α-Synuclein Lowers p53-dependent Apoptotic Response of Neuronal Cells

ABOLISHMENT BY 6-HYDROXYDOPAMINE AND IMPLICATION FOR PARKINSON’S DISEASE*

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We have examined the influence of α-synuclein on the responsiveness of TSM1 neuronal cells to apoptotic stimulus. We show that α-synuclein drastically lowers basal and staurosporine-stimulated caspase 3 immunoactivity and activity. This is accompanied by lower DNA fragmentation and reduced number of terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL)-positive neurons. Interestingly, α-synuclein also diminishes both p53 expression and transcriptional activity. We demonstrate that the antia apoptotic phenotype displayed by α-synuclein can be fully reversed by the Parkinson’s disease-associated dopamine derivative 6-hydroxydopamine. Thus, 6-hydroxydopamine fully abolishes the α-synuclein-mediated reduction of caspase 3 activity and reverses the associated decrease of p53 expression. 6-Hydroxydopamine triggers thioflavin T-positive deposits in α-synuclein, but not mock-transfected TSM1 neurons, and drastically increases α-synuclein immunoreactivity. Altogether, we suggest that α-synuclein lowers the p53-dependent caspase 3 activation of TSM1 in response to apoptotic stimuli and we propose that the natural toxin 6-hydroxydopamine abolishes this antia apoptotic phenotype by triggering α-synuclein aggregation, thereby likely contributing to Parkinson’s disease neuropathology.

Parkinson’s disease (PD)1 is one of the most common age-related neurodegenerative disease (1, 2). At the neuropathological level, this disease is mainly characterized by neuronal intracellular inclusions named Lewy bodies (3), the major component of which is a 140-amino acid-long protein called α-synuclein (4, 5). Most of PD cases are of sporadic origin, but relatively recently, it was reported that rare autosomal dominant forms of PD were due to two mutations borne by α-synuclein (6, 7). That these mutations accelerate the onset and evolution of the disease, but also increase the propensity of α-synuclein aggregation, thereby likely contributing to Parkinson’s disease neuropathology.

Type and PD-related mutated α-synucleins aggregation was associated with increased cell death (10, 12, 13), likely explaining the important apoptosis and neuronal degeneration observed particularly in the dopaminergic pathway in the PD-affected brains (14, 15). This pathogenic phenotype could be due to intrinsic proapoptotic properties associated with α-synuclein that would be exacerbated by the aggregating process. Alternatively, the cell death associated with α-synuclein fibrillation could be due to the abolishment of an antia apoptotic tonus corresponding to the physiological function of α-synuclein.

We have recently demonstrated that wild-type α-synuclein could protect neuronal cells from staurosporine-induced toxicity (16). We also established that wild-type α-synuclein drastically inhibited the caspase 3 activation triggered by several apoptotic stimuli, including staurosporine, C2-ceramide, and etoposide in TSM1 transfected neuronal cells expressing wild-type α-synuclein (16). Of most interest was the observation that the A53T mutation related with familial PD abolished this inhibitory control of caspase 3 activation (16). Here we have further characterized the antia apoptotic function of α-synuclein, and we show for the first time that α-synuclein inhibition of the staurosporine-induced caspase activation in TSM1 neurons is associated with a drastic lowering of p53 expression and transcriptional activity. Furthermore, we establish that the dopamine-related and PD-associated toxin 6-hydroxydopamine (6OH-DOPA) abolishes the antia apoptotic tonus of α-synuclein by apparently triggering its aggregation. The implication of these observations for PD neuropathology is discussed.

MATERIALS AND METHODS

Cell Systems—HEK293 cells expressing wild-type α-synuclein were obtained after transfection with 2 μg of wild-type or A53T-α-synuclein cDNA in pcDNA3 obtained as detailed previously (17). Transfectants were screened for their α-synuclein-like immunoreactivity as described below. TSM1 neurons expressing α-synuclein were obtained and cultured as detailed previously (16).

Western Blot Analyses—Equal amounts of protein (50 μg) were separated on 12% SDS-PAGE gels for the detection of β-tubulin, α-synuclein, active caspase 3, and p53 and then wet-transferred to Hybond-C (Amersham Biosciences) membranes. After transfer, membranes were blocked with nonfat milk and incubated overnight with the following primary antibodies: anti-p53 (mouse monoclonal, Santa Cruz), anti-active caspase 3 (rabbit polyclonal, R & D System), anti-α-synuclein (rabbit polyclonal, Affiniti Laboratory). Immunological complexes were revealed with either an anti-rabbit peroxidase (Immunotech) or with anti-mouse peroxidase (Amersham Biosciences) antibodies depending on the host used for obtention of the primary antibodies described above, followed by electrochemoluminescence (Amersham Biosciences). All protein concentrations were determined as described previously (18).

*p53 Transcriptional Activity—The PG13-luciferase and pG13(-17)-Luciferase p53 gene reporter constructs (provided by Dr. B. Vogelstein) have been described previously (19, 20). One μg of PG13-luciferase or

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1 The abbreviations used are: PD, Parkinson's disease; 6OH-DOPA, 6-hydroxydopamine; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick end labeling; STS, staurosporine.
**RESULTS**

Stably transfected TSM1 neurons overexpressing wild-type α-synuclein (Fig. 1A) reduce by 40 and 60%, respectively, the basal and staurosporine (STS)-induced caspase 3-like activity (Table I). We established that these observations were not cell-specific as these results were also observed in HEK293 cells overexpressing α-synuclein (Table I). α-Synuclein-associated reduced caspase activation in TSM1 neurons was accompanied by a drastically lower immunoreactivity of active caspase 3 in both basal and STS-stimulated conditions (Fig. 1B). This α-synuclein-induced lowering of caspase activity and immunoreactivity was indeed associated with a reduction of apoptotic cell stigmata. Thus, α-synuclein expression clearly reduced the number of apoptotic nuclei as shown by TUNEL analysis (Fig. 2A) in both basal and STS-induced conditions. In the latter condition, α-synuclein reduces the number of TUNEL-positive neurons by about 75% (Fig. 2B). This was correlated by an about 50% lowering of propidium iodide incorporation (Fig. 3), indicating a reduced DNA fragmentation in α-synuclein-expressing TSM1 cells.

We examined whether the diminished caspase 3 activation and neuronal cell death triggered by α-synuclein could be associated with a modulation of p53 expression and/or transcriptional activity as this oncogene has been shown to contribute to proapoptotic pathways ultimately leading to caspase 3 activation. By means of a specific p53 reporter gene construct (19), we established that α-synuclein-expressing cells display lower p53 transcriptional activity (Fig. 4B). This was corroborated by a concomitant decrease in the promotor activation of p21<sup>^waf1</sup>.

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**FIG. 1.** α-Synuclein (α-syn) expression lowers active caspase 3 immunoreactivity in TSM1 neurons. Mock- and α-synuclein stably transfected TSM1 neurons (A) were treated for 2 h in absence (Ct) or in the presence of 1 μM STS, and then active caspase 3 (Casp3) immunoreactivity was monitored as described under “Materials and Methods” (B).

**FIG. 2.** α-Synuclein (α-syn or α-SYN on figure) expression decreases the number of basal and staurosporine-stimulated TUNEL-positive TSM1 neurons. Mock- and α-synuclein stably transfected TSM1 neurons were treated for 16 h in absence (Basal) or in the presence of 0.5 μM STS and then analyzed by TUNEL as described under “Materials and Methods.”

**TABLE I**

| α-Synuclein (α-Syn) lowers basal and staurosporine-stimulated caspase 3 activity in TSM1 neurons and HEK293 cells |
|--------------------------------------------------|
| HEK293 | |
| Mock | a-Syn (%) |
| Basil | 23,735 ± 2632 | 15,036 ± 1206 (63.3) |
| STS | 52,109 ± 3952 | 34,924 ± 2997 (67) |
| TSM1 | a-Syn (%) |
| Mock | |
| Basil | 29,530 ± 5563 | 29,530 ± 5563 |
| STS | 127,759 ± 3397 | 127,759 ± 3397 |
| a-Syn | 51,042 ± 4052 (40) | 51,042 ± 4052 (40) |

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*p21<sup>^waf1</sup>-luciferase cDNA were co-transfected with 1 μg of a β-galactosidase transfection vector (to normalize transfection efficiencies) in TSM1 neurons. Forty-eight hours after transfection, luciferase and β-galactosidase activities were measured as described previously (18). Caspase Activity Measurements—Caspase-3-like activity was fluorometrically measured in absence or in the presence of the caspase 3 inhibitor, c-DEVD-al, as extensively detailed previously (18). Flow Cytometry Analysis of Propidium Iodide Incorporation—TSM1 cells were grown in six-well plates and incubated for 16 h at 37 °C in the presence or absence of 0.5 μM staurosporine. Cells were rinsed and incubated for 1 min with 1 ml of phosphate-buffered saline (PBS) containing 50 μg/ml propidium iodide. Cells were then rinsed twice with PBS, harvested, and gently resuspended in PBS, placed on ice, and analyzed using a fluorescence-activated cell sorter scan flow cytometer (program CellQuest, Becton Dickinson). Red fluorescence due to propidium iodide staining of DNA was expressed on a logarithmic scale simultaneously to the forward scatter of the particles. A hundredthousand events were counted on the scatter gate. The number of apoptotic cells is expressed as a percentage of the total number of events as extensively described previously (18).

TUNEL Analysis—For DNA nick end labeling, cells were fixed for 20 min in 4% paraformaldehyde (in PBS), rinsed in PBS, left overnight in 70% ethanol, and then processed for the dUTP nick end labeling TUNEL technique according to manufacturer's recommendations (Roche Molecular Biochemicals). Staining was assessed with a peroxidase-conjugated antibody and revealed with 3,3′-diaminobenzidine substrate. The DNA label corresponds to black spots. All cells were additionally labeled with erythrosine B.

Fluorescence Cell Staining—For thioflavin T (Sigma) labeling, stably wild-type α-synuclein and mock-transfected cells were cultured on coverslips and treated or not with 0.2 μM 6-OH-DOPA. After 8 h of treatment, cells were fixed with 4% paraformaldehyde/PBS solution for 30 min, incubated 8 min with a 0.05% thioflavin T solution, and washed three times with PBS before mounting with a VectaShield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA). Images were analyzed using a Leitz Aristoplan Wetzlar Germany fluorescence microscope coupled to a numeric Nikon camera photo apparatus.

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Fig. 3. α-Synuclein (α-Syn) expression reduces basal and staurosporine-stimulated DNA fragmentation in TSM1 neurons. Mock- and α-synuclein (α-Syn)-stably transfected TSM1 neurons were treated for 16 h in absence (CT) or in the presence of 0.5 μM STS, and then iodide propidium incorporation was monitored as described under “Materials and Methods.”

Fig. 4. α-Synuclein (α-Syn) expression lowers p53 expression and transcriptional activity in TSM1 neurons. Mock- and α-synuclein (α-Syn)-expressing TSM1 neurons were transiently transfected with β-galactosidase and PG13 (B) or p21/waf-1 (C)-luciferase-cDNA. Forty-eight hours after transfection, luciferase and galactosidase activities were monitored as described under “Materials and Methods.” A, corresponds to the p53-like immunoreactivity monitored (see “Materials and Methods”) in mock- or α-synuclein-expressing TSM1 neurons.

(Fig. 4C), a well characterized downstream effector gene of p53 (20). Interestingly, α-synuclein overexpression was also associated with a diminished p53-like immunoreactivity (Fig. 4A), indicating that both p53 expression and transcriptional activity were reduced by α-synuclein expression in TSM1 neurons.

Several lines of evidence indicate that 6OH-DOPA, a natural oxidized derivative of dopamine, could act as a cell toxin and could contribute to the neuronal degenerescence occurring notably in the substantia nigra of PD-affected brains. We established that 6OH-DOPA increased caspase 3 activity in a time- and dose-dependent manner in mock-transfected cells (Fig. 5A). Interestingly, 6OH-DOPA abolishes the inhibitory control of caspase 3 activity by wild-type α-synuclein (Fig. 5B). It should be noted here that we confirm that the A53T pathogenic mutation abolishes the α-synuclein-mediated inhibitory control of caspase 3 activity in both basal and staurosporine-stimulated conditions (Table II). However, this mutation does not appear to further potentiate the 6OH-DOPA-induced increase of caspase 3 activity observed in mock-transfected cells (Table II).

Interestingly, 6OH-DOPA reverses the α-synuclein-induced decrease in p53 immunoreactivity (Fig. 5C). This effect was accompanied by a time- and dose-dependent increase of α-synuclein-like immunoreactivity (Fig. 6). It is important to note that staurosporine appeared unable to modify the α-synuclein immunoreactivity (Fig. 6). 6OH-DOPA treatment of TSM1 cells also led to thioflavin T-positive deposits in α-synuclein, but not mock-transfected TSM1 cells (Fig. 7), suggesting that the dopaminergic toxin could trigger α-synuclein aggregation, as suggested by previous reports (21, 22). Overall, our data suggest that α-synuclein displays neuronal p53-dependent control of caspase 3-like activity and that 6OH-DOPA could abolish this antiapoptotic phenotype by triggering α-synuclein aggregation.

DISCUSSION

Because dopaminergic neurons undoubtedly die by apoptosis in Parkinson’s disease, the role of proteins involved in the control of cell death and related with this pathology remains of first importance. α-Synuclein is the main component of Lewy bodies, the intracellular inclusions that characterize Parkinson’s disease-affected brains (4). This protein, when mutated,
appears responsible for a subset of autosomal dominant forms of the disease (6, 7).

Several lines of evidence have suggested that α-synuclein could modulate cell death, but data are somewhat puzzling in the field. For instance, it appears that α-synuclein expression is increased in the target injury model (23), but is it a cell compensatory response, or alternatively is α-synuclein expression related with/responsible for the observed cellular toxicity? In line with an antiapoptotic physiological function of α-synuclein, it has been reported that α-synuclein was virtually always associated with normal neurons but not with those exhibiting apoptotic stigmata (24). Furthermore, Lee et al. (25) demonstrated that wild-type, but not mutated, α-synuclein delayed cell death triggered by serum withdrawal. Finally, Hashimoto et al. (26) observed that α-synuclein protected neuronal cells against oxidative stress.

We have shown previously that in neurons, α-synuclein could protect cells from various proapoptotic stimuli and, more precisely, could drastically diminish the caspase 3 activation triggered by several proapoptotic effectors (16). Particularly interesting was the fact that this antiapoptotic phenotype was abolished by the A53T mutation responsible for some familial cases of Parkinson’s disease (16).

Here, we further establish that α-synuclein lowers the staurosporine-induced caspase activation, not only in neurons but also in human HEK293 cells. This lower cellular susceptibility to apoptotic effectors was also illustrated by a reduced DNA fragmentation and decreased number of apoptotic cells as shown by tunel analysis. We demonstrate for the first time that the antiapoptotic function of α-synuclein is mediated by a drastic decrease in both p53 immunoreactivity and transcriptional activity. The latter paradigm was further documented by the ability of α-synuclein to almost totally block the transcription of p21^waf1, a well established downstream target of p53 transcriptional activity (20).

The involvement of p53 in the control of cell death in other neurodegenerative diseases such as Alzheimer’s disease is striking. Thus, p53 expression is associated with a decrease in the expression of the antiapoptotic protein presenilin 1 (27). Conversely, we showed that presenilin 2 and its mutated counterpart trigger p53-dependent caspase 3 activation in the same cell systems (18). Therefore, the p53 oncogene could be seen as a common denominator that mediates the cell death pathway that can be up- or down-regulated according to the physiological/pathological situation.

It is interesting to underline the fact that each time α-synuclein was associated with a proapoptotic phenotype, it was in experimental conditions where the protein was highly overexpressed, i.e. in transgenic animals or in affected brains displaying Lewy bodies (13, 28–30). This is in agreement with the fact that several physiological functions of α-synuclein appeared impaired by aggregation as was shown for its chaperonizing properties and ability to bind to vesicles (for reviews, see Refs. 31 and 32). The fact that aggregation appears exacerbated by Parkinson’s disease-associated pathogenic mutations clearly reinforces the view that aggregation is closely associated with the pathology. That this also impairs a physiological function controlled by α-synuclein remained a matter of speculation. Therefore, our demonstration that 6OH-DOPA abolished the antiapoptotic tonus triggered by α-synuclein (Fig. 5) appears of interest. It should be noted here that the extent of

### Table II

|          | Mock       | α-Syn     | A53T-α-Syn |
|----------|------------|-----------|------------|
| Basal    | 61,991 ± 1733 (n = 4) | 39,149 ± 1380 (n = 4) | 64,807 ± 1176 (n = 4) |
| STS      | 197,048 ± 18,449 (n = 3) | 61,195 ± 915 (n = 3) | 275,584 ± 9720 (n = 3) |
| 6OH-DOPA | 139,280 ± 6042 (n = 6) | 153,908 ± 11,210 (n = 6) | 121,352 ± 13,212 (n = 6) |

**Fig. 6.** 6OH-DOPA, but not staurosporine, increases α-synuclein (α-Syn)-like immunoreactivity in TSM1 neurons. α-Synuclein-expressing TSM1 cells were treated for 8 h with the indicated concentrations of 6OH-DOPA (upper lanes), with 0.2 mM 6OH-DOPA for the indicated times (middle lanes), with 1 μM staurosporine for 2 h or with the indicated concentrations of 6OH-DOPA (lower lanes). Then α-synuclein-like immunoreactivity was monitored as described under “Materials and Methods.”

**Fig. 7.** 6OH-DOPA triggers thioflavin T-positive aggregates in α-synuclein-expressing TSM1 neurons. Mock- and α-synuclein-expressing TSM1 neurons were treated for 8 h in the absence (control) or in the presence of 0.2 mM 6OH-DOPA and then fixed, incubated with thioflavin T solution, washed, and examined for fluorescence as described under “Materials and Methods.”

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activation of caspase by 6OH-DOPA and staurosporine is similar in mock-transfected cells but that only 6OH-DOPA is able to reverse the antiapoptotic phenotype triggered by α-synuclein. The similar extent of caspase activation therefore indicates that the 6OH-DOPA-mediated effect is not due to a nonspecific toxic hyperactivation of caspase 3 that would have led to abolish α-synuclein inhibitory tonic. This is further emphasized by the fact that 6OH-DOPA, but not staurosporine, increases α-synuclein-like immunoreactivity (see “Results”).

6OH-DOPA is a selective catecholaminergic neurotoxin that is not only used as a pharmacological agent able to trigger PD-like stigmata (33–36) but also likely corresponds to a natural dopaminergic catabolite that accumulates in Parkinson’s disease-affected brains (37) and that appears to strongly contribute to this pathology (38, 39). 6OH-DOPA abolishes in a time- and dose-dependent manner the α-synuclein-induced lowering in caspase 3 activity and p53 expression (Fig. 5). This was accompanied by a selective occurrence of thioflavin T-positive aggregates in α-synuclein-expressing neurons but not in mock-transfected cells (Fig. 6). Therefore, we propose that the physiological function of α-synuclein would be to control the level of caspase 3 activity through the regulation of p53 expression and transcriptional activity. We suggest that in Parkinson’s disease, accumulation of 6OH-DOPA leads to α-synuclein aggregation, thereby impairing its physiological function and contributing to subsequent proapoptotic phenotype observed in this disease. The mechanism by which 6OH-DOPA abolished the antiapoptotic phenotype of α-synuclein remains to be established. However, it has been shown that 6OH-DOPA produces hydrogen peroxide upon autooxidation (21) and that this oxidant could generate free radicals able to trigger α-synuclein aggregation (22). Another theoretical possibility could be the nitrification of α-synuclein due to 6OH-DOPA reaction with nitric oxide (40). However, the latter hypothesis remains unlikely, because, in contrast to the in vitro conditions, the low physiological O2 concentration should theoretically lead to nonsignificant above reaction (40).

Particularly interesting was a recent paper indicating that α-synuclein toxicity could be selectively associated with dopaminergic cells in vivo, while the protein appears neuroprotective in other brain areas (41). Our data agree with this hypothesis, since α-synuclein displays an antiapoptotic phenotype in TSM1 that is of neocortical origin (42), i.e. not dopaminergic neurons. However, these cells could also respond to exogenous toxic stimulus such as 6OH-DOPA as likely dopaminergic neurons do. Thus, the cellular machinery underlying α-synuclein physiological function appears to occur in all neuronal cells. However, only neurons of the dopaminergic pathway altered in their dopamine/catabolites would lead to the selective neurodegenerative features occurring in Parkinson’s disease. Whether the alteration of α-synuclein physiological function could contribute to other degenerative diseases such as Alzheimer’s disease, where in some cases, Lewy bodies enriched in α-synuclein are numerous in nondopaminergic pathways, remains a possibility.

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