calpain I [21]. If α-synuclein is cleaved by Calpain I in the N-terminal segment after amino acid 57, the size of remaining C-terminal fragment would be about 10 kDa. Therefore, it is possible that the C-terminal fragment detected in nuclei is generated by Calpain I as a protective mechanism against oxidative stress. Further studies will be necessary to clarify this point.

The physiological role of α-synuclein is still not well-characterized despite its abundance in the CNS [13]. Given the genetic association of α-synuclein with PD, many

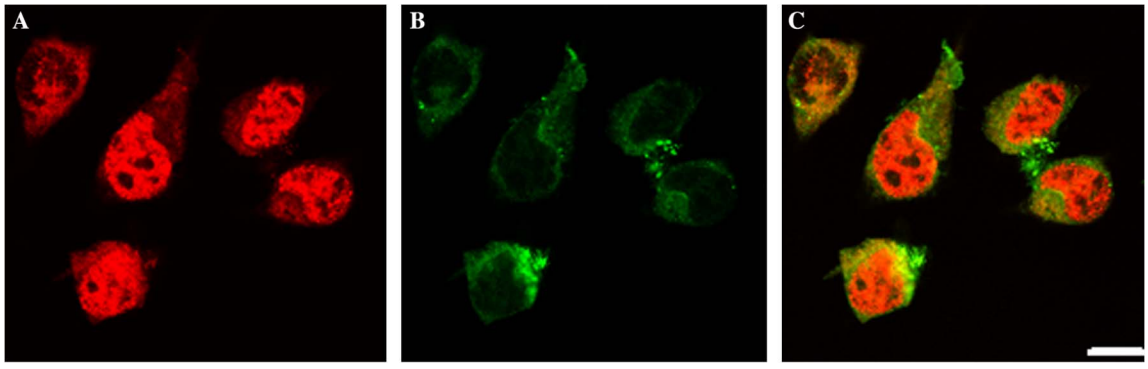

Fig. 5. H_2O_2-treated cells are immunofluorescent double stained with anti-α-synuclein antibody 3D5 shown in red (A) and MDV2 shown in green (B). The α-synuclein positive staining mainly accumulated in nuclei, and weak staining is found in cytosol with 3D5 (A) after the treatment for 4 h. The positive staining only appeared in cytosol with MDV2 (B). The merged image (C) shows that nuclei are stained by 3D5, and cytosols are stained by both MDV2 and 3D5. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

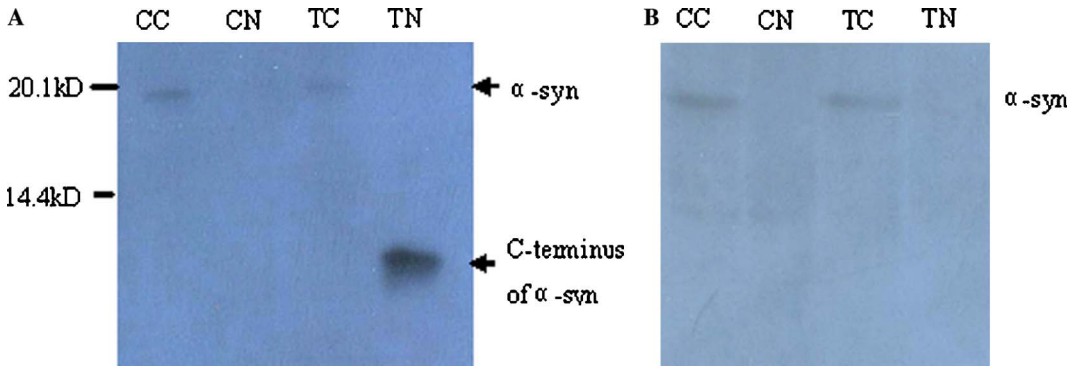

Fig. 6. The translocation of α-synuclein from cytoplasm to nucleus detected by Western blotting. (A) Western blotting of α-synuclein with 3D5 antibody that recognizes amino acids 114–120 of α-synuclein. Full-length α-synuclein is recognized as a ca. 18 kDa band in cytoplasmic fractions. The C-terminus of α-synuclein is stained as a ca. 10 kDa band in the nuclear fraction of H_2O_2-treated cells. (B) Immunostaining of α-synuclein with MDV2 antibody that recognizes N-terminus of α-synuclein. No positive band is found in the nuclear fraction of H_2O_2-treated cells, indicating that the fragment of α-synuclein in nuclei lacks the N-terminus of α-synuclein. CC, cytoplasmic fraction of control cells; CN, nuclear fraction of control cells; TC, cytoplasmic fraction of 4 h H_2O_2-treated cells; TN, nuclear fraction of 4 h H_2O_2-treated cells.

investigators have looked for and found a correlation between α-synuclein and cell death both in vivo and in vitro [3]. However, other studies have suggested that α-synuclein may function as part of a survival response in dopaminergic cells [22,23]. For example, it was shown that α-synuclein levels were increased in healthy, non-apoptotic neurons in the substantia nigra of mice treated with MPTP [7]. Our study also showed that the overexpression of human α-synuclein played a protective role against neurotoxin insult and oxidative stress even in dopaminergic neuronal cells (manuscript in preparation). The present study indicated that those cells where α-synuclein is significantly accumulated in nuclei express more TH than those where α-synuclein is not accumulated in nuclei. The expression level of TH in the former cells is as high as that of untreated cells. In conclusion, our data have shown that H_2O_2 treatment induces the translocation of the C-terminal fragment of α-synuclein from cytosol to nuclei and implied that the translocation might have a protective role in the response of dopaminergic neurons to oxidative stress.

Acknowledgments

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