Proteasome Inhibitors Protect Against Degeneration of Nigral Dopaminergic Neurons in Hemiparkinsonian Rats

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Abstract. Parkinson’s disease is characterized by dopaminergic neuronal death and the presence of Lewy bodies in the substantia nigra pars compacta (SNpc). α-Synuclein and ubiquitin are components of Lewy bodies, but the process of Lewy body formation and the relationship between inclusion formation and dopaminergic neuronal death have not been resolved. In this study, unilateral intranigral microinjection of 6-hydroxydopamine caused a significant loss of tyrosine hydroxylase-immunopositive neurons in both the substantia nigra and striatum and apomorphine-induced contralateral rotation. The co-administration of proteasome inhibitors, such as lactacystin or carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), significantly prevented both dopaminergic neurodegeneration and apomorphine-induced rotational asymmetry. Proteasome inhibitors markedly formed intracellular protein inclusions labeled by thioflavin-S in the SNpc. Inclusion-like immunoreactivities for α-synuclein and ubiquitin were detected after 4 weeks. These results suggest that proteasome plays an important role in both the early phase of dopaminergic neuronal death and inclusion body formation.

Keywords: proteasome inhibitor, neuroprotection, dopaminergic neuron, substantia nigra, Parkinson’s disease

Introduction

Parkinson’s disease (PD) is one of the most prevalent neurodegenerative disorders. Its pathological characteristics include selective death of mesencephalic nigral dopaminergic neurons and the presence in the substantia nigra of intracytoplasmic inclusions, known as Lewy bodies, which are consistently immunostained with antibodies to α-synuclein and ubiquitin (1, 2). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism (AR-JP) (3, 4), and mutations in the α-synuclein (A53T and A30P) and ubiquitin carboxy-terminal hydrolase L-1 (UCH-L1) genes cause familial PD, which is inherited in an autosomal dominant fashion (5, 6). However, the pathogenesis of these disorders is still not clear. It was recently revealed that parkin is a ubiquitin ligase E3 and that mutant parkin loses E3 activity (7, 8). These data present a hypothesis that dopaminergic neuronal death in familial PD is related to impairment of the ubiquitin-proteasome system (9). Furthermore, in patients with sporadic PD, proteasome activity is well preserved in the striatum but reduced in the midbrain (10). Therefore, it has been assumed that insufficient function of the ubiquitin-proteasome system plays an important role in the pathogenesis of PD.

6-Hydroxydopamine (6-OHDA) was the first agent used to produce an animal model of PD (11–13). The common technique involves a unilateral injection of 6-OHDA into the substantia nigra or the medial forebrain bundle, which leads to rapid cell death with a time course (within 1–3 days) similar to that in the acute model using 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) (2). The toxicity of 6-OHDA is thought to be mediated by selective uptake through the transporter for dopamine (DA). 6-OHDA is an effective neurotoxin in rats, mice, cats, and primates, and it has been used predominantly to produce unilateral lesions (2, 12). In rats, the extent of DA depletion can then be
assessed by examining rotary behavior in response to apomorphine (13–15). Thus, this model offers a quantifiable motor deficit (rotation) and has been shown to be useful in the pharmacological screening of drugs that affect DA and its receptors (15, 16). In the present study, we examined the effect of proteasome inhibitors on dopaminergic neuronal death in a rat PD model using 6-OHDA. Furthermore, we investigated whether proteasome inhibition caused the appearance of $\alpha$-synuclein and ubiquitin inclusions in the substantia nigra.

Materials and Methods

Materials

6-OHDA, apomorphine, and thioflavin-S were purchased from Sigma (St. Louis, MO, USA). Lactacystin and carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) were purchased from Calbiochem (San Diego, CA, USA). Primary antibodies included rabbit polyclonal antibodies to tyrosine hydroxylase (TH) and ubiquitin from Chemicon (Temecula, CA, USA), and mouse monoclonal anti-$\alpha$-synuclein antibody (Transduction Lab., Lexington, KY, USA). The ABC Elite kit from Vector Laboratories (Burlingame, CA, USA) was also used.

Rat model

Male Wistar rats (SLC, Inc., Hamamatsu) weighing approximately 280 g were used. The rats were fasted overnight with free access to water and food. For stereotaxic microinjection, rats were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.) and immobilized in a Kopf stereotaxic frame. Subsequently, rats were simultaneously injected with 6-OHDA (8 $\mu$g) in the presence or absence of lactacystin (0.12 $\mu$g) or MG-132 (0.19 $\mu$g) in a final volume of 4 $\mu$l of sterilized physiological saline containing 1% dimethyl sulfoxide (DMSO). The microinjection coordinates (4.8 mm anterior-posterior, 1.8 mm left lateral, 7.8 mm ventral from the bregma) were according to a rat brain atlas (17), and injection was given by a motor-driven 10-$\mu$l Hamilton syringe using a 26-gauge needle. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and The Japanese Pharmacological Society.

Assay of rotational behavior

After the intranigral injection, apomorphine-induced rotational asymmetry was assessed every week. Rotational behavior was tested in rotometer bowls (11, 16). The number of full-body turns (360° rotations) in the ipsilateral and contralateral directions was counted for 60 min after an intraperitoneal administration of apomorphine (0.6 mg/kg).

Immunohistochemistry

After 12 h, 24 h, and 4 weeks, treated rats were perfused through the aorta with 150 ml of 10 mM phosphate-buffered saline (PBS), followed by 300 ml of a cold fixative consisting of 4% paraformaldehyde, 0.35% glutaraldehyde, and 0.2% picric acid in 100 mM phosphate buffer (PB), under deep anesthesia with pentobarbital (100 mg/kg, i.p.). After perfusion, the brain was quickly removed and postfixed for 2 days with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4°C. Brain sections were cut 20-$\mu$m-thick using a cryostat and collected in 100 mM PBS containing 0.3% Triton X-100 (PBS-T). After several washes, the slices were incubated with primary antibody to TH (diluted 1:20,000), $\alpha$-synuclein (1:2,000) or ubiquitin (1:2,000) for 3 days at 4°C. The antibody was detected with an ABC Elite kit using 3,3’-diaminobenzidine (DAB) with nickel enhancement.

Confocal analysis

After 4 weeks, nigral slices were co-incubated with primary antibodies against TH (1:5,000) and $\alpha$-synuclein (1:500) for four days at 4°C. The antibody was detected using rhodamine-labeled anti-mouse IgG antibody and fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody. Fluorescence for rhodamine (red) and FITC (green) was observed with a laser scanning confocal microscope LSM410 (Carl Zeiss, Jena, Germany).

Thioflavin-S staining

To detect the formation of protein $\beta$-sheet structure (an index of protein fibrillization), we performed thioflavin-S staining (18). Briefly, nigral slices were mounted on glass slides, incubated with 1% thioflavin-S in 70% ethanol for 10 min, and then rinsed with 70%, 95%, and 100% ethanol. Thioflavin-S was visualized at a specific filter setting using a laser scanning confocal microscope.

Statistical evaluation

Numbers of apomorphine-induced rotations and nigral TH-immunopositive neurons are given as the mean ± standard error of the mean (S.E.M.). The significance of differences was determined by an analysis of variance (ANOVA). Further statistical analysis for post hoc comparisons was performed using the Bonferroni/Dunn test (StatView; Abacus Concepts, Berkeley, CA, USA).
Results

Effects of proteasome inhibitors on apomorphine-induced rotational behavior

We examined the effect of proteasome inhibitors on dopaminergic neuronal death in vivo. Stereotaxic micro-injection with 6-OHDA alone into the unilateral (left) mesencephalon caused behavioral dysfunction (Fig. 1) and dopaminergic neuronal loss in the ipsilateral substantia nigra (Fig. 2). Administration of apomorphine to animals that had been lesioned unilaterally with 6-OHDA induced movement contralateral to the injection site (Fig. 1: A and B).

The effect of the simultaneous administration of proteasome inhibitors was investigated to determine the role of the proteasome system in dopaminergic neurodegeneration. Rotational behavior was significantly reduced at 1 week by the co-administration of lactacystin or MG-132 with 6-OHDA. Furthermore, this inhibitory effect of lactacystin or MG-132 on apomorphine-induced rotational asymmetry was maintained even after 4 weeks (Fig. 1). Apomorphine-induced rotational behavior was not induced by an injection of proteasome inhibitor alone (Fig. 1: C and D) or the vehicle control (Fig. 1: A and B).

Effects of proteasome inhibitors on TH immunoreactivity in the substantia nigra and striatum

We further assessed the immunoreactivity for TH in the substantia nigra and striatum of rats injected with 6-OHDA in the presence or absence of proteasome inhibitors. TH immunoreactivity was markedly decreased in the ipsilateral SNpc of rats injected with 6-OHDA alone (Fig. 2: A – F). Semi-quantitative analysis of nigral TH-immunopositive cells showed that the micro-injection of 6-OHDA alone caused a significant loss of dopaminergic neurons in a time-dependent manner (Fig. 3). After 4 weeks, the number of TH-immunopositive dopaminergic neurons in the SNpc decreased to less than 10% of the pretreatment value in rats injected with 6-OHDA alone (Figs. 2F and 3).

The 6-OHDA-induced loss of dopaminergic neurons was significantly inhibited by simultaneous injection with proteasome inhibitors, such as lactacystin and MG-132 (Figs. 2: G – L and 3). Similarly, in the ipsilateral striatum, although TH immunoreactivity was markedly decreased, this immunoreactivity was restored by the co-administration of lactacystin or MG-132 (Fig. 4).

Fig. 1. Proteasome inhibitors prevent apomorphine-induced rotational asymmetry. Rats received a single intranigral injection of vehicle control (sterilized physiological saline containing 1% DMSO, open symbol) or 8 µg of 6-OHDA (closed symbol) in the presence of vehicle (open circle, n = 6; closed circle, n = 6), 0.12 µg lactacystin (open triangle, n = 3; closed triangle, n = 4), or 0.19 µg MG-132 (open square, n = 3; closed square, n = 4). A, C: Rotational behavior induced by apomorphine (0.6 mg/kg, i.p.) was assessed every week. The number of full 360° rotations in the contralateral direction was counted for 60 min after the administration of apomorphine. Significance (Bonferroni /Dunn post hoc comparisons after ANOVA): †P<0.01, ††P<0.001 vs the vehicle control at each time point. B, D: Time-course of changes in the number of apomorphine-induced contralateral rotations at 4 weeks in hemiparkinsonian rats injected with vehicle control, or 6-OHDA with lactacystin or MG-132.
Neither lactacystin nor MG-132 alone had a significant effect on the number of dopaminergic neurons in the SNpc (Fig. 3).

**Effect of proteasome inhibitors on thioflavin-S staining in the substantia nigra**

The co-administration of lactacystin or MG-132 with 6-OHDA may promote protein conformational changes and subsequent aggregation in the SNpc. To test this hypothesis, brain sections from vehicle-, 6-OHDA-, or proteasome inhibitor-treated rats were stained with the fluorescence dye thioflavin-S, which binds to the protein β-sheet structure and amyloid fibrils. Thioflavin-S-labeled intracellular protein inclusions were significantly detected at 12 h after injection with 6-OHDA alone. In addition, the co-administration of lactacystin or MG-132 with 6-OHDA further increased intracellular protein inclusions compared to 6-OHDA alone (Fig. 5: A and B).

**Fig. 2.** Representative photomicrographs of the substantia nigra with TH immunohistochemistry. 6-OHDA in the absence (D – F) or presence of lactacystin (G – I) or MG-132 (J – L) was microinjected into rat substantia nigra. Sterilized physiological saline containing 1% DMSO (A – C) was injected in parallel with the corresponding vehicle control. After 12 h, 24 h, or 4 weeks, treated rats were fixed and nigral slices were prepared. Midbrain slices were immunostained by anti-TH antibody. Abbreviations: SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata. Scale bar, 250 μm.

**Fig. 3.** Semi-quantitative analysis of TH-immunopositive neurons in the substantia nigra. 6-OHDA caused significant neuronal loss which was blocked by the co-administration of lactacystin or MG-132. Each value is the mean ± S.E.M. based on the number of TH-immunopositive neurons in ipsilateral nigral slices from operated rats (each group, n = 5). Significance: *P<0.05, **P<0.01, ***P<0.001 vs the number of TH-immunopositive neurons with the vehicle alone. ††P<0.01, †††P<0.001 vs the number of TH-immunopositive neurons with 6-OHDA alone.
Intracellular inclusion body-like immunoreactivities for α-synuclein and ubiquitin in the substantia nigra

We further examined immunoreactivities for α-synuclein and ubiquitin in the SNpc. TH immunoreactivity was strongly detected in the vehicle SNpc, but was almost completely lost by the injection of 6-OHDA alone (Fig. 2). On the other hand, α-synuclein immunoreactivity was undetectable in the SNpc of rats injected with vehicle or 6-OHDA (Fig. 6: A and B). Interestingly, in laser scanning confocal analysis, marked α-synuclein immunoreactivity was detected in some surviving TH-immunopositive neurons in rats that had been injected with 6-OHDA in the presence of lactacystin or MG-132 (Fig. 6: C and D). In addition, dotted α-synuclein immunoreactivity, like intracellular inclusion bodies, was detected in the cytoplasm and peri-nucleus by DAB staining with nickel enhancement (Fig. 7A). Intracellular dotted ubiquitin immunoreactivity was also detected in surviving SNpc neurons in rats that had been injected with 6-OHDA in the presence of lactacystin or MG-132 (Fig. 7B).

Discussion

Unilaterally 6-OHDA-lesioned rats are suitable for evaluating in vivo behavioral recovery in studies of neuroprotective drugs, gene therapy, and the regeneration of transplants from neural or embryonic stem cells (15, 16, 19). Since 6-OHDA is strongly neurotoxic in the in vivo rat nigro-striatal DA pathway, the injection of 6-OHDA alone has been shown to reduce the number of TH-immunopositive neurons in the SNpc or the level

Figure 4. Proteasome inhibitors prevent 6-OHDA-induced loss of TH immunoreactivity in the striatum. Rats were injected with vehicle control (A) or 6-OHDA in the presence of vehicle (B), lactacystin (C), or MG-132 (D). After 4 weeks, treated rats were fixed and brain slices were prepared. Subsequently, striatal slices were immunostained by anti-TH antibody. Scale bar, 2 mm.

Figure 5. Proteasome inhibitors induce thioflavin-S-positive inclusions in the substantia nigra. A: Confocal images of neurons in the substantia nigra at 12 h after microinjection with vehicle or 6-OHDA in the presence of vehicle, lactacystin, or MG-132. Midbrain slices were stained by thioflavin-S. Scale bar, 150 μm. B: The number of thioflavin-S-positive neurons in the substantia nigra. Each value is the mean ± S.E.M. of four operated rats. *P<0.05, **P<0.01, ***P<0.001 vs the number of thioflavin-S-labeled neurons with vehicle alone.
of TH protein in the striatum by more than 90% (14, 16). In addition, marked DA depletion causes the supersensitivity of DA receptors in the striatum, and the administration of apomorphine then induces contralateral rotation (11, 13, 15). In the present study, proteasome inhibitors, such as lactacystin and MG-132, significantly rescued apomorphine-induced rotational asymmetry and prevented the 6-OHDA-induced decrease in nigral TH immunoreactivity. Thus, since proteasome inhibitors protect against 6-OHDA-induced loss of the dopaminergic neuronal pathway in the ipsilateral substantia nigra and striatum, dopaminergic supersensitivity and apomorphine-induced contralateral rotation may not occur.

Although 6-OHDA is one of the most common neurotoxins used to experimentally model nigral degeneration in vivo as well as in vitro, it has also been reported that 6-OHDA is present in both rat and human brains as well as in the urine of L-DOPA-treated PD patients (20). Under physiological conditions, 6-OHDA is rapidly and non-enzymatically oxidized by molecular oxygen to form hydrogen peroxide and the corresponding p-quinone (21, 22). Various downstream mechanisms for its toxicity have been proposed, including the production of reactive oxygen species (ROS), the interaction of one or more of its oxidation products with nucleophilic groups of several intraneuronal proteins, or with peptides like glutathione (21, 22), and the inhibition of mitochondrial complexes I and IV (20, 23). After the injection of 6-OHDA into the SNpc, dopaminergic neurons undergo degeneration within 24 h. In this study, the injection of 6-OHDA alone reduced the number of SNpc TH-immunopositive neurons by about 35% at 12 h and by 60% at 24 h. Although the co-administration of lactacystin or MG-132 with 6-OHDA reduced the number of TH-immunopositive neurons
similar to with 6-OHDA alone at 12 h, proteasome inhibitors inhibited the further loss of dopaminergic neurons at 24 h. In contrast, high concentrations of proteasome inhibitor alone caused neuronal death in the in vitro cultured neurons (24). It is reported that proteasome activity was reduced in PD midbrains (10). Recent studies indicated that massive loss of nigral dopaminergic neurons was not detected in parkin-deficient mice (25, 26). Besides parkin, numerous molecules are reported to have E3 ubiquitin ligase activity in mammalian cells (27). Therefore, null mutation of only the parkin gene may not cause the change in total proteasome activity and the cell death of mouse dopaminergic neurons. In our study, a unilateral injection of lactacystin or MG-132 alone did not cause loss of dopaminergic neurons in the ipsilateral SNpc and apomorphine-induced rotational asymmetry, while simultaneous addition of these proteasome inhibitors rescued 6-OHDA-induced dopaminergic neurodegeneration and behavioral dysfunction. It may indicate that proteasome inhibition by single administration of lactacystin or MG-132 is temporary or moderate, but not permanent or strong. Based on these observations, there is a possibility that proteasome inhibitors can temporarily prevent a 6-OHDA-induced neurodegenerative episode at the early phase (Fig. 8). Thus, reduction of proteasome activity may be a compensatory response to escape death of nigral dopaminergic neurons in PD.

Recent evidence suggests that failure of the ubiquitin-proteasome system leading to protein accumulation contributes to the degeneration of dopaminergic neurons and Lewy body formation in the SNpc in both familial and sporadic forms of PD (10). Autosomal recessive

![Fig. 7. Intracellular inclusion body-like immunoreactivities for α-synuclein and ubiquitin in the SNpc after the co-administration of 6-OHDA and proteasome inhibitors. Nigral slices following the co-administration of 6-OHDA and lactacystin or MG-132 were immunostained by antibodies to α-synuclein (A) and ubiquitin (B). Intracellular α-synuclein-immunopositive or ubiquitin-immunopositive inclusion bodies were detected (arrows). Scale bars, 10 μm.](image)

![Fig. 8. Hypothetical model of 6-OHDA-induced neurotoxicity and proteasome inhibitor-dependent neuroprotection. ROS, reactive oxygen species.](image)
juvenile parkinsonism (AR-JP) is one of the most common forms of familial PD and is characterized by juvenile onset, a recessive mode of inheritance, and selective loss of dopaminergic neurons in the substantia nigra. However, Lewy bodies have not been detected in AR-JP patients (28). Despite the absence of Lewy bodies, dopaminergic neurodegeneration occurs earlier in patients with AR-JP than in those with sporadic PD. Whether Lewy bodies are cytotoxic or cytoprotective to neuronal cells is not yet clear (29, 30). Proteasome is well known to normally regulate the quality control of intracellular proteins. In sporadic PD midbrains, proteasome activity is reduced (10), and α-synuclein is intracellularly accumulated and aggregated. In rare occurrences of autosomal dominant PD, missense mutations in the gene encoding α-synuclein have been shown to produce proteins that are prone to misfold and aggregate (31). The accumulation of aggregated α-synuclein is thought to contribute to the pathophysiology of PD. In particular, the intermediate oligomeric protofibrillar form has been suggested to be the most toxic species (32, 33). It is known that thioflavins (such as S and T) can bind the β-sheet structure in both small oligomers (protofibrils) and large molecular fibrils, but not in monomers (34 – 36). In this study, we could not measure the amounts of small oligomers and large molecular fibrils distinctively. Therefore, Figure 5 shows that the number of thioflavin-S-positive SNpc neurons might be significantly increased by both the injection of 6-OHDA alone and the co-injection of 6-OHDA and proteasome inhibitors (Fig. 5). In contrast, dopaminergic neurons in the SNpc were almost completely lost with 6-OHDA alone, but were protected by lactacystin and MG-132 (Figs. 1 – 3). After 4 weeks, some of the surviving SNpc neurons overexpressed α-synuclein (Fig. 6) and bore intracellular dotted inclusions that were immunoreactive to α-synuclein and/or ubiquitin (Fig. 7). Based on these observations, we presume that i) 6-OHDA-induced dopaminergic neurodegeneration in the SNpc is caused by ROS production and the formation of neurotoxic small oligomers (protofibrils), and ii) proteasome inhibitors may facilitate the formation of large molecular inclusions, probably including α-synuclein and ubiquitin (Fig. 8). To avoid the neurotoxicity of ROS and/or small oligomers, inclusion body formation by proteasome inhibition may play a key role in neuroprotection. In fact, Tanaka et al. recently suggested that aggresome-associated α-synuclein is cytoprotective (30). In addition, proteasome inhibitors prevent either cytochrome c release or caspase-3 activation (37 – 39) and reverse the dephosphorylation of mitogen-activated protein kinase (MAPK) (40). Thus, proteasome inhibition results in neuroprotection and α-synuclein inclusion-bearing neurons may be present in parkinsonian SNpc.

In conclusion, we investigated the effect of proteasome inhibitors on dopaminergic neuronal death in a PD animal model using 6-OHDA. Proteasome inhibitors, such as lactacystin and MG-132, significantly prevented apomorphine-induced rotational asymmetry and nigral neurodegeneration in rats. Furthermore, proteasome inhibition caused the appearance of thioflavin S, α-synuclein and ubiquitin inclusions in the substantia nigra. Thus, proteasome inhibition plays an important role in both the dopaminergic neuroprotection and inclusion body formation.

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