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Dopamine facilitates α-synuclein oligomerization in human neuroblastoma SH-SY5Y cells

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ABSTRACT

Parkinson's disease is characterized by selective loss of dopaminergic neurons in the substantia nigra and by the appearance of Lewy bodies. Fibrillar α-synuclein is the main component of Lewy bodies. Previous studies have suggested that dopamine promotes α-synuclein oligomerization and that partially aggregated or oligomeric α-synuclein could be cytotoxic. To confirm this hypothesis using cell cultures, we performed size exclusion chromatography as a pretreatment method prior to Western blotting to more clearly detect a small amount of α-synuclein oligomers in wild-type α-synuclein-overexpressing SH-SY5Y cells. Using this method, we confirmed that stable overexpression of α-synuclein in SH-SY5Y cells indeed increased the amounts of α-synuclein oligomers in these cells and exposure of the cells to dopamine for 6 h facilitated α-synuclein oligomerization. These dopamine-induced α-synuclein oligomers continued to exist for the following 24 h. However, the dopamine-treated cells did not undergo cell death or apoptosis in spite of the presence of increased oligomeric α-synuclein. Our data may contribute to the understanding of the mechanisms underlying α-synuclein oligomer formation and its suspected cytotoxicity toward dopaminergic neurons.

Introduction

Parkinson's disease (PD) is among the most prevalent neurodegenerative disorders. It is characterized by selective loss of dopaminergic neurons in the mesencephalic substantia nigra and by the presence of cytoplasmic inclusions, namely Lewy bodies.

One of the key molecules involved in the pathogenesis of PD is α-synuclein, the major component of Lewy bodies. α-Synuclein is a 140-amino acid protein, which exists predominantly in presynaptic nerve terminals in the brain [1,2]. Physiologically, it adopts a soluble, unfolded structure [3]; however, in the pathological state, it is supposed to aggregate and form fibrillar deposits within Lewy bodies [4].

Mutations [E46K, A53T], duplication or triplication of the α-synuclein gene increase the propensity of α-synuclein to aggregate and are linked to autosomal–dominant-inherited PD [4]. These findings again imply that aggregation and accumulation of α-synuclein are critical in the pathogenesis of PD. α-Synuclein polymerizes from soluble monomers, to dimers, trimers or oligomers, eventually becoming fibrillar aggregates. It has been hypothesized that, among these aggregated α-synuclein species, α-synuclein oligomers might be toxic to cells [5].

Previous studies have shown that dopamine promotes aggregation of monomeric α-synuclein and stabilizes oligomeric species of α-synuclein both in cell-free systems [6,7] and cultured cells [8,9]. However, in cultured cells, it is technically difficult to detect α-synuclein oligomers only by Western blotting (WB), especially when these are of wild-type α-synuclein. The purpose of this study was to determine whether dopamine promotes wild-type α-synuclein aggregation and whether aggregated α-synuclein has cytotoxicity in cultured cells. Because wild-type α-synuclein exists mainly as a soluble monomer in cells, and the amount of α-synuclein oligomers is thought to be far less than the amount of monomer, we separated cell lysates by size exclusion chromatography (SEC) prior to WB to differentiate α-synuclein oligomers from monomers. Using this method, we studied the interactions between dopamine and α-synuclein aggregation and cytotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells.

Materials and methods

Materials. α-Synuclein, GBR 12935, anti-α-tubulin antibody and protease inhibitor cocktail (P8340) were purchased from Sigma

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(St. Louis, MO). Dopamine hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). Gel filtration standards were purchased from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-α-synuclein antibody was purchased from Santa Cruz Biotechnology (sc-7011R, Santa Cruz, CA). Anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA) was used to detect caspase-3 activation. The lactate dehydrogenase (LDH) release assay was carried out using the Cytotoxicity Detection LDH kit from Kyokuto Pharmaceutical Industrial (Tokyo, Japan).

Plasmid construction. Plasmid construction was performed as previously reported [10]. α-Synuclein cDNA was amplified from postmortem human brain samples by RT-PCR using *Kpn*I- and *Xba*I-tailed primers complementary to the human α-synuclein coding region (GenBank AY049786). The nucleotide sequences of sense and antisense primers were as follows: 5′-CGGGGTACCTG GCCATTCGACGACAGTGT-3′ (sense), 5′-TGCTCTAGAGGATGGAACAT CTGTCAGCA-3′ (antisense). Appropriate restriction digestion of the PCR-amplified product was performed and the wild-type sequence was then cloned into the pcDNA3.1(+) vector (Invitrogen, San Diego, CA). Plasmid DNA was subsequently transformed into DH5α cells (Toyobo, Osaka, Japan). The resulting colonies were screened by PCR, and positives were selected, grown in liquid medium, and sequenced to verify the wild-type α-synuclein sequence (data not shown).

Stable transfection. The human dopaminergic neuroblastoma cell line SH-SY5Y was maintained at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. When cells reached 80% confluency in 60-mm culture dishes, the medium was changed to Opti-MEM I (Invitrogen) and cells were transfected with 8 μg of wild-type α-synuclein DNA constructs using 10 μg of Lipofectamine 2000 (Invitrogen). After incubating the cells for 24 h, positive clones were selected using G418 (1300 μg/mL) for 2 weeks. Single cells were cloned in 96-well tissue culture plates. Stable transfectants established from these clones were evaluated for α-synuclein expression using Western blot analysis and immunocytochemistry.

Cell culture. SH-SY5Y cells were maintained in Opti-MEM I supplemented with 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded in 24-well culture plates for the LDH release assay, or in 100-mm dishes for WB or SEC. Cells were grown for 72 h and then exposed to drugs.

LDH release assay for determination of viability of SH-SY5Y cells. The viability of SH-SY5Y cells was determined by LDH release assay, as described previously [11]. The activity of LDH released into the medium during the exposure to drugs was measured using a LDH assay kit. Aliquots (25 μL) of culture supernatants were mixed with 75 μL of the LDH substrate mixture contained in the assay kit. After incubation for 1 h at room temperature, the reaction was stopped by adding 100 μL of 1 N hydrochloride (HCl) and the absorbance was measured at 570 nm. LDH release from the cultured cells was evaluated as a percentage of the total LDH released following exposure to 1 mM hydrogen peroxide for 24 h.

HPLC-ED analysis of dopamine. Dopamine concentrations were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ED). SH-SY5Y cells were washed twice with cold phosphate-buffered saline (PBS) and scraped in 1 mL of PBS. Cells were then centrifuged at 5000 rpm for 5 min, and homogenized in 300 μL of solubilizing buffer (0.1 N perchloric acid, 10 mM sodium disulfite, 1 mM ethylene diamine tetraacetic acid–disodium salt (EDTA–2Na) by sonication. After centrifugation at 15,000*g* for 15 min at 4 °C, the supernatants were collected for HPLC. Aliquots (10 μL) of the supernatants were analyzed by HPLC-ED. The HPLC system (HTEC-500, Eicom, Kyoto, Japan) consisted of a pump, a degasser, a column oven and an electrochemical detector with a graphite working electrode. The working electrode was maintained at an oxidative potential of +450 mV vs. an Ag/AgCl reference electrode to detect dopamine. The separation of dopamine from other endogenous compounds was achieved on a reversed-phase column (Eicompak CA-5ODS, Eicom) using a mixture of 0.1 M phosphate buffer (pH 6.0) and methanol (88:12, v/v) containing 600 mg/L sodium 1-octanesulfonate and 50 mg/L EDTA–2Na as a mobile phase. The column temperature was kept at 25 °C and the mobile phase was delivered at a flow rate of 0.23 mL/min.

Size exclusion chromatography and subsequent Western blotting. SH-SY5Y cells were washed twice with cold PBS and scraped in 1 mL of PBS. Cells were then centrifuged at 5000 rpm for 5 min, and homogenized in PBS by sonication. After centrifugation at 15,000*g* for 30 min at 4 °C, supernatants were filtered through a 0.2 μm hydrophilic PTFE disposable membrane (Millex-LG, Millipore). Protein concentrations were determined using Bradford's assay (Bio-Rad) and some amounts (2000–3000 μg) was loaded onto Shodex SEC columns (Showa Denko, Tokyo, Japan). The columns were equilibrated with mobile phase buffer (0.01 M Tris–HCl, pH 7.0, 0.15 M NaCl and 0.2% N-lauroylsarcosine) before analysis. Samples were eluted at a flow rate of 0.25 mL/min, and fractions were collected 1 mL per tube. The absorbance value at OD 280 nm of each fraction was used to draw the elution profile. For WB, equal volumes (10 μL) of each fraction were mixed with 2× sample buffer (0.125 M Tris–HCl, 20% glycerol, 4% sodium dodecyl sulfate (SDS), bromophenol blue), without boiling, loaded onto a 5–20% polyacrylamide gradient gel (e-PAGEL, ATTO, Tokyo, Japan) for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon–P^{SQ}, Millipore). The membranes were then incubated in 5% dry milk in Tris-buffered saline with Tween 20 (TBS-T, 20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) at room temperature for 1 h and incubated with α-synuclein antibodies (1:1000 dilution) in blocking solution (3% bovine serum albumin, 0.05% sodium azide in PBS) overnight at 4 °C. The membranes were then washed four times, 10 min each, in TBS-T, followed by 1 h incubation in horse radish peroxidase (HRP) conjugated secondary antibody (1:2000 dilution). The membranes were next washed six times, 10 min each, in TBS-T before incubation in chemiluminescence reagents (ECl plus, GE Healthcare) and then exposed to film. All procedures were performed on ice.

The SEC system consisted of a system controller (SCL-$10A_{VP}$, Shimadzu, Kyoto, Japan), a degasser (DGU-14A, Shimadzu), a solvent delivery (LC-$10AT_{VP}$, Shimadzu), an auto-sampler (SIL-$10AD_{VP}$, Shimadzu), a column oven (CTO-$10AC_{VP}$, Shimadzu), a guard column (KW-G; Showa Denko), Shodex silica-based columns (KW-802.5, KW-803, Showa Denko), a photodiode array detector (SPD-$M10A_{VP}$, Shimadzu), a fraction collector (FRC-10A, Shimadzu) and a sample cooler. For better separation, three silica-based columns were lineated (from the upper side, KW-803, KW-803, and KW-802.5 in this sequence). Data acquisition and processing were carried out using Class VP software (Shimadzu). The SEC conditions were as follows: injection volume 500 μL (including 2000–3000 μg of protein), flow rate 0.25 mL/min, column temperature 25 °C, sample cooler temperature 4 °C.

Western blotting for cleaved caspase-3. SH-SY5Y cells were harvested as described above and homogenized in lysis buffer (0.02 M Tris, pH 7.0, 25 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, with a 1% protease inhibitor cocktail) by sonication. After centrifugation at 15,000*g* for 30 min at 4 °C, supernatants were collected and protein concentrations were determined. Twenty micrograms of each sample was mixed with 2× sample buffer (0.125 M Tris–HCl, 20% glycerol, 4% SDS, 31 mg/mL dithiothreitol, bromophenol blue), boiled for 5 min, loaded on a 5–20% polyacrylamide gradient gel for SDS–PAGE and transferred to PVDF

membranes. The membranes were incubated with anti-cleaved caspase-3 antibody (1:1000 dilution) and appropriate HRP-conjugated secondary antibodies (1:2000 dilution). Immunoreactive proteins on membranes were visualized with ECl plus.

Statistical analysis. The statistical significance of the differences among three or more groups of individual data was analyzed by one-way analysis of variance (ANOVA) and a post hoc multiple comparison using Dunnett's test. Statistical significance was defined as $P < 0.05$. Data are expressed as means ± SEM.

Results

Detection of α-synuclein oligomers by size exclusion chromatography and subsequent Western blotting

The silica-based columns used here gave linear elution profiles of a set of protein standards having molecular weights of 1.35–670 kDa, indicating efficient column separation (Fig. 1A). The elution profiles of wild-type α-synuclein-overexpressing SH-SY5Y cells for monomeric and oligomeric α-synuclein are shown in Fig. 1B. α-Synuclein monomer, dimer, trimer, and tetramer bands were detected mainly in fractions 27, 25, 24, and 23, respectively (Fig. 1B). Monomeric bands were also detected in these oligomers fractions, suggesting that SDS dissolved the oligomeric α-synuclein to monomer. A higher molecular weight smear was also detected (Fig. 1B). By contrast, the elution profiles of untransfected cells showed smaller amounts of endogenous α-synuclein monomer with only slightly detectable oligomer bands (Fig. 1C).

Dopamine facilitated α-synuclein oligomerization and its effect is suppressed by a DAT inhibitor

Next we examined the effect of dopamine on α-synuclein oligomerization in wild-type α-synuclein-overexpressing SH-SY5Y cells. To this end, we first investigated the cellular content of dopamine when cells were exposed to dopamine. Wild-type α-synuclein-overexpressing cells were exposed to 100 μM dopamine for 45 min in the presence or absence of 3.2 μM GBR 12935, a dopamine transporter (DAT) inhibitor. Dopamine concentrations in cell lysates were measured by HPLC. While no detectable amount of dopamine was found in untreated cells, it was detected in dopamine-treated cells (Fig. 2A). In addition, co-administration of 3.2 μM GBR 12935 with dopamine significantly reduced the cellular content of dopamine, indicating that extracellular dopamine was taken up by cells via the dopamine transporter. We examined the elution profiles of dopamine-treated cells for α-synuclein. Wild-type α-synuclein-overexpressing cells were incubated in the presence or absence of 100 μM dopamine for 6 h and cell lysates were separated by SEC. To compare the subtle difference in the amount of α-synuclein oligomer, equal volumes (10 μL) of fractions 19–25 of sham- and dopamine-treated samples were analyzed by SDS–PAGE in a single 5–20% gradient gel, and immunoblotted for α-synuclein. When the elution profiles of dopamine-treated cell lysates (Fig. 2B, right half of the gel) were compared with that of sham treatment (Fig. 2B, left half of the gel), the intensities of oligomeric α-synuclein bands in fractions 21–23 were apparently increased (Fig. 2B and C). Furthermore, the intensities of monomeric α-synuclein bands in fraction 21–23 were also slightly increased (Fig. 2B, right half of the gel), indicating that a portion of dopamine-induced oligomers was denatured by SDS (Fig. 2B and C). These changes, however, could not be detected when the same samples were immunoblotted without SEC, suggesting that SEC worked as an efficient pretreatment method before WB to detect small amounts of dopamine-induced α-synuclein oligomers (Fig. 2D). When the cells were co-treated with dopamine and GBR 12935, the increase in the amount of α-synuclein oligomers was suppressed (Fig. 2E). Overall, these findings suggested that dopamine was taken up by the dopamine trans-

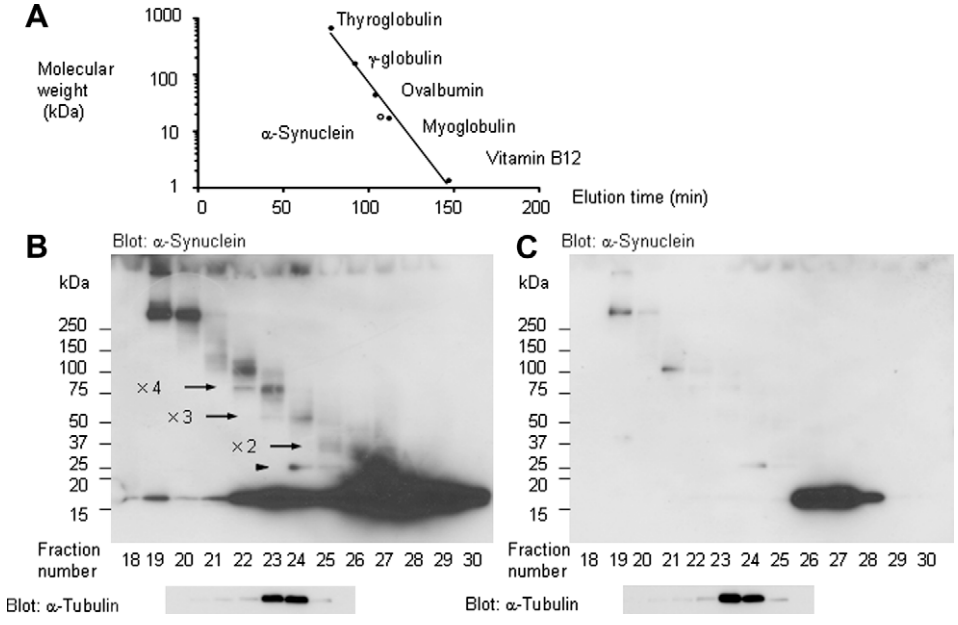

Fig. 1. Elution profiles of SEC (A) and detection of α-synuclein oligomers in SH-SY5Y cells (B, C). (A) Calibration curve for SEC. The linearity of the calibration curve indicates efficient column separation. Protein size markers and molecular weights are as follows: thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) (filled circles). Recombinant monomeric α-synuclein (18 kDa) is shown as an open circle. (B, C) Detection of α-synuclein oligomeric bands in SH-SY5Y cells. Equal amounts (2000 μg of protein) of the PBS-soluble cell lysates were separated by SEC and 10 μL of each collected fraction was analyzed by SDS–PAGE in 5–20% gradient gels. The PVDF blots were analyzed for α-synuclein (upper panel) and re-blotted for α-tubulin as a loading control (lower panel). (B) Cell lysates from wild-type α-synuclein-overexpressing SH-SY5Y cells were analyzed. Films were exposed for long enough to detect trace oligomeric bands, so the monomeric α-synuclein bands were saturated. α-Synuclein dimer, trimer, and tetramer were detected (arrow). The 25-kDa band in fraction 24 (arrowhead) was a non-specific band. Higher molecular species were also detected. (C) Cell lysates from untransfected SH-SY5Y cells were analyzed. The film exposure time was the same as in (B). Note, endogenous α-synuclein monomer was detected in fraction 27.

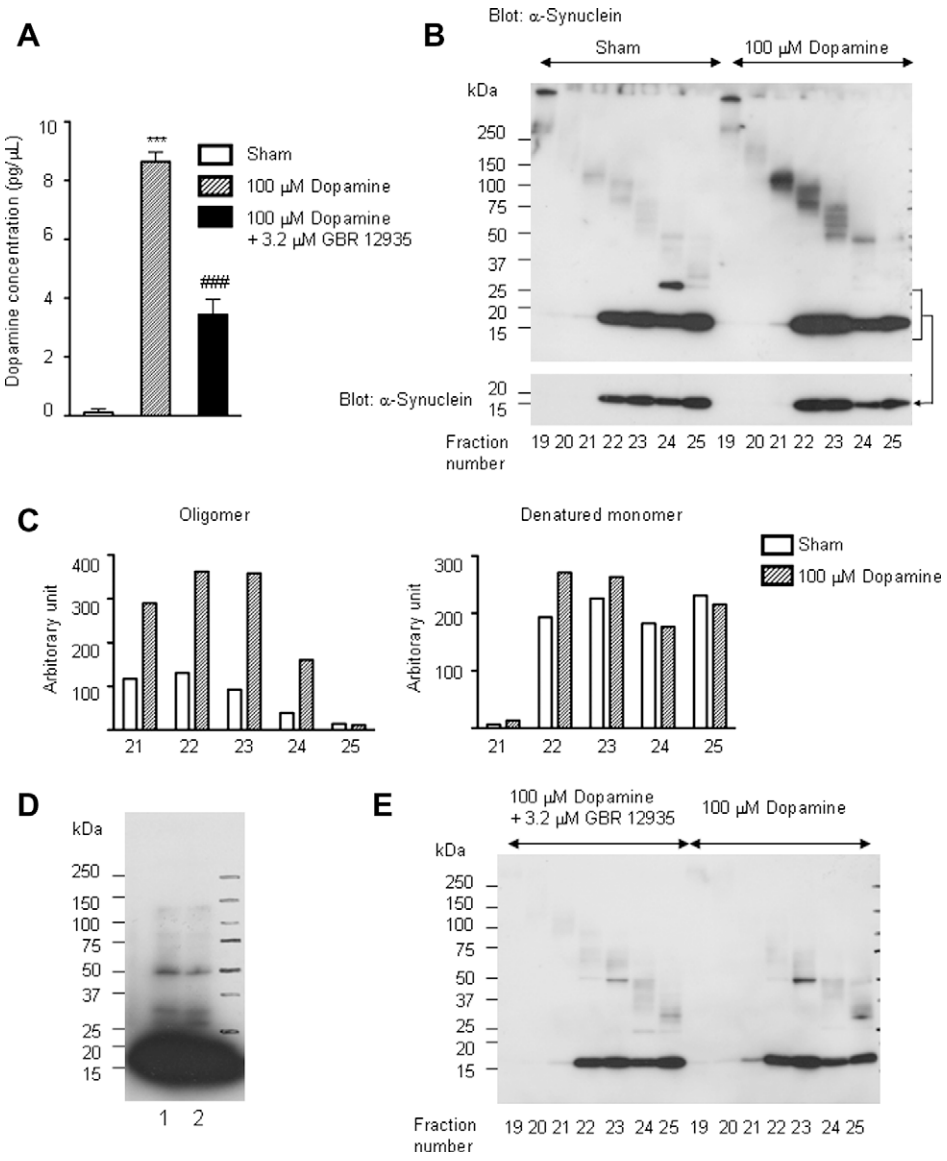

Fig. 2. Dopamine content (A) and α-synuclein oligomerization (B–E) after dopamine treatment. (A) Intracellular uptake of dopamine via the dopamine transporter. Wild-type α-synuclein-overexpressing SH-SY5Y cells were exposed to 100 μM dopamine for 45 min in the presence or absence of GBR 12935. Cells were collected in PBS and homogenized in 300 μL of solubilizing buffer. After centrifugation at 15,000*g* for 15 min at 4 °C, supernatants were analyzed for dopamine content by HPLC. $^{***}P < 0.001$ compared with sham treatment. $^{\#\#\#}P < 0.001$ compared with dopamine alone. $n = 3$ dish/experiment. Data were expressed as means ± SEM. (B) Dopamine facilitated α-synuclein oligomerization in wild-type α-synuclein-overexpressing SH-SY5Y cells. Cells were exposed to 100 μM dopamine for 6 h and equal amounts (3000 μg of protein) of cell lysate from the PBS-soluble fraction were separated by SEC. Ten-microliter aliquots of each of fractions 19–25 from sham and dopamine treatments were analyzed by SDS–PAGE in a single 5–20% gradient gel. The PVDF blots were analyzed for α-synuclein. Compared with sham treatment (left half of the gel), the amounts of α-synuclein oligomers were increased by dopamine treatment (right half of the gel). Lower panel shows monomeric α-synuclein bands with the film exposed for a shorter time than in the upper panel. The results are representative of three independent experiments. (C) The blots shown in (B) were quantified using NIH ImageJ software for the relative amounts of α-synuclein oligomers and their denatured monomers. White bars represent sham treatment. Striped bars represent dopamine treatment. (D) The same samples as shown in (B), before SEC, were analyzed by WB for α-synuclein. Lane 1, sham treatment. Lane 2, 100 μM dopamine treatment for 6 h. WB without SEC could not detect any difference in the amount of α-synuclein oligomer. (E) When cells were exposed to dopamine in the presence of GBR 12935, formation of α-synuclein was suppressed. Cells were exposed to 100 μM dopamine with or without 3.2 μM GBR 12935 for 6 h, and same amount (2000 μg of protein) of PBS-soluble cell lysate was separated by SEC. Ten-microliter aliquots of each of fractions 19–25 from dopamine-treated cells, with or without GBR 12935, were analyzed by SDS–PAGE in the same 5–20% gradient gel. The PVDF blots were stained for α-synuclein. Compared with dopamine treatment alone (right half of the gel), the amounts of α-synuclein oligomers were decreased by dopamine treatment with 3.2 μM GBR 12935 (left half of the gel).

porter and facilitated wild-type α-synuclein oligomerization in cells.

α-Synuclein oligomer continued to exist in cells up to 24 h after washing out dopamine-containing medium but did not cause cell death

Because it has been hypothesized that α-synuclein oligomers are cytotoxic, we investigated the effect of dopamine-induced α-synuclein oligomers on cell viability. To this end, wild-type α-synuclein-overexpressing SH-SY5Y cells were exposed to 100 μM dopamine for 6 h and incubated in dopamine-free medium for a further 24 h. The elution profile of dopamine washout treatment showed that almost the same amount of α-synuclein oligomer was still found in the cells after 24 h of washout time, compared with no washout treatment (Fig. 3A). An LDH release assay revealed that cells were viable after 24 h washout time (Fig. 3B). In addition, though staurosporine-induced apoptotic cells

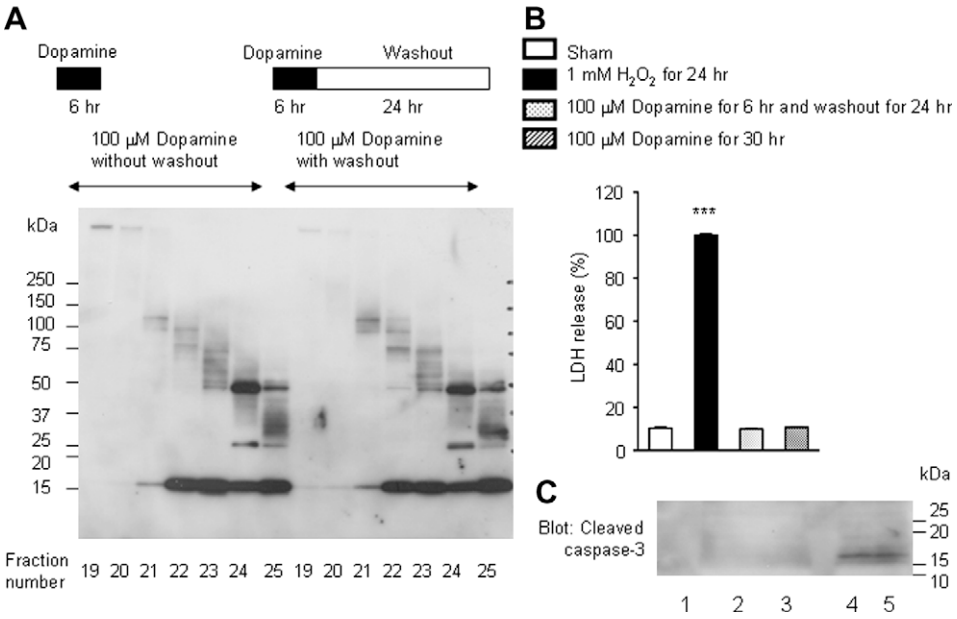

Fig. 3. Twenty-four hours-persistent oligomers in cells and their cytotoxicity (A–C). (A) Dopamine-induced α-synuclein oligomers existed in cells after removal of dopamine from the medium for 24 h. α-Synuclein-overexpressing SH-SY5Y cells were exposed to 100 μM dopamine for 6 h. After that, cells were incubated in dopamine-free medium for a further 24 h. The same amount (2000 μg of protein) of cell lysate was separated by SEC and 10 μL aliquots of each of fractions 19–25 from dopamine-treated cells with or without 24 h washout treatment were analyzed for α-synuclein. Almost the same amount of α-synuclein oligomer still existed after removal of dopamine for 24 h (right half of the gel), compared with dopamine treatment for 6 h (left half of the gel). (B) An LDH assay showed that the dopamine-treated cells with oligomeric α-synuclein did not undergo cell death within the following 24 h. Wild-type α-synuclein-overexpressing SH-SY5Y cells were exposed to 100 μM dopamine for 6 h. After that, the cells were incubated in dopamine-free medium for further 24 h. No difference in LDH release was found between sham treatment and dopamine treatment. ***$P < 0.001$ compared with sham treatment. $n = 4$ dish/experiment. Data were expressed as means ± SEM. (C) Caspase-3 was not activated in dopamine-treated cells with oligomeric α-synuclein. α-Synuclein-overexpressing SH-SY5Y cells were treated with dopamine or staurosporine and analyzed by WB for cleaved caspase-3. Cleaved caspase-3 levels did not increase following dopamine treatment. Lane 1, control. Lane 2, treatment with 100 μM dopamine for 6 h. Lane 3, treatment with 100 μM dopamine for 6 h with 24 h washout time. Lane 4, treatment with 0.25 μM staurosporine for 3 h. Lane 5, treatment with 0.25 μM staurosporine for 6 h.

caused cleavage of caspase-3, this was not observed in dopamine-treated cells (Fig. 3C). These findings suggested that neither cell death nor apoptosis had occurred, in spite of the fact that α-synuclein oligomers still existed in the cells.

Discussion

In this study, we adopted the combined methods of SEC and WB to detect wild-type α-synuclein oligomers in cells. Although we could not distinguish α-synuclein oligomers from α-synuclein bound to other proteins, their molecular weights and sequential separation by SEC suggested that these bands with oligomeric molecular weights included α-synuclein oligomers. Previous studies have analyzed cell lysates of α-synuclein-overexpressing cells by combining SEC and WB [8,12]; however, only monomeric wild-type α-synuclein bands, but not oligomeric bands, were detected in these studies. In our study, oligomeric α-synuclein bands themselves, especially tetramers were detected. It is said that in most cell models the recovered α-synuclein is predominantly monomeric on SDS–PAGE gels [13]. We suspect that this might reflect, in part, the nature of α-synuclein oligomers that are sensitive to SDS or heating. Therefore, in this study, the non-boiling sample preparation condition may have helped make the detection of α-synuclein oligomer bands easier. Another means of detecting oligomeric α-synuclein is delipidation treatment. Sharon et al. [14] reported that α-synuclein oligomers could also be detected by simple WB with non-boiled samples (from mouse brains) when heat was added to blotted PVDF membranes or samples were delipidated, and therefore, that some α-synuclein oligomers were not detected by WB because they were bound to lipid in cells. Therefore, lipid-bound α-synuclein was not detected in this study.

Exposure of α-synuclein-overexpressing cells to dopamine promoted oligomerization of α-synuclein. These data were consistent with the findings of previous studies using recombinant α-synuclein [6,7] or cultured cells [8,9]. They reveal that dopamine promotes the formation of SDS-resistant α-synuclein oligomers within 5 min [7] to 3 days [6] in a cell-free system or within 3–5 days [8,9] in cells. We demonstrated that intracellular oligomerization was facilitated by dopamine within 6 h, and that the oligomers were stable for at least 24 h.

A previous study using A53T mutant- and tyrosine hydroxylase mutant-overexpressing SH-SY5Y cells revealed that α-synuclein oligomers were innocuous [8]. These results are consistent with our study. However, it has been controversial whether over-expressed α-synuclein, some part of which may be oligomerized as shown in Fig. 2, is cytotoxic or innocuous [13]. Therefore, the cytotoxicity of oligomeric α-synuclein requires confirmation in further experiments.

In conclusion, we demonstrated that dopamine promotes wild-type α-synuclein oligomerization in cells and that these α-synuclein oligomers did not cause cytotoxicity, at least up to 24 h. Further study is needed to elucidate the relationship between α-synuclein oligomerization and Parkinson's disease.

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