Wild-type but Not Parkinson’s Disease-related Ala-53 → Thr Mutant α-Synuclein Protects Neuronal Cells from Apoptotic Stimuli*

Cristine Alves da Costa‡§, Karine Ancolio‡, and Frédéric Checler‡¶

From the ‡Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, UPR411, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

Recent works suggest that α-synuclein could play a central role in Parkinson’s disease (PD). Thus, two mutations were reported to be associated with rare autosomal dominant forms of the disease. We examined whether α-synuclein could modulate the caspase-mediated response and vulnerability of murine neurons in response to various apoptotic stimuli. We established TSM1 neuronal cell lines overexpressing wild-type (wt) α-synuclein or the PD-related Ala-53 → Thr mutant α-synuclein. Under basal conditions, acetyl-Asp-Glu-Val-Asp-aldehyde-sensitive caspase activity appears significantly lower in wt α-synuclein-expressing cells than in neurons expressing the mutant. Interestingly, wt α-synuclein drastically reduces the caspase activation of TSM1 neurons upon three distinct apoptotic stimuli including staurosporine, etoposide, and ceramide C₂ when compared with mock-transfected cells. This inhibitory control of the caspase response triggered by apoptotic agents was abolished by the PD-related pathogenic mutation. Comparison of wild-type and mutated α-synuclein-expressing cells also indicates that the former exhibits much less vulnerability in response to staurosporine and etoposide as measured by the sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzensulfonic acid assay. Altogether, our study indicates that wild-type α-synuclein exerts an antiapoptotic effect in neurons that appears to be abolished by the Parkinson’s disease-related mutation. We have taken advantage of the design of a clonal cell line from neocortical origin (TSM1 cells (10)) to examine the possible influence of α-synuclein in the control of the neuronal apoptotic response and to establish a putative modulation of such a function by the Parkinson’s disease pathogenic mutation. We set up TSM1 neurons stably overexpressing wild-type α-synuclein or its Parkinson’s disease-associated Ala-53 → Thr mutant to examine their caspase response to various apoptotic stimuli. We show here that wild-type α-synuclein displays antiapoptotic properties that are abolished by the Parkinson’s disease-related mutation.

EXPERIMENTAL PROCEDURES

α-Synuclein Cloning and Mutagenesis—A cDNA library was prepared from total RNA derived from human adult cortex. A polymerase chain reaction product was obtained by means of the following primers, 5’-CGCAAACTTAGGAATTAGCCATGGATGTATTCAT-3’ containing the HindIII restriction site and 5’-TTTCTGAGATTTCT- TAGGCTCAGGTTGTCAT-3’ containing the XhoI site. The polymerase chain reaction fragment was cut with HindIII and XhoI and then subcloned in pcDNA3 vector either empty or encoding wild-type or Ala-53 → Thr α-synucleins. Transfectants were screened by Tris-Tricine gel analysis and Western blotting (see below). Positive clones overexpress a 18–19-kDa immunoreactive protein in agreement with a previous study (4). TSM1 Culture and Stable Transfection—TSM1 neuronal cells were cultured as described (13). TSM1 cells were stably transfected with superfect agent (Qiagen) containing 2 μg of pcDNA3 vector either empty or encoding wild-type or Ala-53 → Thr α-synucleins. Transfectants were screened by Tris-Tricine gel electrophoresis and Western blotted as described (15). Nitrocellulose sheets were heated in boiling phosphate buffer and then capped with 5% skim milk in phosphate-buffered saline. Membranes were then rinsed and incubated with a 1:5000 dilution of anti-human α-synuclein

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‡ Recipient of a grant from Aventis Pharma.
¶ To whom correspondence should be addressed. Tel.: 33 4 93 95 77 60; Fax: 33 4 93 95 77 08/04; E-mail: checler@ipmc.cnrs.fr.

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; Ac-DEVAD-al, acetyl-Asp-Glu-Val-Asp-aldehyde; 7AMC, 7-amino-4-methylcoumarin; XTt, sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid; β-APP, β-amylloid precursor protein; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Wild-type but Not A53T α-Synuclein Protect Neuronal Cells

RESULTS

Mock-transfected TSM1 neurons were examined for the modulation of their caspase activity in response to various apoptotic stimuli. In basal conditions, TSM1 neurons display an Ac-DEVD-7AMC hydrolyzing activity that is virtually fully abolished by prior treatment with the caspase inhibitor, Ac-DEVD-al (Fig. 1, N.St). Ac-DEVD-al-sensitive activity was drastically enhanced after treatment of mock-transfected TSM1 neurons with staurosporine, etoposide, and ceramide C2 (Fig. 1), three classical pro-apoptotic effectors.

We set up stably transfected TSM1 neurons overexpressing wild-type and Ala-53 → Thr α-synucleins. As expected from the use of antibody specificities against the human species, mock-transfected TSM1 neurons do not display any α-synuclein-like immunoreactivity (Fig. 2). We obtained several positive clones expressing various levels of an 18–19-kDa immunoreactive protein in agreement with the expected molecular weight of α-synuclein (4). We have selected two clones expressing virtually identical amounts of wild-type (clone T1) and mutated (clone K1) α-synucleins (Fig. 2) for further analysis.
TABLE I

| Effector | Mock | K1 | T1 |
|----------|------|----|----|
|          | DEVD | + | - | DEVD | + | - | DEVD | + | - | DEVD | + | - |
| Basal    | 1669 ± 65 | 336 ± 9.6 | 1333 | 1868 ± 85 | 396 ± 33 | 110 | 680 ± 93 | 260 ± 46 | 31 |
| STS      | 10068 ± 223 | 508 ± 54 | 9561 | 10399 ± 664 | 395 ± 58 | 104 | 2751 ± 300 | 409 ± 61 | 25 |
| ETO      | 11985 ± 1010 | 476 ± 49 | 11499 | 11400 ± 1045 | 618 ± 52 | 99 | 4296 ± 227 | 332 ± 50 | 35 |
| CER      | 9925 ± 1546 | 269 ± 18 | 9656 | 6359 ± 551 | 329 ± 20 | 62 | 2459 ± 298 | 326 ± 15 | 22 |

Mock-transfected TSM1 neurons (Mock) and K1 or T1 clones were cultured without (−) or with (+) Ac-DEVD-al in the absence (basal) or in the presence of staurosporine (STS, 1 μM, 2 h), etoposide (ETO, 50 μM, 24 h) or ceramide C2 (CER, 100 μM, 24 h). After incubations, caspase activity was assayed as detailed under “Experimental Procedures.” Δ corresponds to the Ac-DEVD-al-sensitive Ac-DEVD-AMC-hydrolyzing activity. % are the Δ obtained with T1 and K1 cells expressed as the percent of Δ obtained with mock-transfected TSM1 neurons. Values are the mean ± S.E. of duplicate determinations of 3–12 independent experiments.

Wild-type but Not A53T α-Synuclein Protect Neuronal Cells

T1 clone exhibits a drastically lower basal Ac-DEVD-al-sensitive caspase activity than mock-transfected cells, suggesting that wild-type α-synuclein exerts an inhibitory control on basal caspase activity (Fig. 3). Interestingly, another clone (T6) displaying lower wild-type α-synuclein-like immunoreactivity than T1 clone also displays a reduced basal caspase activity, although to a lower extent (not shown). Very strikingly, this inhibitory tonus was not observed with the K1 clone, the basal caspase activity of which resembles that measured in mock-transfected cells (Fig. 3).

Fig. 4 indicates that the treatment of T1 and K1 clones with the Ac-DEVD-al did not not modify the α-synuclein-like immunoreactivities, indicating that the distinct basal apoptotic caspase-mediated response of K1 and T1 clones could not be accounted for a distinct susceptibility of wild-type and mutated α-synucleins to caspase proteolysis. This agrees well with previous studies showing that α-synucleins are long-lived proteins in various cell types including PC12 and HEK293 cells (14) and that both wild-type and Ala-53 → Thr α-synucleins resist proteolysis by the proteasome in TSM1 neurons (11).

We further examined the caspase activation of TSM1 transfectants upon stimulation by various apoptotic stimuli. Table I indicates that T1 clone responsiveness to staurosporine, etoposide, and ceramide C2 was 22–35% of those observed with mock-transfected TSM1 neurons. Here again, the K1 clone displays a caspase response close to that observed with the mock-transfected cells. Of most importance was the fact that the Ac-DEVD-al-sensitive hydrolyzing activities were virtually abolished by Ac-DEVD-al whatever the stimulus examined (Table I). It should be noted here that apoptotic stimuli do not modify the immunoreactivity of wild-type and α-synucleins in TSM1-transfected cells (not shown), excluding the possibility that the distinct responses could be due to a modulation of α-synuclein expression by apoptotic agents.

All apoptotic effectors activate the Ac-DEVD-al-sensitive caspase activity in a dose-dependent manner (Fig. 5). At all concentrations examined, the T1 clone displays a caspase response drastically lower than those exhibited by mock-transfected cells or K1 clone (Fig. 5). Time course analysis of caspase activation upon apoptotic effectors further confirms the much lower Ac-DEVD-al-sensitive activity detectable at any time of the kinetics in cells expressing wild-type α-synucleins when compared with other transfectants (Fig. 6).

The measurement of Ac-DEVD-al-sensitive caspase activity is a specific cell response that can be likely ascribed to programmed cell death. To examine the global response, we studied the etoposide and staurosporine-induced vulnerability of mock-transfected TSM1 neurons and compared it with those of T1 and K1 clones. Both effectors trigger an ~50% decrease in cell viability of mock-transfected neurons (Fig. 7). Wild-type α-synuclein clearly enhances neurons viability in response to staurosporine (71.2%, n = 14, p < 0.001 compared with mock) and etoposide (75.8%, n = 8, p < 0.001, see Fig. 7, A and B). Interestingly, K1 clone viability is highly affected by both agents and appears even more susceptible...
than mock-transfected cells (32% staurosporine, n = 14, p < 0.01 and 23% etoposide, n = 8, p < 0.001, see Fig. 7, A and B).

**DISCUSSION**

A dense network of histological and biochemical evidence indicates that programmed cell death could contribute to Parkinson’s disease neuropathology. Thus, Mochizuki et al. (15) reported on the presence of nick end-labeled apoptotic stigmata in the midbrains of late and early onset affected patients. This was confirmed by a morphological study showing typical degenerating neurons in the nigro-striatal area (16, 17). Several animal models used to study Parkinson’s disease pathology led to the in situ detection of apoptotic nuclei (18) as it can also be evidenced in several cell models including human neuroblastoma (19), PC12 (20, 21), or primary cultures of mesencephalic neurons (21).

Several biochemical clues of a link between Parkinson’s disease and actors of the apoptotic pathways have also been reported. Bcl2 expression is modulated in Parkinson’s disease-affected brains (22, 23). Prostate apoptosis response-4 levels increase in neurons of the dopaminergic pathway after exposure of mice or monkeys to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (24), a neurotoxin classically used to elicit dopaminergic neurons cell death. Furthermore, oxidative stress seems to contribute to the Parkinson’s disease pathogenesis (25), as corroborated by subsequent works demonstrating that a mitochondrial impairment (26) and ceramide-dependent apoptosis (27) occurred in the neuronal cell line NS20Y as well as in PC12 cells.

We previously used TSM1 neurons to establish a protein-kinase A-regulated $\alpha$-secretase cleavage of $\beta$-amyloid precursor protein ($\beta$-APP) (28, 29). This cell model also allowed us to confirm the unusual phenotypic alteration triggered by a novel mutation of $\beta$-APP associated with familial form of Alzheimer’s disease in agreement with that seen in HEK293 cells (13). Altogether, these data indicate that TSM1 neurons represent a most suitable cell system from central origin to examine the putative function of protein candidates involved in neurodegenerative disease. This cell model was used to demonstrate that $\alpha$-synuclein could negatively control the Ac-DEVD-al-sensitive caspase activation of TSM1 neurons in response to various stimuli, the pharmacological spectrum of which strongly suggests an apoptotic rather than necrotic mechanism. Of most interest is the observation that this negative control of caspase activity is fully abolished by the Ala-53→Thr mutation of $\alpha$-synuclein responsible for autosomal dominant forms of the disease (5). The cellular mechanism underlying the ant apoptotic function of $\alpha$-synuclein still remains to be elucidated. However, one could postulate on the involvement of the chaperoning property of $\alpha$-synuclein. Thus, $\alpha$-synuclein has been shown to bind tau proteins (30) and to display the ability to interact with brain vesicles, a property that is abolished by Parkinson’s disease mutations (30). Most interesting was the recent report (31) indicating that $\alpha$-synuclein exhibits a 40%
homology with members of the 14-3-3 chaperone protein family. 14-3-3 proteins interact with BAG, a pro-apoptotic oncogene that remains active when sequestered in the cytosol (32). To explain the antiapoptotic function of α-synuclein by its chaperone activity, one could therefore envision that, under physiological conditions, wild-type α-synuclein interacts with cellular intermediates of the apoptotic pathways. In the pathology, α-synuclein accumulates and aggregates as has been documented by the high concentration of the protein in Lewy bodies invading Parkinson’s disease brains. Under these pathological conditions, the wild-type α-synuclein-mediated inhibitory tonus on caspase activity could be abolished, thereby contributing to increased cell death. This hypothesis is in agreement with the observation that aggregated α-synuclein triggers cell death in human neuroblastoma cells (8). In this context, mutated α-synucleins could accelerate the pathogenesis because of the absence of neuroprotection to apoptotic stimuli (our study), its higher susceptibility to aggregation (7), and its ability to trigger apoptotic cell death when aggregated (8). It should be noted in support of this hypothesis that at a cellular level, α-synuclein is almost exclusively found to be associated with normal neurons but not with those exhibiting an apoptotic phenotype (9). Some authors (33) demonstrated that in the target injury model, α-synuclein expression was up-regulated, suggesting that this could correspond to a compensatory response of neurons designed to promote their survival, in agreement with a physiological antiapoptotic function.

It is interesting to emphasize the parallels between Parkinson’s disease and Alzheimer’s disease pathology. Thus, familial Alzheimer’s disease cases are mostly due to mutations located on two proteins, namely the β-amyloid precursor protein and presenilin 1 (for reviews see Refs. 34 and 35). It has been demonstrated that wild-type presenilin 1 displays antiapoptotic function that is abolished by presenilin 1 bearing familial Alzheimer’s disease mutations (for reviews see Refs. 36 and 37). Identical observations indicate that β-APP confer resistance to p53-induced cell death, but the familial Alzheimer’s disease-associated V717I β-APP did not fit (38).

Our work opens a possible track to slow down or stop the progression of the neurodegeneration taking place in Parkinson’s disease. Thus, one can envision the design of peptides or chemically designed agents displaying α-synuclein anti-aggregating properties. According to our hypothesis, such effectors may prevent α-synuclein deposits and should maintain it as a physiological antiapoptotic modulator.

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