Article

A novel molecular mechanism for nitrated α-synuclein-induced cell death

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Although previous studies have demonstrated the involvement of nitrated α-synuclein in neurodegenerative disorders (synucleinopathies), the effects of nitrated α-synuclein and the molecular mechanisms underlying its toxicity are still unclear. In the present study, nitrated α-synuclein with four 3-nitrotyrosines (Tyr^{39}, Tyr^{125}, Tyr^{133}, and Tyr^{136}) was obtained non-enzymatically by incubation with nitrite. The nitrated protein existed as a mixture of monomers, dimers, and polymers in solution. The nitrated α-synuclein could induce cell death in a time- and concentration-dependent manner when SH-SY5Y cells (a human neuroblastoma cell line) were incubated with the dimers and polymers. Treatment with anti-integrin α5β1 antibody partially rescued the SH-SY5Y cells from the cell death. Dot blotting and immunoprecipitation revealed that the nitrated protein bound to integrin on the cell membranes. Level of nitric oxide (NO) and calcium-independent inducible NO synthase (iNOS) activity increased during the initial stages of the treatment. The expression of phosphorylated focal adhesion kinase (FAK) decreased in the cells. Subsequently, an increase in caspase 3 activity was observed in SH-SY5Y cells. Our results demonstrate that activation of iNOS and inhibition of FAK may both be responsible for the cell death induced by nitrated α-synuclein. These data suggest that the cytotoxicity of nitrated α-synuclein is mediated via an integrin-iNOS/-FAK signaling pathway, and that the nitration of α-synuclein plays a role in neuronal degeneration.

Keywords: nitric oxide, nitration, neuronal death, toxicity, focal adhesion kinase, calcium-independent inducible nitric oxide synthase

Introduction

α-synuclein is a relatively small (140 amino acids in length), abundant, natively unfolded, soluble protein found in cells throughout the nervous system and is particularly enriched in presynaptic nerve terminals (Maroteaux et al., 1988; Martin et al., 2004). Abnormal accumulated α-synuclein is the major component of Lewy bodies in α-synuclein-related diseases (synucleinopathies) such as Parkinson's disease and Alzheimer's disease. Dysfunction of α-synuclein remains a common denominator in each of these diseases although synucleinopathies manifest diverse pathological features.

Many factors can induce intraneuronal aggregation of α-synuclein into filamentous lesions. This pathway is accompanied by post-translational modifications of α-synuclein including nitration (Duda et al., 2000), phosphorylation (Kim et al., 2006), and glycation (Luth et al., 2005; Chen et al., 2010). Aggregation of nitrated α-synuclein has been found in the brain tissues of mice with synucleinopathies, indicating that nitration plays an important role in the process of neurodegenerative diseases (Benner et al., 2008). However, the molecular mechanism of cytotoxicity induced by the nitrated protein is unclear and needs to be investigated.

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Nitration, an event known to influence protein function, is a salient feature of diverse synucleinopathies (Ischiropoulos, 2003). Reactions involving nitrite ions are increasingly recognized as a means through which nitric oxide (NO) exerts negative effects on human tissues. Wide distribution of nitrated α-synuclein has been found in brain tissues of patients with diverse types of synucleinopathies (Giasson et al., 2000). Nitrite has been shown to modify tyrosine residues in type I collagen to form 3-nitrotyrosine (Wang et al., 2007), and nitrated α-synuclein can accelerate degeneration of nigral dopaminergic neurons (Benner et al., 2008). 3-nitrotyrosine increases markedly in the rat midbrain under fasting conditions (Liu and He, 2010). Modification of tyrosine residues, which affects the phosphorylation and dephosphorylation of tyrosine, is a significant mechanism of cell regulation.

Besides the synuclein located in cells, there is also extracellular α-synuclein particularly in plasma in normal subjects. Cells normally secrete α-synuclein into their surrounding media, both *in vitro* and *in vivo* (El-Agnaf et al., 2003). Full-length α-synuclein is also present in cerebrospinal fluid from Parkinson's disease subjects (Borghi et al., 2000). It is also reported that intravesicular localization and secretion are part of normal life cycle of α-synuclein and might also contribute to pathological function of this protein (Lee et al., 2005). Recently, it was found that

Lewy bodies can spread from cell to cell (Desplats et al., 2009). Nitrated α-synuclein was found in Lewy bodies and neuritis in synucleinopathies but diffusely in the cytoplasm of scattered neurons in PiD, a frontotemporal dementia disorder (Dalfo et al., 2006). Above all, it is important to investigate the effects of extracellular α-synuclein or nitrated α-synuclein on neuronal cells.

Integrins, acting as the heart of many human diseases (Hynes, 2002), are the major metazoan receptors for cell adhesion to extracellular matrix proteins. Wright et al. (2007) have reported that amyloid β peptide binds to α2β1 and α5β1 integrins, activating Pyk2, which further leads to phosphorylation of paxillin and induces neurotoxicity. It has been demonstrated that fibrillar bovine serum albumin (BSA) causes cellular apoptosis by binding to integrin α5β1 and leads to the dephosphorylation of focal adhesion kinase (FAK; Tyr^{397}) and glycogen synthase kinase-3β (Huang et al., 2009). Sugiura et al. (2009) found that 3-nitrotyrosine significantly inhibits chemotaxis towards fibronectin through suppression of integrin α5β1 expression, while 3-nitrotyrosine enhances the expression of calcium-independent inducible NO synthase (iNOS) and NO release. Therefore, the relationship between nitrated α-synuclein and integrin α5β1/iNOS requires further investigation.

Here, we used a non-enzymatic method which mimics physiological processes at neutral pH (Wang et al., 2007) to generate nitrated α-synuclein and investigated the underlying mechanisms of nitrated α-synuclein-induced cell death. Our results show that the nitrated protein bound to integrin α5β1 on cell membranes, further inhibiting the phosphorylation of FAK, and increasing the activity of iNOS (Lerouet et al., 2002), resulting in a marked increase in caspase 3 activity and cell apoptosis. These findings provide insights revealing how nitrative lesions form in synucleinopathies.

Results

Nitration induces α-synuclein to form amorphous protein aggregations

In order to study the chemical modification of α-synuclein in the presence of nitrite, purified human recombinant α-synuclein was incubated with 200 mM $NaNO_2$ at 37°C and aliquots were taken for both SDS-PAGE and immunoblotting with Syn211, an anti-α-synuclein mouse monoclonal antibody (Figure 1A) at different time intervals. A protein band with a molecular mass of ~34 kDa was detected by SDS-PAGE. This band is most likely α-synuclein dimers formed during incubation with nitrite. Western blotting confirmed formation of the dimer. In the absence of nitrite, this dimer band was not detectable by either SDS-PAGE or western blotting (Figure 1B). These results indicate that α-synuclein is dimerized in the presence of nitrite, and are in agreement with the report of Souza et al. (2000) that under oxidative/nitrative conditions, short oligomers of α-synuclein may be covalently cross-linked by dityrosine formation, which makes the polymerization process irreversible.

To determine the effective range of nitrite concentration for the above effects, α-synuclein was incubated with 100, 200, 400, and 800 mM $NaNO_2$. Exposure to $NaNO_2$ led to dimerization and a limited amount of polymerization in a concentration- and time-dependent manner (Figure 2A and B). Dimerization and

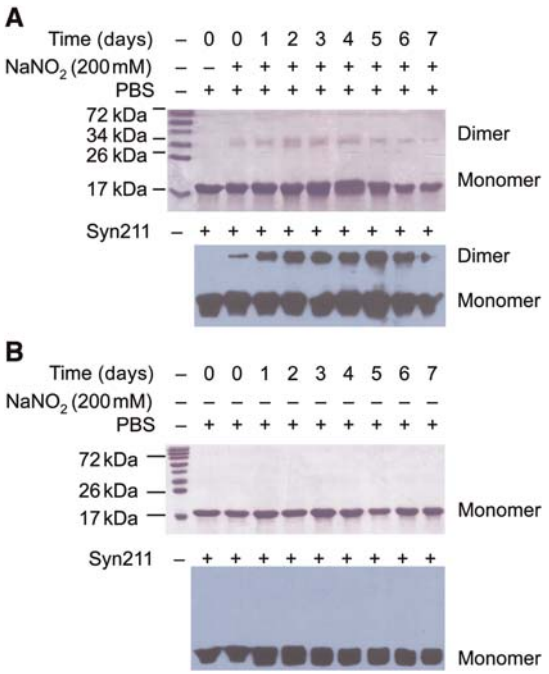

Figure 1 Effects of nitration on α-synuclein in the presence of sodium nitrite. (**A** and **B**) Purified human recombinant α-synuclein protein (1 mg/ml) was treated with (**A**) or without (**B**) 200 mM $NaNO_2$ for up to 7 days, aliquots were taken at different time intervals and analyzed by 15% SDS-PAGE following with Coomassie brilliant blue stain (upper panels) or western blotting (lower panels) using Syn211, an anti-α-synuclein monoclonal antibody.

polymerization of α-synuclein in the presence of 100 mM $NaNO_2$ was undetectable. In contrast, 800 mM nitrite resulted in many protein aggregations that caused problems in SDS-PAGE and western blotting analyses (data not shown). Therefore, 200 and 400 mM $NaNO_2$ concentrations were used in subsequent α-synuclein nitration experiments.

Monoclonal antibody nSyn12, which recognizes 3-nitrotyrosines at positions 125 and 136 in α-synuclein, was used to check whether α-synuclein was nitrated (Figure 2C and D). Nitration of the protein was detected in both the 200 and 400 mM nitrite treatments (37°C). Monomers, dimers, and polymers of nitrated α-synuclein were observed after the 3-day incubation (Figure 2E–G), indicating that $NaNO_2$ efficiently induced α-synuclein nitration and polymerization under these conditions. Interestingly, analysis of nitration efficiency showed that nitration was more efficient at a $NaNO_2$ concentration of 200 mM rather than 400 mM (Figure 2H) in the 3- and 4-day incubations. This may be because higher concentrations of nitrite induced more protein aggregation. Thus, we subsequently used 200 mM nitrite in all experiments unless stated otherwise. The average molecular mass of nitrated α-synuclein increased by ~180 Da, equal to that of four nitro groups, compared with that of the native protein detected by MALDI-TOF mass spectrometry (data not shown). As there are four tyrosinyl residues (Tyr^{39}, Tyr^{125}, Tyr^{133}, and Tyr^{136}) in α-synuclein, the increase in the molecular mass of the nitrated protein may be due to the modification of all four residues.

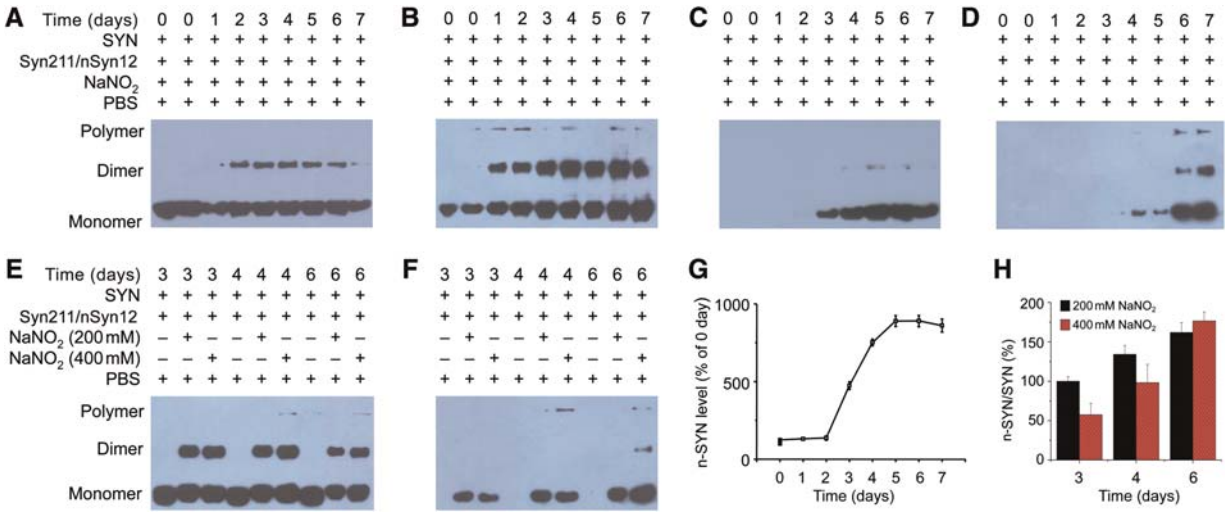

Figure 2 Nitration and oligomerization of α-synuclein. (**A**–**F**) Recombinant α-synuclein was reacted with 200 mM (**A** and **C**) and 400 mM (**B** and **D**) $NaNO_2$ at 37°C for the durations indicated and then probed with Syn211 (**A**, **B**, and **E**) or nSyn12 (**C**, **D**, and **F**), an anti-nitro-α-synuclein antibody. Equal amounts of protein were loaded in each lane. (**G** and **H**) The time course of changes in nitrated α-synuclein (**G**) and the relative nitration of α-synuclein at different concentrations of $NaNO_2$ (**H**) are shown in gray scale. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

To investigate the effect of nitration on the structure of α-synuclein, we determined its conformation by measuring changes in protein absorbance, circular dichroism and fluorescence. A significant increase in absorbance appeared at 430 and 350 nm when α-synuclein was nitrated (Supplementary Figure S1A), in agreement with previously published results (Crow and Ischiropoulos, 1996; van der Vliet et al., 1996). 3-nitro-L-tyrosine was used as a standard and gave an absorption peak at 430 nm. The emission peak present at 350 nm possibly indicates protein polymerization. The presence of a peak at 430 nm confirms that α-synuclein is nitrated under these experimental conditions.

We then measured protein intrinsic fluorescence and observed a marked decrease in the emission intensity of nitrated α-synuclein compared with that of the control (Supplementary Figure S1B). The fluorescence of nitrated α-synuclein was as low as that of 3-nitro-L-tyrosine, indicating that nitration quenched the fluorescence of tyrosinyl residues. Thus, decreases in intrinsic fluorescence observed here cannot be used to determine conformational changes in α-synuclein. Circular dichroism spectrum measurements of secondary structure showed that nitration did not result in a significant difference in secondary structure between nitrated and native α-synuclein (Supplementary Figure S1C).

As nitrated α-synuclein polymerizes by itself, we used thioflavin T (ThT) fluorescence to examine polymer characteristics and found that the ThT fluorescence intensity of nitrite-treated α-synuclein increased throughout the incubation compared with that of native α-synuclein (Supplementary Figure S1D). To investigate the morphology of nitrated α-synuclein, aliquots were taken at different time intervals and observed by electron microscopy (Supplementary Figure S2). As was the case for results from SDS-PAGE and western blotting, amorphous protein aggregations could be observed under the electron microscope during the incubation. However, we did not observe any α-synuclein fibrils. This finding is consistent with a previous report that nitration of a tyrosinyl residue in α-synuclein can prevent fibril formation (Norris et al., 2003). Taken together, our results show that nitration causes α-synuclein to form amorphous aggregations.

Nitrated α-synuclein is cytotoxic

To gain some insight into the function of nitrated α-synuclein, human neuroblast cell line SH-SY5Y cells were treated with nitrated α-synuclein and cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Nitrated α-synuclein inhibited cell viability in a dose-dependent manner (Figure 3). The concentration of nitrated α-synuclein for half-inhibition of cell viability was 2.6 ± 0.5 μM under our experimental conditions (Figure 3A). Nitrated α-synuclein at 10 μM decreased cellular viability to ~10% of the control. $NaNO_2$ used as a positive control (Figure 3B) had a much higher half-inhibition dose (150 mM) than nitrated α-synuclein, and native α-synuclein as a negative control exhibited little inhibition of cell viability. This demonstrates that nitrated α-synuclein significantly inhibits cell viability (Figure 3C).

To clarify which component of nitrated α-synuclein protein is cytotoxic, we isolated monomers after chemical modification and detected the effect of different component on cells by CCK-8 assay. No changes in cell viability were observed in the presence of monomers (Figure 3D). However, the mixture of nitrated products still showed marked cytotoxicity to SH-SY5Y cells under the experimental conditions used. The viability of cells treated with native α-synuclein (negative control) and $NaNO_2$ (positive control) supports the above results. This suggests that nitrated α-synuclein becomes cytotoxic when protein dimers and polymers are formed.

Flow cytometry was used to quantitatively estimate both nitrating agents and nitrated α-synuclein-induced apoptotic cell death. Using an annexin V-FITC apoptosis detection kit, we found that nitrated α-synuclein induced cell death in a time-dependent manner (Figure 4). About $5.42\% \pm 1.03\%$ of the cells underwent

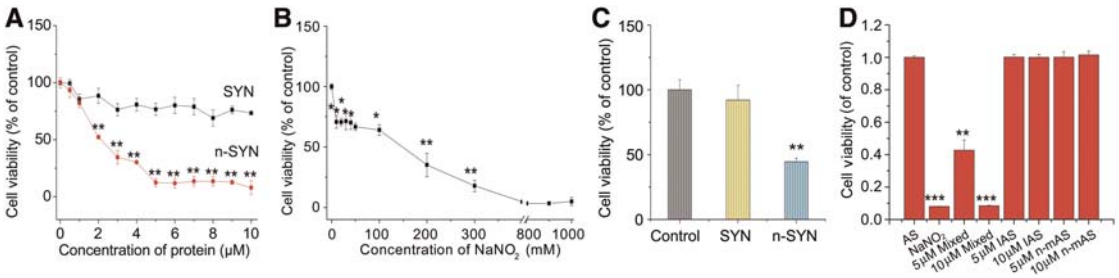

Figure 3 Inhibitory effects of nitrated α-synuclein on cell viability. (**A** and **B**) SH-SY5Y cells were incubated with different concentrations of nitrated α-synuclein (**A**) or different concentrations of $NaNO_2$ (**B**) for 24 h before measuring cell viability. (**C**) SH-SY5Y cells were treated with 5 μM α-synuclein or nitrated α-synuclein (untreated cells as the control) for 24 h, and then collected for whole cell viability measurements. Cell viability values are expressed as percentages of the control. (**D**) Cells were incubated with nitrated α-synuclein monomers, isolated α-synuclein monomers, mixed nitrated α-synuclein, $NaNO_2$, and native α-synuclein, and cell viability was assayed using the CCK-8 assay. AS, native α-synuclein; mixed, mixed nitrated α-synuclein; IAS, isolated α-synuclein monomer; n-mAS, nitrated α-synuclein monomer. All values are mean ± SD, $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

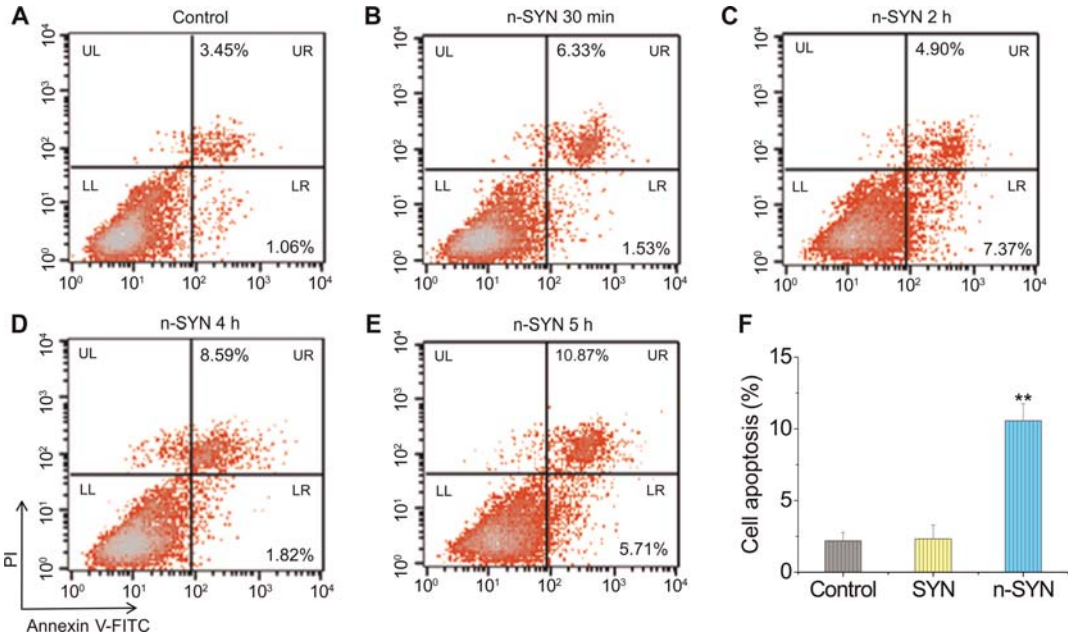

Figure 4 Nitrated α-synuclein induces apoptosis in SH-SY5Y cells. (**A**–**E**) SH-SY5Y cells were treated for analysis of cell apoptosis using flow cytometry, with 5 μM nitrated α-synuclein and collected at different time intervals (**B**–**E**), 5 μM native α-synuclein was used as a control (**A**). Data are representative of three separate experiments. (**F**) Cells treated with 5 μM nitrated α-synuclein were compared with those treated with native protein (untreated cells were used as a control) for 4 h. Representative cytograms of cells stained with annexin V-FITC and PI. LL, viable cells; LR, cells in early apoptosis; UR, cells in late apoptosis; UL, cells in necrosis. Data are mean ± SD, $n = 3$. $^{**}P < 0.01$ versus control. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

cell apoptosis at the early stages (up to 2 h) of exposure to nitrated α-synuclein. An increase in cell apoptosis was observed after cells were treated with nitrated α-synuclein for longer than 2 h. After treatment with nitrated α-synuclein for 4 h, more SH-SY5Y cells (10.57% ± 1.18%) were apoptotic compared with the control (2.19% ± 0.99%). Native α-synuclein used as a negative control had no significant effect on the induction of cell death. About 50% of SH-SY5Y cells treated with 200 mM $NaNO_2$ for 2 h, as a positive control, showed chromatin condensation and nuclear fragmentation under fluorescence microscopy using Hoechst 33342 as a DNA stain (data not shown). These results show again that nitrated α-synuclein is toxic to SH-SY5Y cells.

Changes in integrin α5β1 and phosphorylation of FAK in the presence of nitrated α-synuclein

Huang et al. (2009) reported that the integrin/FAK/caspase 3-signaling pathway is involved in modulating apoptosis caused by BSA. They found that fibril BSA could bind to integrin α5β1 and cause cellular apoptosis. To explore the underlying mechanism of cell death in the presence of nitrated protein, we first investigated whether the apoptotic effects of nitrated α-synuclein were mediated via integrin α5β1. Thus, SH-SY5Y cells were pre-treated separately with IgG and anti-integrin α5β1 antibody, then incubated with nitrated α-synuclein in serum-free medium. MTT assay results revealed that pre-treated SH-SY5Y cells with anti-integrin α5β1 antibody, but not rabbit IgG, diminished the

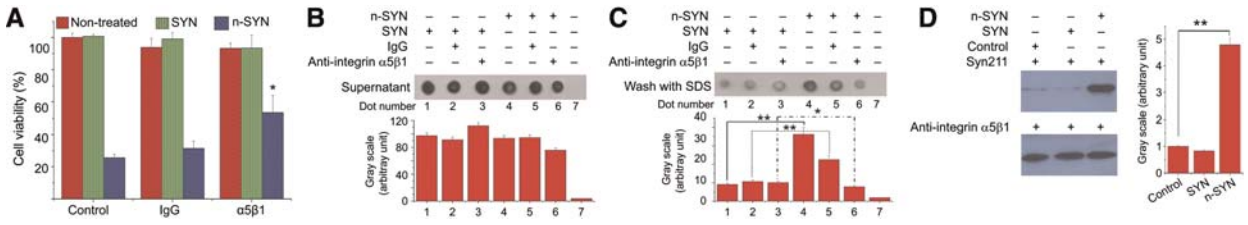

Figure 5 Nitrated α-synuclein interacts with integrin α5β1. SH-SY5Y cells were pre-treated with or without 0.5 μM rabbit IgG and 0.5 μM rabbit anti-integrin α5β1 for 30 min and then incubated with 5 μM nitrated α-synuclein (n-SYN) or 5 μM native α-synuclein (SYN) in serum-free medium for 4 h or 8 h. Untreated cells were used as a control. (**A**) Cells were collected for MTT assay. (**B** and **C**) The culture supernatants (**B**) and bound proteins on the cells (**C**) after 4 h incubation were collected for dot blotting, Syn211 was used as the primary antibody for blotting, and data were statistically analyzed. (**D**) Immunoprecipitation was carried out with anti-integrin α5β1 antibody, and data were statistically analyzed. Data are mean ± SD ($n = 3$), $*P < 0.05$; $**P < 0.01$ versus control. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

cytotoxic effects of nitrated α-synuclein (Figure 5A). Dot blotting results (Figure 5B and C) showed that nitrated protein bound to cells in the absence of the anti-integrin α5β1 antibody, indicating that nitrated α-synuclein associates with α5β1 on cell membranes. Immunoprecipitation results also revealed that nitrated α-synuclein could directly interact with integrin (Figure 5D). However, native α-synuclein showed no significant interaction with integrin α5β1. Taken together, these results suggest that nitrated α-synuclein leads to cell apoptosis by binding to integrin α5β1 in SH-SY5Y cells.

We then investigated whether molecules such as FAK and caspase 3, which are involved in the integrin signaling pathway cascade, were affected by nitrated α-synuclein. Results from western blotting and immunoprecipitation using an anti-FAK monoclonal antibody did not show a significant increase in the expression of FAK in the presence of nitrated α-synuclein (Figure 6A). However, the activation of FAK detected by immunoprecipitation with anti-p-Tyr antibody (PY99, recognizes phosphotyrosine) revealed a decrease in the FAK activation of cells treated with nitrated α-synuclein at the early stages of treatment (before 30 min), and then an increase after 1 h of treatment (Figure 6B). $NaNO_2$ used as a positive control also decreased the activity of FAK in cells. Native α-synuclein as a negative control had no significant effect on the phosphorylation of FAK. These results suggest that FAK is activated in response to treatment with nitrated α-synuclein.

Changes in NO production and iNOS activity in cells treated with nitrated α-synuclein

It has been suggested that NO plays an important role in the process of cell death (Decker et al., 2008; Bolton et al., 2009; Golli Bennour et al., 2009). Previous research has shown that NO can result in the activation of FAK, which leads to tyrosine phosphorylation in proteins (Garcia-Benito et al., 2000; Gemba et al., 2002). Using 3-amino,4-aminomethyl-2′,7′-difluorescein, diacetate (DAF-FM DA) to detect the production of NO in cells, we observed that nitrated α-synuclein promoted NO production in SH-SY5Y cells compared with control cells (Figure 7A), and this was confirmed by quantitative analysis (Figure 7B). The concentration of NO in cells treated with nitrated α-synuclein was 151.14 ± 3.40 μM, much higher than that in untreated cells (36.63 ± 1.89 μM) or control cells treated with native α-synuclein (32.89 ± 4.16 μM) (Figure 7C).

Caspase 3 activity of cells treated with nitrated protein was then investigated. Results showed that caspase 3 activity

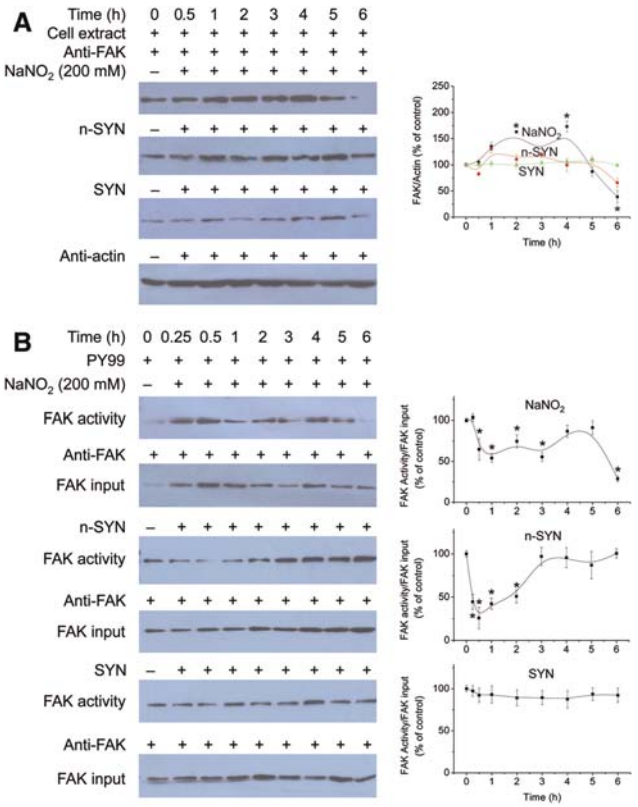

Figure 6 Effect of the nitration of α-synuclein on FAK activation in SH-SY5Y cells. Cells were incubated with 200 mM $NaNO_2$, nitrated α-synuclein, and native α-synuclein, respectively. (**A** and **B**) Aliquots were taken at different time intervals for western blotting with mouse anti-FAK monoclonal antibody (**A**) or immunoprecipitation carried out with anti-FAK monoclonal antibody, followed by western blotting with mouse monoclonal antibody p-Tyr (PY99) (**B**), and data were statistically analyzed. Data are mean ± SD, $n = 3$, $*P < 0.05$ for comparisons with the control. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

increased significantly when cells were treated with nitrated α-synuclein compared with control cells treated with native α-synuclein and BSA (Figure 7D).

To investigate whether this increased NO production was induced by nitrated α-synuclein, SH-SY5Y cells were treated with 5 μM nitrated α-synuclein and then collected for iNOS production

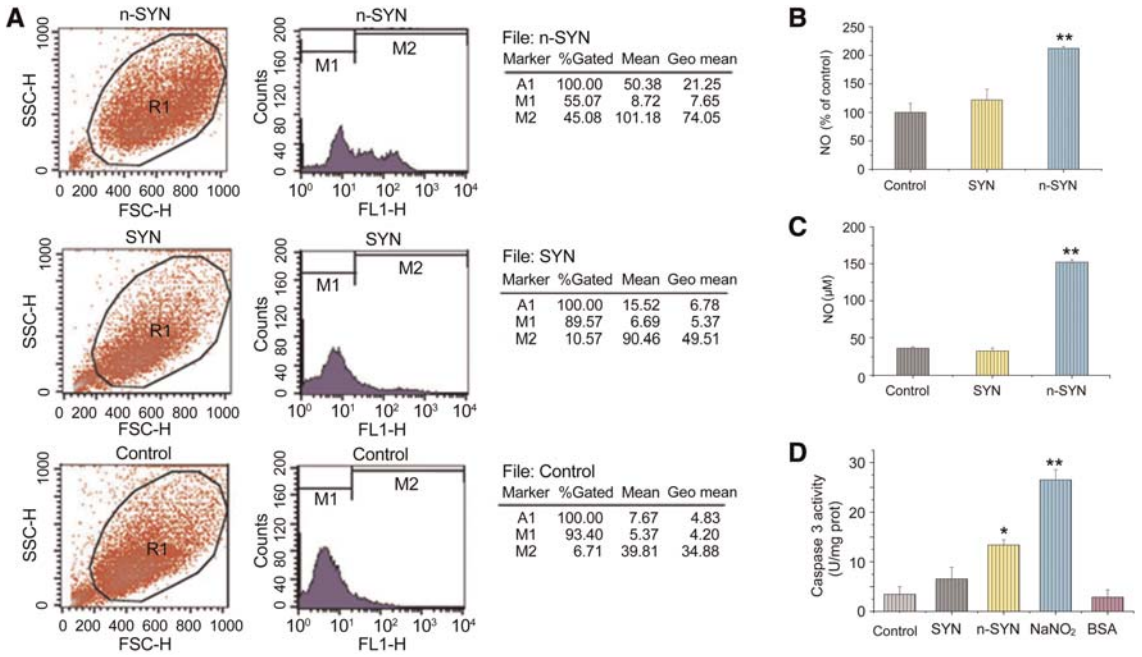

Figure 7 Nitration of α-synuclein enhances NO production in SH-SY5Y cells. (**A** and **B**) Cells were exposed to 5 μM nitrated α-synuclein for 2 h, labeled with DAF-FM DA for 20 min and then analyzed using flow cytometry. Native α-synuclein alone was used as a control. M1 and M2 represent the numbers of cells with negative and positive DAF-FM DA signals, respectively. The M2 geo mean of the control was regarded as 100% to calculate the relative NO content for the cells incubated with nitrated α-synuclein and native α-synuclein. (**C**) Cells were exposed to 5 μM nitrated α-synuclein for 24 h and then collected to determine the content of NO in cells using a NO detection kit. (**D**) Caspase 3 activity was detected by flow cytometry. Data are mean ± SD, $n = 3$. $^*P < 0.05$; $^{**}P < 0.01$ for comparisons with normal cells. SYN, α-synuclein; n-SYN, nitrated α-synuclein.

analysis. The level of cellular iNOS increased significantly in SH-SY5Y cells in the presence of nitrated α-synuclein (5 μM) (Figure 8B). When cells treated with native α-synuclein were used as a control, no marked increase in iNOS expression was detected (Figure 8C). Since previous reports suggest that nitrite increases the level of cellular iNOS (Ucar et al., 2007; Jiang et al., 2009), we investigated the effect of various concentrations of $NaNO_2$ on iNOS expression. Western blotting revealed that after 24 h of $NaNO_2$ (0–100 mM) treatment, the production of iNOS increased when the concentration of $NaNO_2$ was <30 mM (Figure 8A). Higher concentrations of $NaNO_2$ did not elevate this production further but rather inhibited iNOS production in SH-SY5Y cells, showing that the production of iNOS decreased as the concentration of $NaNO_2$ increased to >30 mM. Cells were then treated with a higher concentration of $NaNO_2$ (200 mM) for various lengths of time as indicated, and the inhibitory effect of iNOS production was observed in cells (Figure 8D).

To confirm that nitrated α-synuclein was able to induce iNOS production, we detected the iNOS activity of cells using an iNOS activity detection kit. Consistent with results from western blotting, there was a higher level of iNOS activity in cells treated with nitrated α-synuclein than in cells treated with native α-synuclein during the first 3 h of incubation (Figure 8E and F). It is of note that iNOS activity increased during the early stages of treatment (within 15 min). Control cells treated with 200 mM $NaNO_2$ did not show a significant increase in iNOS activity, unlike cells treated with nitrated α-synuclein. This suggests that nitrated α-synuclein induces the expression of cellular iNOS.

As described above, FAK activity increased during the first 3 h of treatment with nitrated α-synuclein. Interestingly, when we compared FAK and iNOS activity, we found that there was an inverse correlation between FAK and iNOS activity (Figure 8G). These results suggest that there is more than one pathway to cell death when cells are treated with nitrated α-synuclein.

Discussion

Abnormal post-translational modifications including phosphorylation, glycosylation, ubiquitination, and glycation have previously been reported to play different roles in the development of α-synucleinopathies. In addition to the post-translational modifications described above, other reports provide evidence of nitration of α-synuclein in pathological inclusions of neurodegenerative diseases (Duda et al., 2000; Giasson et al., 2000), raising the possibility that nitrative damage may have a direct role in the initiation and progression of these diseases.

Several studies have shown that chemical and metabolic modifiers can alter the kinetics of α-synuclein fibril formation as well as the biophysical properties of the fibrils (Souza et al., 2000; Kim et al., 2007; Martinez-Vicente et al., 2008; Paleologou et al., 2008). Tyrosine nitration is an important post-translational modification of α-synuclein and has been shown to occur *in vivo* in several human pathological conditions. Previous studies showed that nitrating agents such as peroxynitrite and nitrogen dioxide are strong oxidants capable of promoting not only nitration but also oxidation of tyrosine and other amino acids

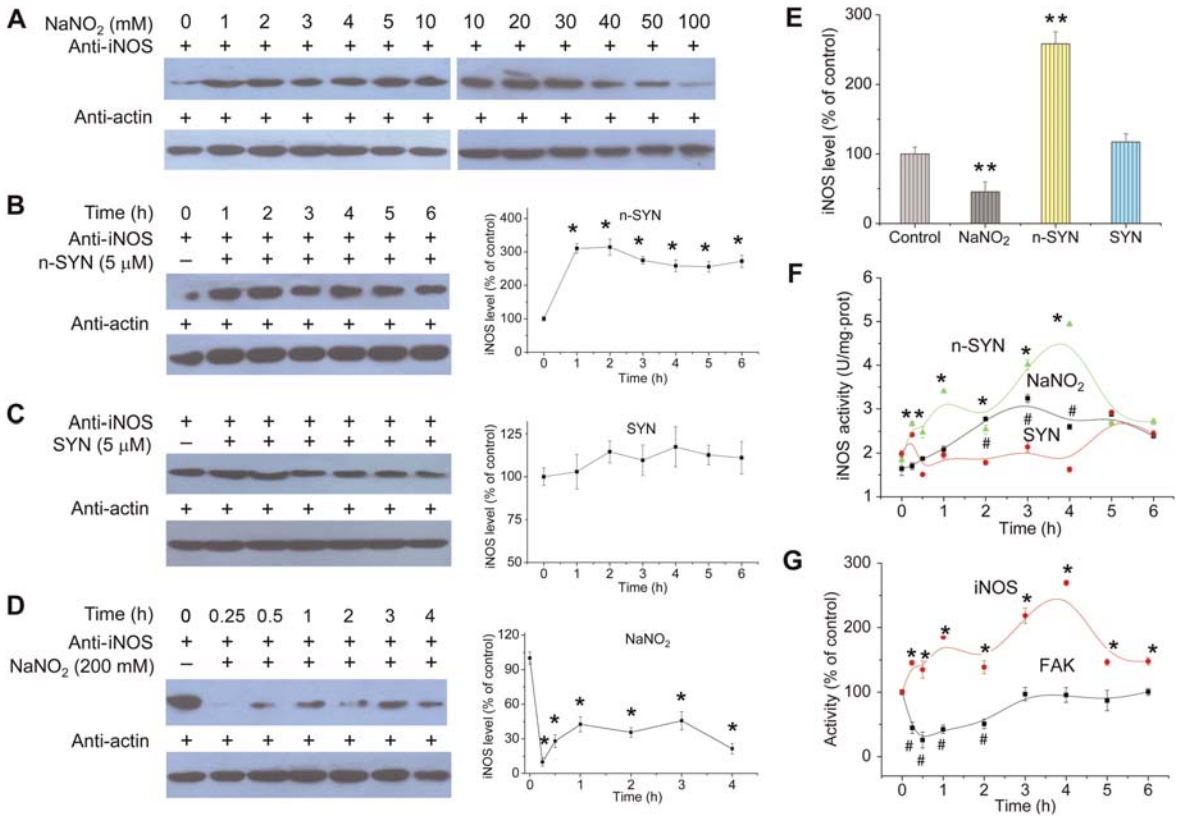

Figure 8 iNOS production in SH-SY5Y cells. (**A**) The content of iNOS in cells was detected using western blotting on 10% SDS-PAGE. Cells were treated with different concentrations of $NaNO_2$ for 24 h and then collected for determination of iNOS production by western blotting on 10% SDS-PAGE. (**B**–**F**) Cells were treated with 5 μM nitrated α-synuclein (**B**), 5 μM α-synuclein (**C**), or 200 mM $NaNO_2$ (**D**) and then collected for measurements of iNOS level (**E**) and activity (**F**) with corresponding detection kits. (**G**) Comparison of FAK activity and iNOS activity. Data are mean ± SD, $n = 3$. $^{*,\#}P < 0.05$ versus control; $^{**}P < 0.01$ for comparisons with normal cells. SYN, α-synuclein; n-SYN, nitrated α-synuclein; iNOS, calcium-independent inducible nitric oxide synthase.

(Paxinou et al., 2001; Ischiropoulos, 2003; Zhang et al., 2006). However, these studies have used peroxynitrite as an inducer for short durations, and the pH values of the reaction buffers used were lower than physiological conditions. During the pathogenesis of many diseases, tissues may be exposed to elevated nitrite levels for prolonged periods under physiological conditions. Thus, conducting experiments at neutral pH is more likely to mimic *in vivo* processes involving long-lived extracellular proteins. On the basis of these considerations, and in contrast to the studies previously noted, we have focused primarily on nitrite-induced modifications to proteins at neutral pH. The rate of reactions at neutral pH is slow, and may thus mimic the true situation of protein nitration in living cells.

Since these modifications accumulate over decades in living organisms, it is necessary to use nitrite concentrations that are higher than those found under physiological conditions. In the present study, we used $NaNO_2$ (200 mM) as an inducer to generate nitrated α-synuclein at neutral pH for different lengths of time. We found that nitrated α-synuclein was successfully generated after incubation with $NaNO_2$ for 3 days and that nitrated α-synuclein was maintained at a high level for up to 7 days as determined by western blotting analysis (Figure 1). Furthermore, all four tyrosinyl residues can be nitrated in the presence of 200 mM $NaNO_2$. Increased concentrations of $NaNO_2$ (400 mM) induced the production of more dimers and polymers of α-synuclein (Figure 2), but resulted in protein aggregates.

In order to study conformational changes in the modified protein, we measured the intrinsic fluorescence and the absorbance of the protein. As shown above, the protein intrinsic fluorescence was quenched when α-synuclein was nitrated. We then investigated the secondary structure of nitrated α-synuclein by recording circular dichroism spectra. Conformational changes in the modified protein were not detected. To clarify whether the nitrated protein formed aggregates, ThT staining was performed and the fluorescence was measured. Consistent with previous observations from our laboratory (Wei et al., 2009), modified protein aggregates, namely amyloid aggregations, were detected by ThT positive fluorescence (Supplementary Figure S1D). The results of electron microscopy further clarified that nitrated α-synuclein had formed amorphous aggregations. These results corroborated previous findings that nitrated α-synuclein is incapable of forming fibrils (Hodara et al., 2004).

The effects of nitrated α-synuclein *in vivo* and intracellular conditions that favor iNOS production are not completely understood. Using SH-SY5Y cells as a model, we studied the effect of nitrated α-synuclein on cell death. We found that nitrated

α-synuclein induced cell death in a time-dependent manner. Cells observed under the microscope were round and swollen with shrunken nuclei, showing some typical apoptotic features (data not shown). In contrast, cells treated with native α-synuclein maintained their normal shape and function under the experimental conditions used. Assessment of cell mitochondrial function using the MTT and CCK-8 assays indicated that cell viability was significantly reduced in the presence of nitrated α-synuclein (nitrated monomers having no cytotoxicity). That is to say, α-synuclein becomes cytotoxic after the protein was nitrated and converted to dimers and polymers. Furthermore, the results from the annexin V-FITC assay showed that cell apoptosis could be induced by nitrated α-synuclein in a time-dependent manner.

Next, we investigated how nitrated α-synuclein induces cell death. As described by Huang et al. (2009), albumin fibrillization causes cellular apoptosis by binding to integrin α5β1 although nitrated α-synuclein has not been reported as a ligand for integrin. Wright et al. (2007) have reported that Aβ binds to α2β1 and α5β1 integrin to activate Pyk2 and phosphorylated paxillin and induces neurotoxicity. Here, we found that anti-integrin α5β1 antibody can inhibit nitrated α-synuclein-mediated cell apoptosis (Figure 5). Furthermore, the association between the nitrated protein and the integrin suggests that nitrated α-synuclein activates the pathway related to integrin. However, integrin contains other subtypes such as α3β1 and α5β3 which are reported to play roles in apoptosis (Montgomery, 1992; Tsuji, 2004). Our work reveals that integrin α5β1 may be one of the important factors involved in nitrated protein-mediated cell apoptosis.

We then detected the expression and cellular FAK activity of cells in the presence of nitrated α-synuclein. A rapid decrease in the activation of FAK (p-FAK; Figure 6B) was observed in comparison with native α-synuclein within 15–30 min of incubation. However, the expression level of FAK did not change significantly under the experimental conditions. Thus, incubation with nitrated α-synuclein leads rapidly to the inhibition of FAK phosphorylation, but not to the down-regulation of FAK expression.

It is well known that a high concentration of NO induces apoptotic cell death in neuronal cells (Feil and Kleppisch, 2008; Bolton et al., 2009). As shown above, nitrated α-synuclein induced and enhanced the activation of NO production in cells during the initial stages of the incubation (up to 15 min). The up-regulation of the expression of iNOS during the initial stages of the incubation with nitrated α-synuclein also confirmed the increase of NO level in the cells. The increase in NO level associated with activation of caspase 3 may lead to cell apoptosis.

Our results show that high concentrations of NO can activate the phosphorylation of FAK, inducing apoptotic cell death (Figure 8G). This is consistent with a report by Garcia-Benito et al. (2000). Protein tyrosine nitration results in a post-translational modification and is receiving increasing attention as an important component of NO signaling. It has been well documented that NO can inhibit the activity of several enzymes of neuronal cells and that NO reacts with superoxide at a high rate to form peroxynitrite, which is far more reactive and damaging than its precursors (Reynolds et al., 2007; Feil and Kleppisch, 2008; Bolton et al., 2009). In our experiments, iNOS activity increases with a simultaneous decrease in FAK activity, supporting the idea that NOS could be activated via an FAK-independent pathway (Gupta and Vlahakis, 2009).

Taken together, the results of the present study provide new information about α-synuclein nitration. Nitrated α-synuclein was successfully obtained by using $NaNO_2$ at a neutral pH *in vitro*. Nitrated α-synuclein aggregations were toxic to SH-SY5Y cells. Concurrent inhibition of FAK and activation of iNOS activity may be responsible for the cell death induced by nitrated α-synuclein via the integrin pathway. Our data suggest that the key event(s), which are induced by nitrated α-synuclein and trigger cell death, may be related to the enhanced level of NO and may trigger integrin pathway in cells (Tang et al., 2007). As discussed above and shown in Supplementary Figure S3, there is a complex interplay between these proteins, which finally promotes caspase 3-mediated cellular apoptosis. Future work will focus on the presence of nitrated α-synuclein *in vivo* and its potential involvement in the pathogenesis of synucleinopathies.

Materials and methods

Protein purification, nitration, and ultrafiltration

Human wild-type α-synuclein was expressed in *Escherichia coli* BL21(DE3). The recombinant α-synuclein protein was isolated and purified as described previously (Huang et al., 2005). α-synuclein (1 mg/ml) was incubated with or without (control) 200 mM $NaNO_2$ dissolved in 10 mM phosphate-buffered saline (PBS; pH 7.4) in the dark for 1 week at 37°C. Nitrated protein products were separated from unincorporated $NaNO_2$ by ultrafiltration. Ultrafiltration is a membrane process whereby large molecules are retained in the solution in the stage of centrifugation, for the most part, by size exclusion. In the procedure, 1 ml of the mixture was placed in an Amicon ultra-4 tube (Millipore) and diluted with 9 ml phosphate buffer before centrifugation (1906 *g*, 4°C, 20 min). After centrifugation for 20 min, there is ~1 ml solution left in the Amicon ultra-4 tube; such a procedure was repeated three times. For detection of nitrated Tyr residues, α-synuclein was incubated with 200 mM $NaNO_2$ for 6 days, and the nitrated Tyr residues were detected with MALDI-TOF mass spectrometry (Axima-CFR Plus) after the sample was ultrafiltrated four times. Protein concentrations were determined using a BCA™ protein assay kit (Thermo).

To clarify whether nitrated α-synuclein monomers are cytotoxic, the nitrated products were analyzed by 15% SDS-PAGE. The monomer bands in the gel were cut out and extracted (4°C, overnight) with a mini protein gel extraction kit according to the manufacturer's instructions (Tiandz Inc.). The mixture was centrifuged at 10000 *g*, 4°C, for 10 min. The supernatant (200 μl) was mixed with precipitating organic solvent (1 ml). After placing at −20°C for 30 min, the protein samples were centrifuged (12000 *g*, room temperature, 20 min) and pellets were collected. Native α-synuclein was also processed in the same way and then used as a control. Subsequently, the monomers were used in cell viability assays. The nitrated protein (containing monomers, dimers, and polymers), native α-synuclein, and 200 mM $NaNO_2$ were also added, respectively, to the cell media as controls.

Fluorescence and spectrophotometric analysis

Human α-synuclein treated with or without 200 mM $NaNO_2$ for the indicated time period was collected and absorbance was

measured from 200 to 600 nm using a U-2010 spectrophotometer (Hitachi). For fluorescence analysis, the intrinsic fluorescence of recombinant α-synuclein incubated with or without different concentrations of $NaNO_2$ was measured at an excitation wavelength of 280 nm using an F-4500 fluorescence spectrophotometer (Hitachi).

Circular dichroism and thioflavin T staining

The circular dichroism spectra of nitrated α-synuclein and the untreated control were recorded at 25°C using a PiStar kinetic circular dichroism spectrometer (Jasco-720). To analyze the formation of amyloidogenic fibrils, ThT (Sigma) staining was carried out. A solution of ThT (final concentration of 10 μM) was prepared in 90 mM glycine-NaOH buffer (pH 8.5) prior to measurements. α-synuclein protein treated with or without 200 mM $NaNO_2$ at the time points indicated was collected and incubated in a ThT solution (final concentration of 5 μM) for 15 min. Fluorescence intensity was then detected (λ_{ex} = 440 nm; λ_{em} = 480 nm; band pass = 5 nm).

Negative stain electron microscopy

α-synuclein protein reacted with 200 mM $NaNO_2$ for different lengths of time was placed on a formvar-coated EM grid (10 μl, sufficient to cover the grid surface) and was incubated at 25°C for 1 min. The liquid was subsequently dried-off with filter paper. The samples were washed twice with ddH_2O and soaked in 3% uranyl acetate for 1 min. Samples were visualized using a JEM-100CX electron microscope (JEOL Ltd.).

Western blotting

Total proteins (10–30 μg) from cell lysates were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Nitrocellulose membranes were blocked for 1 h in 5% (*w*/*v*) non-fat-dried milk and probed overnight at 4°C with the anti-α-synuclein monoclonal antibody Syn211 (1:1000) (Sigma), anti-nitro-α/β-synuclein nSyn12 (1:1000) (Upstate), rabbit anti-NOS-2 antibody (1:500) (BIOS), anti-FAK antibody (1:1000) (Santa Cruz), monoclonal anti-3-nitrotyrosine antibody (1:1000) (Sigma), p-Tyr (PY99) (1:1000) (Santa Cruz), anti-integrin α5β1 antibody (1:500) (BIOS), and anti-β-actin antibody (1:1000) (Santa Cruz). The membrane was washed three times with Tris-buffered saline containing Tween-20 (TBST), and then incubated with either a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h at 37°C. The blot was washed three times with TBST, which was developed with SuperSignal horseradish peroxidase substrate (Thermo), and chemiluminescence was detected on Kodak XAR film.

Cell culture and exposure to nitrate

To study the effects of nitrated α-synuclein on cells, SH-SY5Y cells were seeded on 6-well (2×10^6 cells/well) or 24-well (1×10^4 cells/well) multi-dishes. Culture medium consisted of phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For treatments, cells, maintained at 37°C in a humidified 5% CO_2/95% air atmosphere, were incubated with additional $NaNO_2$ (200 mM), nitrated α-synuclein (5 μM), or native α-synuclein (5 μM).

Cell viability assay

Cell viability was determined with the MTT (Sigma) reduction assay or CCK-8 colorimetric assay kit (Dojindo Laboratories). 1×10^4 cells were seeded in 96-well plate and incubated for 24 h with various concentrations of $NaNO_2$ or nitrated α-synuclein. Control cells were incubated with the corresponding concentration of native α-synuclein. Following this incubation, cell viability was detected by MTT reduction assay or CCK-8 assay. For MTT reduction assay, cells were treated with 0.5 mg/ml MTT for 4 h followed by exposure to 100% dimethylsulfoxide for 10 min. The optical density (OD) of the dissolved formazan grains within the cells was measured spectrophotometrically at 490 nm. The viability of control cells was set to 100%, and the percentage of viable cells collected from each treatment was calculated relative to the control group. For CCK-8 assay, 10% CCK-8 solution (diluted by DMEM) was added to each well, after 4 h incubation, the OD was measured spectrophotometrically at 450 nm. The viability of control cells was set to 1 and the increase-fold in the OD compared with that of the control was calculated.

NO concentration assays

Cells treated as described above were collected by centrifugation at 800 *g*. After the pellets were dissolved in 0.9% sodium chloride, the media were centrifuged (384 *g*, 4°C, 5 min) and the supernatants were collected. NO level analysis was performed with a NO content detection kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. NO was assayed spectrophotometrically (550 nm) by measuring total nitrate plus nitrite (NO_3^- plus NO_2^-).

iNOS activity assay

iNOS activity assay was performed with an iNOS assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. NOS catalyzes the formation of NO and L-citrulline from L-arginine and molecular oxygen, and NO reacts with a nucleophile to generate colored compounds. One unit of NOS activity was defined as the production of 1 nmol NO per second per milligram cell lysate protein. The activity of iNOS was measured at 530 nm.

Immunoprecipitation and dot blotting

SH-SY5Y cells (2×10^6) were scraped from culture plates into PBS and collected by centrifugation at 500 *g*. Cells were lysed in ice-cold immunoprecipitation buffer (Beyotime Inc.). After centrifuging the homogenate (14000 *g*, 4°C, 15 min), 500 μl supernatant was incubated with 2 μg of anti-FAK antibody (Santa Cruz) or anti-integrin α5β1 antibody (BIOS) per 100–500 μg of total protein overnight at 4°C with gentle shaking. The antigen/antibody immunocomplex was captured by further incubating with 100 μl of PBS-washed protein A/G agarose beads for 2 h and by spinning for 2 min at 800 *g*. The beads were washed three times with PBS and resuspended in 10 μl 4 × sample buffer. After boiling for 5 min in a water bath, the supernatant was collected by centrifugation at 14000 *g* for 1 min. Proteins were then analyzed by western blotting using an anti-FAK antibody, and the same membrane was stripped and pre-probed with p-Tyr (PY99) to detect the phosphorylated FAK (p-FAK). Syn211 was used in detecting anti-integrin α5β1 antibody captured proteins.

For dot blot analysis, cells were pretreated with rabbit IgG (1:500) (Sigma) or anti-integrin α5β1 antibody (1:500) for 30 min before adding 10 μM nitrated α-synuclein to the media. The same concentration of native α-synuclein was used as a control. After incubation for another 4 h, the supernatants were

gently removed and used for dot blotting after centrifugation (500 *g*, room temperature, 10 min). Cells were washed with PBS three times. Then 100 μl of 5 mM Tris buffer (1 mM SDS, 3 mM Na_2HPO_4, 1 mM KH_2PO_4, 38 mM glycine, 110 mM NaCl, 2 mM KCl, pH 7.4) was added to the cell culture. Cells were then put into a shaker and were gently shaken at 37°C for 30 min. Supernatants were collected for dot blotting and monoclonal Syn211 was used as the primary antibody.

Caspase 3 activity assay

Caspase 3 activity was determined by cleavage of the substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-*p*NA; Beyotime Inc.) to generate yellow *p*-nitroanilide (*p*NA) according to the manufacturer's instructions. In brief, cells were harvested and washed twice in PBS, and lysed in a lysis buffer. The lysates were centrifuged (16000 *g*, 4°C, 15 min), and protein concentrations in the supernatants were determined using a Bradford protein assay kit (Beyotime Inc.). Cell lysate (20 μg) was incubated with 2 mM Ac-DEVD-*p*NA at 37°C for 120 min (repeated in triplicate). Cleavage of Ac-DEVD-*p*NA was determined by measuring absorption at 405 nm with an ELISA plate reader.

Flow cytometry

The generation of NO in living cells treated with $NaNO_2$, nitrated α-synuclein, and α-synuclein control were detected by using DAF-FM DA. After treatment with different reagents, cells were collected by centrifugation, washed three times with PBS and then probed with DAF-FM DA (5 μM) at 37°C for 20 min. The level of NO in cells was then analyzed by flow cytometry.

An annexin V-FITC apoptosis detection kit was used to detect cell apoptosis by flow cytometry. Cells (1×10^5) were washed three times with PBS and collected by centrifugation. The pellets were resuspended in 500 μl binding buffer. After addition of 5 μl annexin V-FITC and 5 μl propidium iodide (PI), cells were incubated in the dark at room temperature for 15 min before analyzing by flow cytometry.

Statistical analysis

Statistical analysis was performed with SigmaStat 11.5 (SPSS). Differences between treatments were examined by one-way ANOVA. Pair-wise multiple comparison procedures were applied when significant differences were observed. A probability value of $P < 0.05$ was regarded as significant in all analyses. All the results we showed had been repeated at least three times.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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