6-Hydroxydopamine but Not 1-Methyl-4-phenylpyridinium Abolishes α-Synuclein Anti-apoptotic Phenotype by Inhibiting Its Proteasomal Degradation and by Promoting Its Aggregation*

Received for publication, December 30, 2005 · Published, JBC Papers in Press, February 7, 2006, DOI 10.1074/jbc.M513903200

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We established previously that α-synuclein displayed a protective anti-apoptotic phenotype in neurons, mainly by down-regulating p53-dependent caspase-3 activation (Alves da Costa, C., Ancolio, K., and Checler, F. (2000) J. Biol. Chem. 275, 24065–24069; Alves da Costa, C., Paitel, E., Vincent, B., and Checler, F. (2002) J. Biol. Chem. 277, 50980–50984). This function was abolished by Parkinson disease-linked pathogenic mutations and by the dopaminergic toxin, 6-hydroxydopamine (6OH-DOPA) (Alves da Costa, C., Paitel, E., Vincent, B., and Checler, F. (2000) J. Biol. Chem. 275, 50980–50984). However, the mechanisms by which 6OH-DOPA interfered with α-synuclein function remained unclear. Here we showed that 6OH-DOPA prevents α-synuclein-mediated anti-apoptotic function by altering its degradation. Thus, 6OH-DOPA treatment of TSM1 neurons and SH-SY5Y neuroblastoma cells enhances endogenous α-synuclein-like immunoreactivity and inhibits the catabolism of endogenous and recombinant α-synucleins by purified 20 S proteasome. Furthermore, we demonstrated that 6OH-DOPA directly inhibits endogenous proteasomal activity in TSM1 and SH-SY5Y cells and also blocks purified proteasome activity in vitro. This inhibitory effect can be prevented by the anti-oxidant phenyl-N-butylnitrone. We also established that 6OH-DOPA triggers the aggregation of recombinant α-synuclein in vitro. Therefore, we conclude that 6OH-DOPA abolishes α-synuclein anti-apoptotic phenotype by inhibiting its proteasomal degradation, thereby increasing its intracellular concentration and potential propensity to aggregation, the latter phenomenon being directly exacerbated by 6OH-DOPA itself. Interestingly, 1-methyl-4-phenylpyridinium (MPP⁺), another toxin inducer of Parkinson disease-like pathology, does not affect α-synuclein protective function and fails to trigger aggregation of recombinant α-synuclein. Furthermore, MPP⁺ does not alter cellular proteasomal activity, and only high concentrations of the toxin affect purified 20 S proteasome by a mechanism that remains insensitive to phenyl-N-butylnitrone. The drastically distinct effects of 6OH-DOPA and MPP⁺ on α-synuclein function are discussed with respect to Parkinson disease pathology and animal models mimicking this pathology.

Parkinson disease (PD) is the second most common age-related neurodegenerative disease. This invalidating disease is characterized, at an immunohistochemical level, by the selective destruction of the dopaminergic neurons of the substantia nigra pars compacta. Although the replacement treatment consisting of the administration of the dopamine precursor 3,4-dihydroxy-L-phenylalanine has proven useful to temporarily stabilize patients, it still remains a symptomatic treatment, and the exact etiology of the disease remains a crucial and still unsolved issue.

Parkinson disease cases are generally of sporadic origin, but a few cases have a genetic etiology. These familial PD cases are typically of earlier onset and more aggressive and can be caused by either autosomal dominant or recessive inherited mutations (3). The identification of the protein candidates that, when mutated, contribute to familial PD and the delineation of their associated physiological or PD-related altered function will allow a better understanding of the etiology of the disease.

The α-synuclein (α-syn) protein (4) is one of the major molecules involved in PD neuropathology. Several independent point mutations (5–7) or the duplication/triplication of the α-syn gene (8–11) causes autosomal dominant cases of PD. The α-syn protein is, together with ubiquitin, the main component of Lewy bodies (12), the intracellular inclusions that accumulate as the disease progresses (13). The overexpression of wild-type or mutant α-syn triggers dopaminergic neuronal loss accompanied by exacerbated α-syn aggregation in transgenic animals (14). Conversely, the α-syn depletion leads to nigrostriatal dysfunction (15). Therefore, both the overexpression or deletion of α-syn leads to PD-like functional and morphological alterations. These apparently paradoxical observations could be reconciled by assuming that α-syn has a physiological function that would be abrogated in knock-out mice but also blocked by a concentration-dependent aggregation process associated with overexpression in transgenic animals.

We have reported previously on the ability of α-syn to protect TSM1 neurons and HEK293 cells from staurosporine-induced caspase-3 activation (1). This phenotype was associated with a down-regulation of the p53 pathway (2). Interestingly, the α-syn protective effect was abolished by PD-linked mutations (1) as well as by 6-hydroxydopamine (6OH-DOPA) (2), a natural dopaminergic toxin (16). The abrogation of the α-syn anti-apoptotic phenotype by both genetic and biochemical defects firmly suggested that this protective effect reflected a physiolog-
ical function of the protein, correlating well with the nigrostriatal alteration observed in knock-out mice lacking α-syn (15).

Although the effect of 6OH-DOPA on α-syn function was drastic, the mechanisms by which this toxin abolishes the α-syn-associated protective phenotype in neurons remains largely speculative. Furthermore, whether the modulation of α-syn function was selectively altered by 6OH-DOPA and not by other natural or synthetic neurochemicals affecting dopaminergic neurons remained to be established. In this study we demonstrate that 6OH-DOPA, but not 1-methyl-4-phenylpyridinium (MPP⁺), interferes with the α-syn anti-apoptotic phenotype by both inhibiting its proteasomal degradation and promoting its aggregation.

EXPERIMENTAL PROCEDURES

**Materials and Cells—**Z-Gly-Gly-Leu-7-aminomethylcoumarin, staurosporine, lactacystin, stabilized 6OH-DOPA, phenyl-N-butylinotroten (PBN), and MPP⁺ were all purchased from Sigma. XTT and lactate dehydrogenase (LDH) cell toxicity assay kits were purchased from Roche Applied Science and Promega, respectively. The TSM1 murine neuronal cell line expressing wild-type α-syn was obtained and cultivated as detailed previously (1). SH-SY5Y neuroblastoma cells were cultured as TSM1 neurons.

**Preparation and Purification of Recombinant Human α-Syn—**The human α-syn cDNA sequence was cloned into pRSETB (Invitrogen), overexpressed in the *Escherichia coli* strain BL21(DE3), and purified by acid precipitation followed by anion exchange as described previously (17). The α-syn was dialyzed against phosphate-buffered saline and then lyophilized using a freeze dryer. Purity was determined by SDS-PAGE and Coomassie Brilliant Blue staining.

**Purification of the 20 S Proteasome and Fluorimetric Assay—**Purified bovine pituitary 20 S proteasome (specific activity of about 6 mmol of p-nitroaniline released per h/mg from Z-Gly-Gly-Leu-p-nitroaniline) was obtained as described (18). Proteasomal activity was measured as follows. Briefly, Z-Gly-Gly-Leu-7-aminomethylcoumarin (100 μM) was incubated with 2 μl of the purified enzyme for several time intervals at 37 °C, in a final volume of 100 μl of 25 mM Tris-HCl, pH 7.5 (containing 5 mM MgCl₂, 1 mM dithiothreitol, and 1 mM EDTA), in the absence or in the presence of lactacystin (1 μM). Fluorescence was recorded at 396 and 460 nm for excitation and emission wavelengths, respectively, as described previously (19).

**Endogenous Proteasomal Activity—**TSM1 cells were cultivated in 6-well plates until 70–80% of confluence and then treated for 8 h at 37 °C in the presence and absence of various concentrations of MPP⁺. Sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT)-metabolizing activity was determined as described previously (1). Caspase-3 activity was monitored as described (20).

**LDH Assay—**TSM1 neurons were grown in a 5% CO₂ atmosphere in 96-well plates in a final volume of 100 μl per well and were incubated overnight at 37 °C in the presence and absence of various concentrations of MPP⁺. Sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT)-metabolizing activity was determined as described previously (1). Caspase-3 activity was monitored as described (20).

**RESULTS**

6OH-DOPA but Not MPP⁺ Abolishes the α-Syn Anti-Apoptotic Phenotype—We previously established that wild-type α-syn drastically reduced the staurosporine-induced activation of caspase-3 in HEK293 cells and in TSM1 neurons (1). This anti-apoptotic phenotype was abolished by the dopamine toxin 6OH-DOPA (2). In mock-transfected TSM1 neurons, the pro-apoptotic effector staurosporine (1 μM) drasti-

We examined whether MPP⁺ could modulate the protective effect of α-syn on cellular toxicity. As expected, MPP⁺ elicited a dose-dependent toxic effect on mock-transfected TSM1 neurons (Fig. 2A). At a low MPP⁺ concentration (0.1 μM), TSM1 viability measured by XTT assay was reduced by about 50%, although a maximal effect was observed at 1 μM and plateaued at higher concentrations of MPP⁺ (Fig. 2A). Interestingly, α-syn expression significantly protected TSM1 from MPP⁺-induced toxicity, except at the highest concentration of MPP⁺ tested.
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FIGURE 1. 6OH-DOPA but not MPP⁺ abolishes α-syn inhibitory effect on caspase-3 activation. A, mock-transfected or α-syn-expressing TSM1 neurons were treated for 2 h with staurosporine (STS, 1 μM) or for 8 h with 6OH-DOPA (6OH, 0.2 mM) or MPP⁺ (0.2 mM), and then active caspase-3-like immunoreactivity (Casp-3_act) and tubulin expression were analyzed as described under “Experimental Procedures.” B and C, mock-transfected (black bars) or α-syn-expressing (white bars) TSM1 neurons were treated for 8 h without (C) or with the indicated concentrations of 6OH-DOPA (B) or MPP⁺ (C), and then caspase-3 activity was monitored as described under “Experimental Procedures.” Values represent the means ± S.E. of three independent experiments performed in duplicate.

(See text for further details.)

FIGURE 2. α-Syn protects TSM1 neurons against MPP⁺-induced toxicity. A, mock-transfected (black bars) or α-syn-expressing (white bars) TSM1 neurons were treated overnight with the indicated concentrations of MPP⁺, and then cell viability was monitored by XTT assay as described under “Experimental Procedures.” 6OH-DOPA (6OH, 0.2 mM) or MPP⁺ (5 mM), and then cell viability was monitored by LDH release toxicity assay as described under “Experimental Procedures.” Values represent the means of 2 and 5 independent experiments performed in triplicate and are expressed as percent of control untreated cells taken as 100 percent of cytotoxicity (% release total LDH) for A and B, respectively. *, p < 0.05; **, p < 0.01; ***, p < 0.001; NS, not significant; Wt, wild type. All statistical analysis correspond to comparisons versus control except when indicated by horizontal lines or brackets.

(See text for further details.)
Whereas MPP did not inhibit the activity, even at high concentrations, 6-OH-DOPA induced inhibition of the 20 S proteasome (Fig. 8 A). The same distinct effects were observed in SH-SYSY cells where 6-OH-DOPA but not MPP inhibited endogenous proteasomal activity (Fig. 9 A). To confirm the ability of 6-OH-DOPA but not MPP to inhibit the proteasome, we tested the ability of these compounds to block purified 20 S proteasome. The 6-OH-DOPA inhibited the 20 S proteasome in a dose-dependent manner, and this was prevented by 6-OH-DOPA (Fig. 9 A). As shown in Fig. 6 C, a high concentration of MPP inhibited 20 S proteasome activity, but this inhibition remained insensitive to PBN (Fig. 7 A). Interestingly, PBN also fully prevented the 6-OH-DOPA-induced inhibition of endogenous proteasomal activity in SH-SYSY cells (Fig. 7 B). As described above (Fig. 5 C), MPP did not modulate endogenous proteasomal activity in SH-SYSY cells, and PBN treatment combined with MPP led to a proteasomal activity similar to that observed with MPP alone (Fig. 7 B).

6-OH-DOPA but Not MPP Triggers Aggregation of Recombinant α-Syn—When studying the hydrolysis of recombinant α-syn by purified 20 S proteasome (Fig. 8 A), we unexpectedly observed the disappearance of α-syn immunoreactivity when recombinant protein was exposed to 6-OH-DOPA alone or 6-OH-DOPA and 20 S proteasome (Fig. 8 B, see 6-OH/MPC lane). However, this was not observed with MPP alone (Fig. 8 B). Based on recent reports that dopamine (17) and dopaminergic neurotransmitter (26) can promote α-syn aggregation, we presumed that 6-OH-DOPA, in addition to its effects on proteasome activity, could be directly altering the physical properties of α-syn. Recombinant α-syn alone was stable in vitro, even after 72 h (Fig. 9 A, − lanes), but an 8-h incubation with 6-OH-DOPA caused the total disappearance of α-syn monomer immunoreactivity. 6-OH-DOPA promoted the formation of high molecular weight aggregates within 2 h after exposure to 6-OH-DOPA, which drastically increased with time (Fig. 9 B). Interestingly, MPP did not alter α-syn immunoreactivity, even after 72 h of incubation (Fig. 9 A).

**DISCUSSION**

The majority of PD appears to be of sporadic origin with a few cases associated with either an autosomal recessive or dominant transmission (27, 28). Although the familial forms of PD are generally aggressive and characterized by an earlier age of onset, the sporadic and genetic PD cases appear to share the same clinical features (29) as well as common histological lesions and molecular defects. All PD-affected brains exhibit intracytoplasmic inclusions referred to as Lewy bodies and...
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![Graphs showing the effect of 6OH-DOPA on α-Synuclein catabolism and aggregation.](image)

extensive neurodegeneration of substantia nigra pars compacta, leading to a drastic depletion of dopaminergic transmission (30). The death of dopaminergic neurons appears linked to increased oxidative stress and apoptosis (27, 30–34). Insights from experimental models seem to indicate that cell death could be associated with an exacerbation of the p53-dependent proapoptotic pathway (35–38).

The α-syn protein is a major component of Lewy bodies (12) and was the first gene product identified with a mutation responsible for autosomal dominant PD (5). Either overexpression or deletion of α-syn results in PD-like stigmata in animal models. Overexpression of α-syn leads to its aggregation accompanied by dopaminergic neuronal loss (14), whereas α-syn gene knock-out triggers nigrostriatal dysfunction (15). These discrepant observations could be reconciled by our work (1, 2) and the work of others (39–43) showing that nonfibrillar α-syn exerts an anti-apoptotic effect that is fully abolished by pathogenic mutations (1), which have been shown to elicit α-syn aggregation (44–46).

The α-syn-mediated anti-apoptotic phenotype was abolished by 6OH-DOPA (2), a dopaminergic derivative thought to act as a natural toxin involved in PD pathology (16). Although α-syn immunoreactivity is enhanced by treatment with 6OH-DOPA (2), the mechanisms by which the toxin could block α-syn function remained elusive. This study shows that 6OH-DOPA triggers two neurochemical alterations that directly impact α-syn physiology. First, 6OH-DOPA prevents α-syn degradation, and second, it triggers its aggregation.

The effect of 6OH-DOPA on α-syn degradation was demonstrated both in vivo and in cell cultures by the capability of 6OH-DOPA to inhibit the proteasome. Thus, 6OH-DOPA inhibits both cellular endogenous proteasome (47) and purified 20 S proteasome activities and prevents α-syn degradation by both enzymatic systems. Furthermore, 6OH-DOPA and lactacystin, a rather specific proteasome inhibitor (21), both inhibit the degradation of α-syn in a nonadditive manner, suggesting that both chemicals target the same intracellular activity. More importantly, 6OH-DOPA inhibits cellular proteasomal activity and protected α-syn from degradation in SH-SY5Y. This cell system is widely used and is considered as a particularly relevant model to study the biology of Parkinson disease-related proteins because it is a human dopaminergic cell line (48–51). This also indicates that the 6OH-DOPA effect described in this study is not cell-specific.

Because lactacystin can alter the expression levels of proteins that are driven by cytomegalovirus promoters (52–54), it was important to demonstrate that recombinant α-syn still undergoes 6OH-DOPA-sensitive hydrolysis by the purified 20 S proteasome in vitro. It should be noted that the 26 S proteasome machinery is involved in the cellular degradation of polyubiquitinated proteins, whereas the 20 S proteasome contributes mainly in the breakdown of natively unfolded proteins, a category of proteins to which α-syn belongs (55). These data agree well with the observations that impairment of the ubiquitin-proteasome system causes dopaminergic neurons cell death accompanied by the formation of Lewy bodies and α-synuclein inclusions (56, 57).

The mechanism by which 6OH-DOPA could trigger inhibition of the proteasome is likely related to its pro-oxidant activity. Thus, 6OH-DOPA has been shown to contribute to the production of hydrogen peroxide and reactive oxygen species (27). Accordingly, the effect of 6OH-DOPA on purified 20 S proteasome is prevented by the anti-oxidant PBN. More importantly, PBN reverted the 6OH-DOPA-induced
inhibition of α-syn degradation and proteasome activity in SH-SY5Y neuroblastoma cells.

6OH-DOPA also triggers aggregation of recombinant α-syn in vitro. It is also likely to be due to its pro-oxidant properties as it has been shown that oxidized α-syn is more prone to aggregation (45). It has been shown that protein aggregation significantly contributes to inhibit the cellular proteasomal machinery, and this could be a general process responsible for most of the misfolded proteins-associated neurodegenerate diseases (58). Treatment of cells by 6OH-DOPA first leads to increased α-syn expression without clues of high molecular weight α-syn-related aggregates. It is therefore likely that 6OH-DOPA first enhances cellular α-syn levels via inhibition of the proteasome. Subsequent to this process, one could envision that increased concentrations of α-syn could lead to its aggregation and that this process is further self-potentiated by the ability of 6OH-DOPA to promote α-syn aggregation.

The above data are in good agreement with the observations of aggregated α-syn in Lewy bodies and the fact that PD animal models resulting from 6OH-DOPA intracerebral administration show substantia nigra degeneration, even if none of the current 6OH-DOPA-associated animal models of PD leads to Lewy bodies-like inclusions (3). It is interesting to note that MPTP-linked models of PD share some but not all of the features observed after 6OH-DOPA administration. MPP⁺ triggers mitochondrial defects and oxidative stress (59, 60), and these cellular dysfunctions are very rarely associated with Lewy bodies. However, it should be noted that data on MPP⁺-induced apoptosis are not consensual. Strikingly, unlike 6OH-DOPA, MPP⁺ does not trigger annexin V staining or mitochondrial alterations on dopaminergic cell cultures (61). Furthermore, Choi et al. (62) showed that MPP⁺ triggers p53-independent necrosis rather than apoptosis in the dopaminergic cell line MND9. Finally, Jackson-Lewis et al. (63) failed to confirm apoptotic cells in MPTP-injected mice. Some of the controversial data could be due to the very high doses of MPP⁺ usually examined (61) and in agreement with the following: first, the effect of MPP⁺ on α-synuclein-mediated caspase 3 inhibition that was observed only at a high 5 mM concentration of MPP⁺ (see Fig. 1); second, the very faint and dose-independent effect of MPP⁺ on proteasome activity (see Fig. 6).

Clearly, MPP⁺ and 6OH-DOPA display distinct effects on α-syn. The
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MPP⁺ was unable to inhibit cellular proteasome activity, did not modulate α-syn degradation, and did not affect α-syn aggregation in vitro (see Figs. 5, 6, and 8). The sole effect observed only with high concentrations of MPP⁺ concerns the inhibition of purified 20 S proteasome. However, unlike 6OH-DOPA, MPP⁺ inhibitory effect was not reverted by the antioxidant PBN. This indicates that the mechanisms of proteasome inhibition by 6OH-DOPA and MPP⁺ are clearly distinct. That MPP⁺ did not affect cellular proteasome activity and α-syn degradation suggests that the inhibition of purified proteasome by high concentrations of MPP⁺ is likely irrelevant for cellular biology of α-syn and proteasomal activity.

Therefore, only 6OH-DOPA is directly capable of modulating the expression levels and biophysical properties of α-syn. This reconciles the observed aggregated α-syn in Lewy bodies in PD brains with alterations associated with 6OH-DOPA experimental models. It is important to note that unlike 6OH-DOPA, which is considered by many as a physiological dopaminergic neurotoxin present in the human brain, MPP⁺ is the monoamine oxidase B-derived product of the synthetic drug MPTP. Therefore, it is likely that 6OH-DOPA is more relevant to recapitulate the molecular dysfunctions responsible for PD despite the potential of MPTP models to study nigral degeneration and PD-like symptoms.

6OH-DOPA triggers apoptosis by a p53-dependent pathway (2, 37). Interestingly, α-syn anti-oxidative function involves the down-regulation of p53-mediated caspase-3 activation (2). When 6OH-DOPA inhibits α-syn function, this is accompanied by an augmentation of p53 levels (2). It is striking that β-syn, a member of the syn family, is also anti-oxidative, lowers the p53 pathway, but remains protective even in the presence of 6OH-DOPA (64). Furthermore, β-syn protects α-syn from 6OH-DOPA (64), in agreement with the ability of β-syn or some of its fragments to prevent α-syn aggregation in vitro (65, 66) or to reverse the defects observed in animals overexpressing α-syn alone (67). Interestingly, the levels of β-syn are drastically lower in Lewy bodies, and therefore, one could speculate that, when β-syn levels are reduced, the protection of α-syn by its congener is affected and α-syn susceptibility to 6OH-DOPA toxicity is increased leading to the above-described biochemical consequences.

Our study at least partly explains the topological selectivity of the lesions observed in PD. Thus, α-syn is widely distributed within the central nervous system where the protein exerts its anti-oxidative function. However, only the dopaminergic neurons have the potential of producing the endogenous toxin able to modify α-syn degradation and to modulate its biophysical characteristics. This firmly links α-syn dysfunction to the very narrow anatomical selectivity of the lesions observed in PD. These observations also support the concept that a strategy aimed at preventing α-syn aggregation, either by β-syn-related synthetic fragments or by interfering with 6OH-DOPA-associated toxicity, likely by reducing its pro-oxidative properties might be beneficial.

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APRIL 7, 2006•VOLUME 281•NUMBER 14
JOURNAL OF BIOLOGICAL CHEMISTRY
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