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ORIGINAL ARTICLE

Oxidative Stress, Induced by 6-Hydroxydopamine, Reduces Proteasome Activities in PC12 Cells

Implications for the Pathogenesis of Parkinson's Disease

Hanoch Elkon, Eldad Melamed, and Daniel Offen*

Felsenstein Medical Research Center and the Department of Neurology, Rabin Medical Center, Tel-Aviv University-Sackler School of Medicine, Petah-Tikva, 49100, Israel

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Abstract

Mutations in familial Parkinson's disease (PD) have been associated with the failure of protein degradation through the ubiquitin-proteasome system (UPS). Impairment of proteasome function has also been suggested to play a role in the pathogenesis of sporadic PD. We examined the proteasome activity in PC12 cells treated with 6-hydroxydopamine (6-OHDA), the dopamine synthetic derivate used in models of PD. We found that 6-OHDA treatment increased protein oxidation, as indicated by carbonyl group accumulation, and increased caspase-3 activity. In addition, there was an increase in trypsin-, chymotrypsin-, and postacidic-like proteasome activities in cells treated with 10–100 μ*M* 6-OHDA, whereas higher doses caused a marked decline. 6-OHDA exposure also increased mRNA expression of the 19S regulatory subunit in a dose-dependent manner, whereas the expression of 20S- and 11S-subunit mRNAs did not change. Administration of the antioxidant *N*-acetylcysteine to 6-OHDA-treated cells prevented the alteration in proteasome functions. Moreover, reduction in cell viability owing to administration of proteasome inhibitor MG132 or lactacystin was partially prevented by the endogenous antioxidant-reduced glutathione. In conclusion, our data indicate that mild oxidative stress elevates proteasome activity in response to increase in protein damage. Severe oxidative insult might cause UPS failure, which leads to protein aggregation and cell death. Moreover, in the case of UPS inhibition or failure, the blockade of physiological reactive oxygen species production during normal aerobic metabolism is enough to ameliorate cell viability. Control of protein clearance by potent, brain-penetrating antioxidants might act to slow down the progression of PD.

Index Entries: Parkinson's disease; 6-OHDA; UPS; caspase 3; carbonyl detection (Oxyblot™).

Introduction

Parkinson's disease (PD) is characterized by resting tremor, cogwheel rigidity, bradykinesia, and loss of postural disturbance (Parkinson, 1817; Gibb and Lees, 1988). Pathologically, it is characterized by the loss of neuronal cells in the substantia nigra pars compacta (SNc) and by intracellular neuronal inclusions, that is, the Lewy bodies (LBs) (Lewy, 1912; Forno, 1996). The primary cause of nigral neuronal degeneration is unclear, but progression of the disease might be associated with several contributory factors, including electron transport failure in the mitochondria (Schapira et al., 1990), iron accumulation (Dexter et al., 1987; Youdim et al., 1989), excess free radical generation, low levels of antioxidant

*Author to whom all correspondence and reprint requests should be addressed. E-mail: doffen@post.tau.ac.il

(Perry et al., 1982; Sofic et al., 1992; Sian et al., 1994), and excitotoxicity in the SNc (Beal, 1992; Coyle and Puttfarcken, 1993). The production of reactive oxygen species (ROS) might damage cellular proteins and lipids and impair their functions (Davies, 1987; Dexter et al., 1994). The importance of interference with protein integrity in the pathophysiology of PD is highlighted by the fact that several of the mutations that cause rare familial Parkinson's disease (FPD) cases are associated with protein processing in the ubiquitin-proteasome system (UPS).

The UPS, via degradation and proteolysis, is responsible for the regulation of protein levels that mediate cellular activities. It eliminates the damaged and unwanted proteins that might be toxic to the cell. First, the degraded proteins are marked with a polyubiquitin chain in an ATP-dependent process. Initially, ubiquitin is activated in its C-terminal glycine (Gly76) by the ubiquitin-activating enzyme, El. One of several ubiquitin-conjugating enzymes, E2, then transfers the activated ubiquitin from El to a member of the ubiquitin-protein ligase family, E3, to which the substrate protein is specifically bound. This enzyme catalyzes covalent attachment of ubiquitin to the substrate. Later on, the polyubiquitin chain is synthesized by transfer of additional ubiquitin moieties to Lys48 of the previously conjugated ubiquitin molecule by forming another isopeptide bond (Glickman and Ciechanover, 2002). Polyubiquitinated protein is then degraded by the 26S proteasome, a ubiquitin/ATP-dependent multicomponent enzymatic complex. The 26S proteasome is assembled from the catalytic core 20S proteasome and two other optional regulatory multisubunits, 19S and 11S. The 20S proteasome is composed of 28 subunits arranged in four heptameric-stacked rings to form a cylindrical structure. The 20S proteasome hydrolyzes most peptide bonds present in a protein by several proteolytic activities. The regulatory moiety of the 26S proteasome, known as the 19S particle, contains at least 17 subunits, including ATPases, a de-ubiquitinating enzyme, and polyubiquitin-binding subunits. The proteins are degraded to short peptides, and the ubiquitin molecules are recycled (Coux et al., 1996; Glickman and Ciechanover, 2002). One of the mutated proteins that was found to be associated with FPD is α-synuclein. α-Synuclein is a presynaptic protein with unknown function that was found to be a major component in the LBs in both inherited and sporadic PD (Spillantini et al., 1998). Point mutations in α-synuclein (A53T and A30P) led to the relatively early onset of FPD (Polymeropoulos et al., 1997; Kruger et al., 1998). Although α-synuclein was found to be degraded by the UPS, it appeared that mutated α-synuclein tended to aggregate and resist degradation (Conway et al., 1998; Bennet et al., 1999; Narhi et al., 1999; Goedert, 2001). It was found that α-synuclein is processed by the ubiquitin ligase (E3) of the UPS, called parkin. Interestingly, parkin is another protein in which mutations cause autosomal-recessive FPD (Kitada et al., 1998; Shimura et al., 2000, 2001). Another rare mutation causing an autosomal-dominant form of FPD was also found to be linked to the UPS, identified as the ubiquitin C-terminal hydrolase L1 (UCH-L1) protein. Ubiquitin C-terminal hydrolase L1 (UCH-L1) enzymes, which are also found in LBs (Lowe et al., 1990), belong to a family of de-ubiquitinating enzymes, believed to cleave polymeric ubiquitin to monomers and to hydrolyze bonds between ubiquitin molecules and other small adducts. This missense mutation reduces the catalytic activity, affecting the cleavage and turnover of unknown substrate(s), which leads to its accumulation and aggregation (Leroy et al., 1998). The involvement of a UPS-related mutation with FPD raises the possibility that dysfunction of UPS is a mechanism also for the pathophysiology of sporadic PD. It was suggested that severe oxidative stress causes extensive protein oxidation, which might directly generate the cross-linking of protein fragments that aggregate and become progressively resistant to proteolytic digestion. These aggregates of oxidized proteins actually bind to the 20S proteasome and act as irreversible inhibitors (Davies, 2001). Thus, the possibility that the impairment of clearance of the damaged protein by the UPS plays a key role in the pathogenesis of neurodegenerative diseases, including PD, has already emerged (Jenner and Olanow. 1998; Ding and Keller, 2001; McNaught et al., 2001a).

In previous studies we demonstrated the importance of oxidative stress induced by dopamine for neuronal cell survival (Offen et al., 1996, 1999; Gilgun-Sherki et al., 2002). In this study we focus on proteasomal functions in the presence of 6-hydroxydopamine (6-OHDA), the pro-oxidant derivate of dopamine widely used in animal models of PD. We also examined the contribution of antioxidants in situations of proteasomal impairment.

Materials and Methods

Cell Culture and Viability Assay

PC12 cells were grown in Dulbecco's Modified Eagle Medium (Beit-Haemek, Israel) supplemented with 10% fetal calf serum (FCS), 5% horse serum (Beit-Haemek, Israel) and 1% (v/v) penicillin/streptomycin/nystatin, maintained at 37°C in a humidified 5% CO_2 incubator. One day prior to the experiment, 2×10^6 cells in 8 mL of culture medium were placed in a 10 cm^3 culture dish. After cells were washed and 2% serum medium added, they were treated with 6-OHDA (RBI, Natik, MA) and NAC (Sigma, St. Louis, MO).

Trypan Blue Viability Assay

Treated cells were incubated in phosphate-buffered saline (PBS; pH 7.5) containing 0.4% trypan blue for 5 min and then visualized by light microscope. Cells excluding trypan blue were considered as viable.

Neutral Red Viability Assay

One hundred milliliters of 0.34% neutral red (Sigma), dissolved 1:100 in DCCM-1 (Beit-Haemek, Israel), was added to cultured medium, placed into each well, and incubated for 2 h at 37°C. Wells were then washed with PBS containing 1 m*M* $MgCl_2$, added to 200 mL of Sorenson buffer (0.1 *M* disodium citrate with 0.1 *M* HCl at 1.6:1, respectively), and mixed with 100 mL of ethanol (Borenfreund and Puerner, 1984). Viability was correlated to absorption intensity and measured with the ELISA reader Fluostar (BMG Lab Technologies, Offenberg, Germany) at a wavelength of 590 nm.

Proteasome Activity

Supernatants of treated culture cells were transferred to 14-mL tubes and centrifuged at 1100 rpm to collect disconnected cells. Fresh ice-cold proteolysis buffer [10 m*M* Tris-HCl at pH 7.8; 0.5 m*M* dithiothreitol (DTT); 5 m*M* ATP, and 5 m*M* $MgCl_2$] was added to the culture dish. The attached cells were scraped and combined to the cell pellets and homogenized with a Teflon homogenizer (Thomas). Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). For the proteasome activity assay, 100 µg of protein extract was incubated with specific fluorogenic substrates: 40 µ*M* Z-Leu-Leu-Glu-AMC (Calbiochem, San Diego, CA) for postacidic activity; 100 µ*M* Soc-Leu-Leu-Val-Tyr-AMC (Calbiochem) for chymotrypsin-like activity; and 100 µ*M* Z-Ala-Arg-Arg-AMC (Calbiochem) for trypsin-like activity. Reactions were conducted in triplicate in a final volume of 200 µL in a flat black-fluorescence 96-well plate (Greiner, Germany). Cleavage products were measured using fluorescence plate reader Fluostar (BMG LabThechnologies) with excitation at 380 nm and emission at 460 nm (gain = 12). Reactions were followed for 120 min at 37°C. Enzymatic activity was determined from linear reaction rates and expressed as fluorescence units/min per milligram of protein. Background fluorescence was determined by preincubating the protein extract with 50 µ*M* irreversible proteasome inhibitor, lactacystin (Calbiochem), for 30 min prior to addition of the proteasome substrate.

Caspase-3 Activity

Supernatants of cultured cells were transferred to 14-mL tubes and centrifuged at 1100 rpm to collect disconnected cells. Two hundred microliters of fresh ice-cold lysis buffer (10 m*M* Tris-HCl; 10 m*M* KH_2PO_4/K_2HPO_4 at pH 7.5; 130 m*M* NaCl; 1% Triton X-100; 10 m*M* NaPPi; protease inhibitor cocktail [Boehringer Mannheim, GmbH, Germany]) was added to the culture dish. The attached cells were scraped and combined with cell pellets and sheared with a 25-gauge needle (three to five times). Samples were centrifuged at 15,000*g* for 10 min at 4°C, and the supernatant was collected. Protein concentrations were determined, and 75 µg of protein extract was added to 200 µL with reaction buffer [10 m*M* DTT, 20 m*M* HEPES, 10% glycerol] and incubated with 50 µ*M* Ac-DEYD-AMC (Alexis Biochemical, Carlsbad, CA) as caspase-3 fluorogenic substrate. Background fluorescence was determined by preincubating the protein extract with 50 µ*M* Z-Asp2.6-dichlorobenzoyloxymethyl ketone (Alexis Biochemical), a caspase inhibitor, 30 min prior to the addition of caspase-3 substrate. Cleavage products were measured using the fluorescence plate reader Fluostar with excitation at 380 nm and emission at 460 nm (gain = 20).

Protein Oxidation

Protein oxidation levels were determined by Oxyblot™ oxidation detection kit (Intergen, Purchase, NY), based on immunochemical detection of protein carbonyl groups derived from 2,4-dinitrophenyl hydrazine, according to the manufacturer's instructions.

Northern Blot Analysis

Procedures were followed according to Sambrook et al. (1989). All other reagents/materials were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of mRNA

Total RNA from treated PC12 cells was extracted according to Chomczynski and Sacchi (1987). RNA was quantified by spectrophotometer, and 10 µg of total RNA was separated on 1% agarose formaldehyde–denaturing gel electrophoresis. RNA was then blotted onto Duralon-UV™ membrane (Stratagene, Cedar Creek, TX), and cross-linked by 1200 mJ/cm^2 uv radiation (Hoefer Scientific Instruments, San Francisco, CA).

Primers

Primers were designed as follows: Proteasome 20S (RC6: D30804), 5′-GTTGTAGCCTCT GTCTCAGG-3′ and 5′-CAATGGCATCATCTGT GTAG-3′ allowed the amplification of 339 of 900 bp total gene size; proteasome 19S (p112: AJ006340), 5′-AGTATTGC-GAGTCCTGGTTAAA-3′ and 5′-TCTGGAC-TAGCGTCTGGTGTT-3′ allowed the amplification of 362 of 3600 bp total gene size; proteasome 11S (PA28: D45249), 5′-ATCTCAGAGTTGGATGC-CTTCT-3′ and 5′-AGGTAGTAACCAGGTTGAGTT-3′ allowed the amplification of 291 of 900 bp total gene size; and GAPDH (AB017801), 5′-AAGGT-CATCCCA GAGCTGAA-3′ and 5′-ATGTAGGC-CATGAG GTCCAC-3′ allowed the amplification of 338 of 1242 bp total gene size.

cDNA was generated by RT-PCR reaction (Super Script™ II Reverse Transcriptase, Invitrogen), and probes were prepared by specific primers in the PCR apparatus (MJ Research). Probes were labeled with [^{32}P]dCTP (NEN Life Science Products) and Klenow enzyme (New England Biolabs), and hybridized with the membrane in hybridization buffer, as described in Sambrook et al. (1989). All other reagents/materials were obtained from Sigma-Aldrich.The hybridization signals were measured with a PhosphorImager (Cyclone) and analyzed with OptiQuant™ software. To normalize total RNA levels, membranes were additionally hybridized with the GAPDH probe, a housekeeping gene.

Statistical Analysis

Results are expressed as mean ± SE The statistical significance of differences between the control and treated group was determined by Student's *t*-test. mRNA results were analyzed by linear regression, best-fit standard 95% confidence interval.

Results

6-OHDA Toxicity in PC12 Cells

Exposure of PC12 cells to 0.1–0.3 m*M* 6-OHDA for 24 h demonstrated dose-dependent decrease in cell survival, as measured by trypan blue exclusion assay (up to 80%, Fig. 1A). However, treatment was not toxic for up to 10 h, even with the high dose of 6-OHDA (0.3 m*M*) (Fig. 1B). The 6-OHDA-induced cell death was also demonstrated by the lactate dehydrogenase (LDH) activity assay, which showed a similar pattern of dose- and time-dependant curves (Fig. 1C,D).

Protein Oxidation in 6-OHDA-Treated PC12 Cells

The Oxyblot kit enables the assessment of the presence of carbonyl groups on gel-separated proteins, indicating the level of oxidized proteins. We found extensive carbonyl group formation in the 6-OHDA-treated cell extract (detected from 40–220 kDa). Addition of the antioxidant NAC (10 m*M*), 30 min before 6-OHDA treatment, inhibited the accumulation of oxidized products (Fig. 2).

Untreated cells demonstrate only marginal levels of carbonyl group formation, and exposure to NAC (10 m*M*) alone showed even lower levels of oxidized products (Fig. 2).

Caspase-3 Activity in 6-OHDA-Treated PC12 Cells

To assess the apoptotic state in 6-OHDA-treated PC12 cells, we measured caspase-3 activity using specific fluorogenic substrate, Ac-DEVD-AMC. Caspase-3 fluorogenic substrate breakdown occurred in PC12 cells treated with 0.3 m*M* 6-OHDA up to 24 h. Caspase-3 activity was elevated by approx 450% compared with untreated cells. Administration of NAC (10 m*M*), 30 min before 6-OHDA treatment, prevented the elevation of caspase-3 activity (Fig. 3).

Proteasome Activity in 6-OHDA-Treated PC12 Cells

As UPS is responsible for the elimination of oxidized proteins, we followed its proteolytic processes during the exposure of PC12 cells to 6-OHDA. Trypsin-, chymotrypsin- and postacidic-like proteasome activities were measured with specific fluorogenic substrates. Extracts of PC12

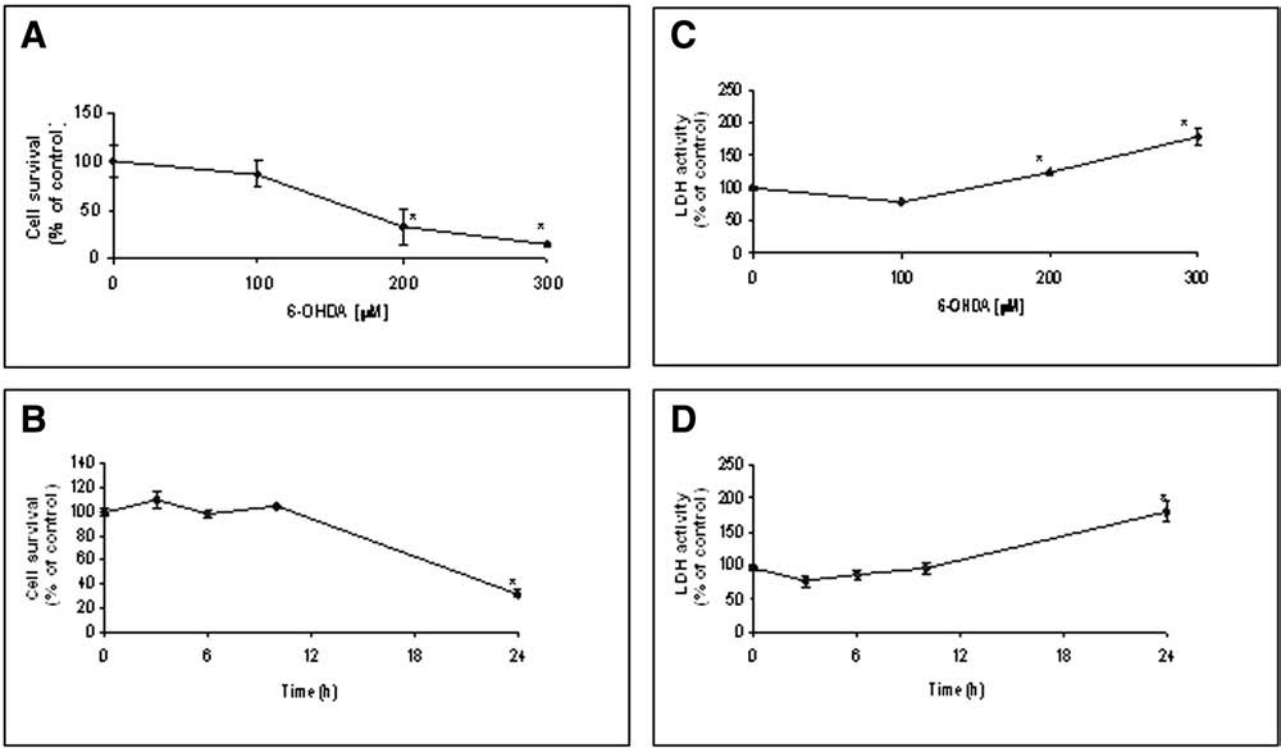

Fig. 1. Survival of PC12 cells treated with 6-OHDA. Exposure to 6-OHDA (0.1–0.3 m*M*) treatment induced cell death, as indicated by trypan blue assay and LDH activity (mean + SE; control vs 6-OHDA [*] $p < 0.01$, $n = 3$).

cells treated for 15 h with 6-OHDA demonstrated elevated trypsin-, chymotrypsin-, and postacidic-like activities in lower 6-OHDA concentrations (<0.1 m*M*), whereas higher doses of 6-OHDA (0.1–0.3 m*M*) caused a decrease in their proteolytic activities (Fig. 4A). Similarly, we found that an extended incubation period (>10 h) with 0.3 m*M* 6-OHDA caused a reduction in proteolytic activity (Fig. 4B). Addition of the antioxidant NAC (10 m*M*) to 6-OHDA cells treated with 0.3 m*M* attenuated the reduction in all three proteolytic activities (Fig. 5).

Proteasome Subunit mRNA Expression in 6-OHDA-Treated PC12 Cells

The 6-OHDA-induced reduction in proteolytic activities might reflect direct inhibition of enzyme activity or down-regulation of their expression. Therefore, we isolated and analyzed the mRNA expression of RC6 (20S), p112 (19S ATP-dependent regulatory subunit of 26S), and PA28 (11S regulatory subunit of 26S) subunits of the proteasome in 6-OHDA-treated PC12 cells. Northern blot analysis revealed that exposure to increased doses of 6-OHDA did not change the mRNA levels of RC6 and PA28 transcripts, whereas the expression of the p112 subunit showed a moderate, but significant, increase (Fig. 6). Thus, the oxidative stress-induced proteolytic activity inhibition seen in high doses of 6-OHDA is probably attributable to post-translational events.

Cell Viability in the Presence of Proteasome Inhibitors and Antioxidants

Several studies have demonstrated that proteasome inhibition is toxic to various cell types. Previously we have shown that PC12 cells are sensitive to proteasome inhibition and to sublethal doses of 6-OHDA (Elkon et al., 2001). In this study we have examined whether the antioxidant-reduced glutathione (GSH) can attenuate the cytotoxic effect of UPS inhibition, even in the absence of exogenous pro-oxidant. Antioxidant-reduced glutathione (GSH) (1 m*M*) was added to PC12 cells treated for 24 h with increasing concentrations of MG132 or lactacystin, potent reversible and irreversible (respectively) inhibitiors of the proteasome. We found that cell survival, as indicated by the neutral red method, markedly decreased in the presence of

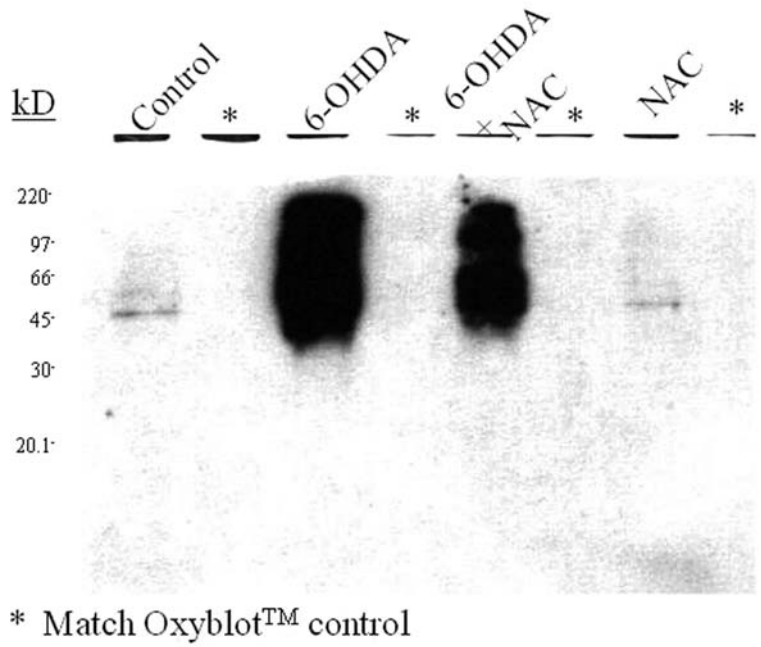

Fig. 2. Detection of oxidized proteins (carbonyl groups) in PC12 cells treated with 6-OHDA alone or in combination with NAC. Treatment with 0.3 m*M* 6-OHDA increased protein oxidation, as indicated by the accumulation of carbonyl groups. Addition of the antioxidant NAC (10 m*M*) diminished protein oxidation. Underivatized protein extracts were used as negative controls ([*] match Oxyblot control).

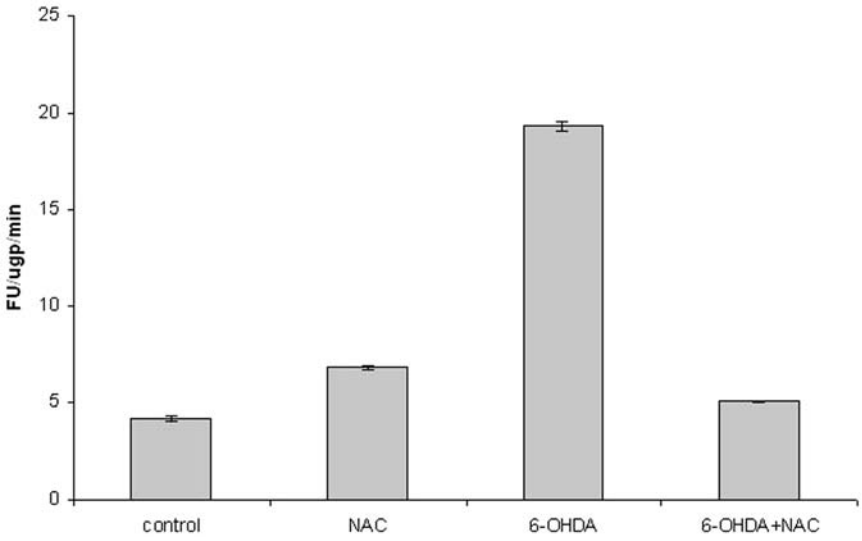

Fig. 3. Caspase 3 activity in PC12 cells treated with 6-OHDA and NAC. Treatment with 6-OHDA (0.3 m*M*) for 18 h increased apoptotic activity by elevating caspase-3 activity, whereas addition of NAC (10 m*M*) inhibited these elevations (mean ± SE; control vs 6-OHDA [*] $p < 0.0001$, $n = 3$).

MG132 (Fig. 7A) and lactacystin (Fig. 7B). Survival of PC12 cells treated with 200 µ*M* MG132 was reduced by 21.8%, whereas the addition of 1 m*M* GSH to the treated cells neutralized MG132 toxicity (Fig. 7A). Moreover, the extensive inhibition of proteasome in PC12 cells treated with 500 µ*M* MG132 decreased cell survival by 62.1%, whereas the addition of GSH attenuated its toxicity to 11.4% (Fig. 7A). Furthermore,

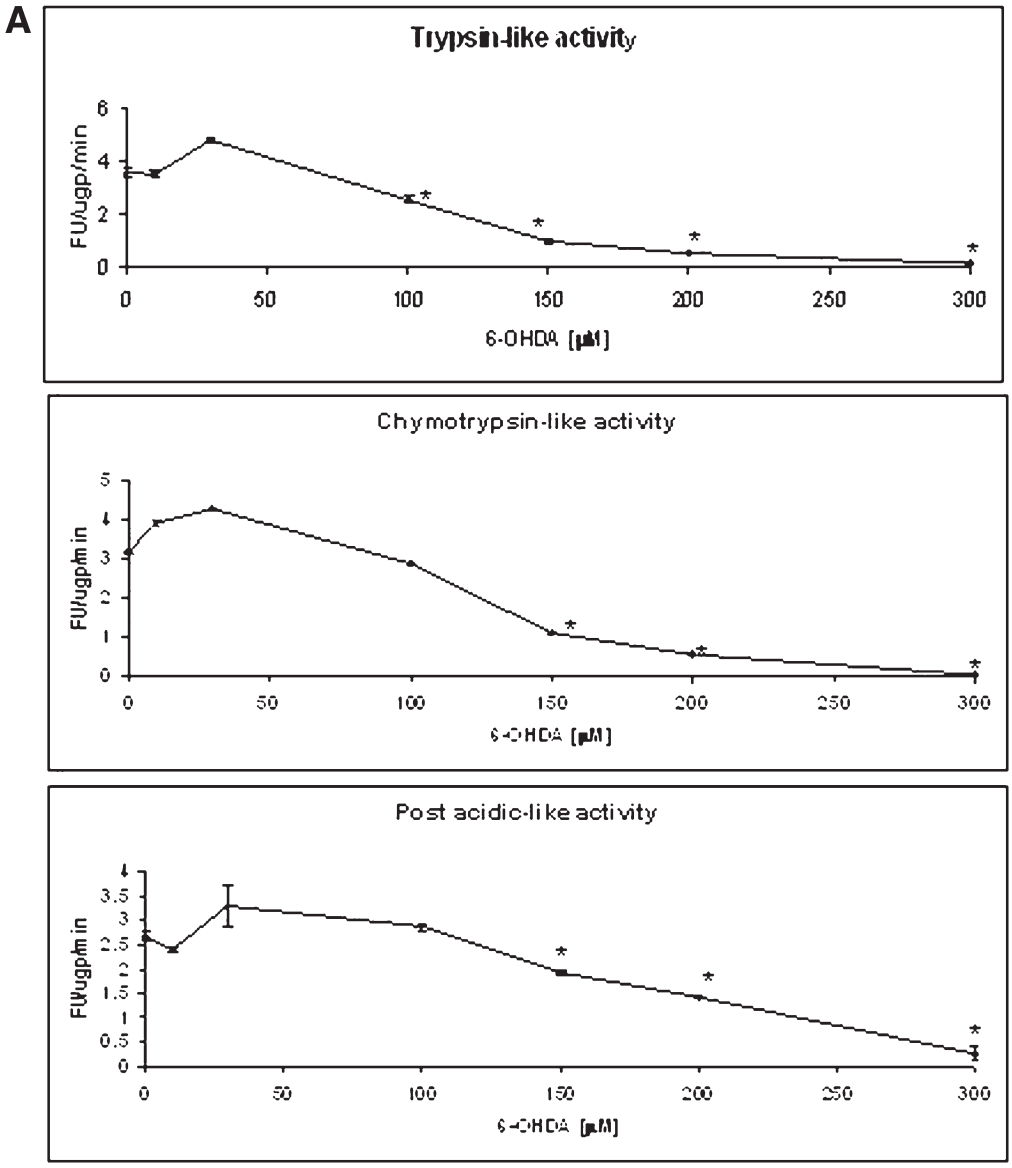

Fig. 4. *(Continued)*

survival of PC12 cells treated with 3 μ*M* lactacystin decreased by 21.9%, whereas the addition of 1 m*M* GSH to the treated cells abolished lactacystin toxicity and improved cell viability (Fig. 7B). When treated with 10 μ*M* lactacystin, survival of PC12 cells decreased by 33.5% whereas the addition of GSH markedly attenuated the lactacystin toxicity (Fig. 7B).

Discussion

Exposure of PC12 cells to 6-OHDA, a dopaminergic neurotoxin used in experimental animal models of PD, decreases cell viability. We found that 6-OHDA treatment markedly increased protein oxidation, as indicated by the extensive formation and accumulation of carbonyl groups. In addition, 6-OHDA treatment induced an increase in apoptosis, as indicated by the elevation of caspase-3 activity. Administration of the antioxidant NAC markedly rescued 6-OHDA-treated cells from protein oxidation and abolished the elevation in caspase-3 activity. Moreover, we found that oxidative stress induced by 6-OHDA affects UPS integrity. In mild oxidative conditions (<0.1 m*M* 6-OHDA) proteasome proteolytic

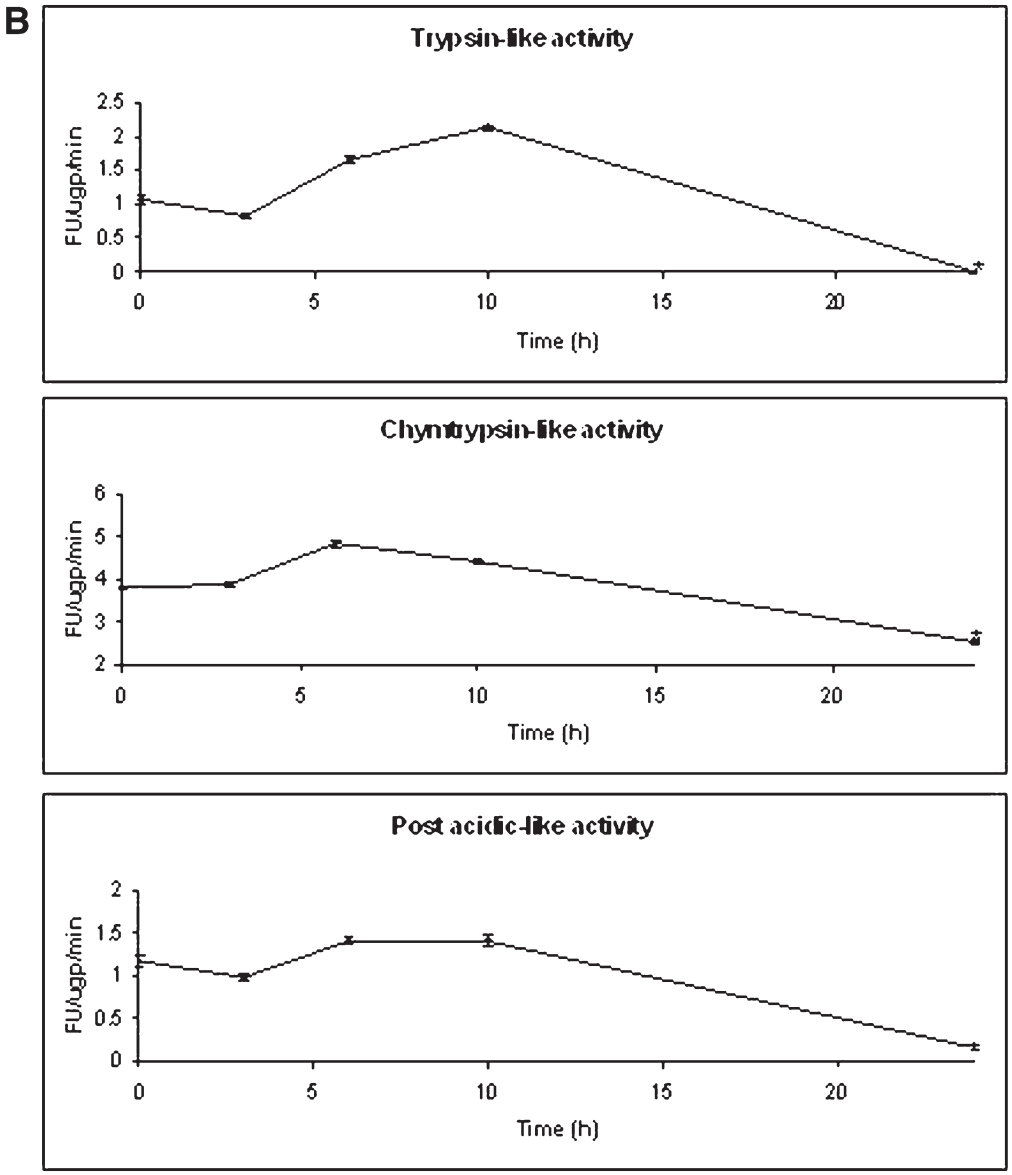

Fig. 4. Proteasome activities in PC12 cells treated with 6-OHDA. **(A)** Low doses of 6-OHDA (0.01–0.1 m*M* for 15 h) elevated the trypsin-, chymotrypsin-, and postacidic-like activities. With high doses of 6-OHDA (0.1–0.3 m*M* for 15 h) trypsin-, chymotrypsin-, and postacidic-like activities were markedly reduced. **(B)** With a high dose of 6-OHDA (0.3 m*M*), short-term incubation (10 h) elevated the trypsin-, chymotrypsin-, and postacidic-like activities; long-term incubation (24 h) markedly reduced these activities (mean ± S.E.; control vs 6-OHDA [*] $p < 0.0001$, $n = 3$).

activities (trypsin-, chymotrypsin-, and postacidic-like) increased, whereas severe exposure (>0.1 m*M* 6-OHDA) caused a massive decline. We also examined the mRNA transcripts of three key components of the 26S proteasome. It was found that mRNA levels of RC6 and PA28 of the subunits 20S and 11S (respectively) did not change with increasing doses of 6-OHDA. In contrast, p112 (subunit 19S) was elevated when exposed to higher 6-OHDA concentrations. Moreover, we found that reduction in cell viability owing to proteasome inhibition by MG132 or lactacystin might be partially prevented by cotreatment with an antioxidant (GSH).

It is known that intranigral injections of 6-OHDA create local oxidative stress in the SNc and thus induce dopaminergic cell death in an experimental

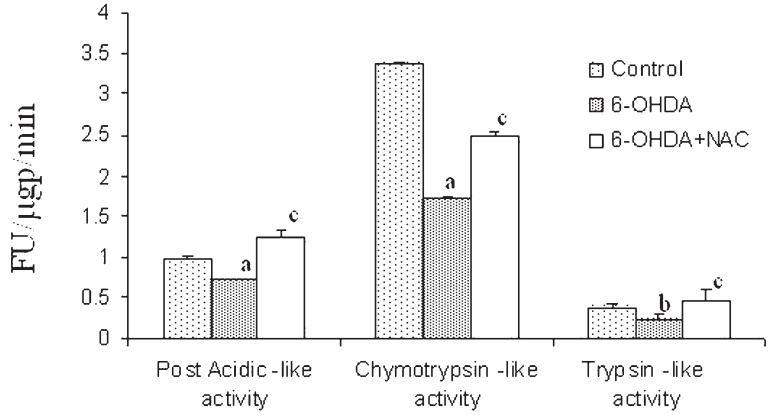

Fig. 5. Proteasome activities in PC12 cells treated with 6-OHDA and NAC. Reduction in proteasome activities in PC12 cells induced by 6-OHDA (0.3 m*M*), were attenuated by the addition of NAC (10 m*M*) (mean ± S.E.; control vs 6-OHDA [a] $p < 0.001$, control vs 6-OHDA [b] $p < 0.02$, 6-OHDA vs 6-OHDA and NAC [c] $p < 0.05$, $n = 3$).

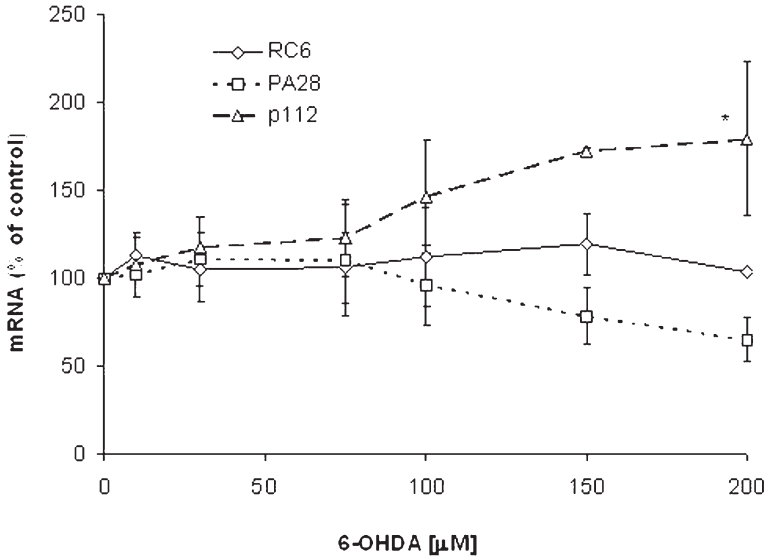

Fig. 6. Proteasome subunit mRNAs of PC12 cells treated with 6-OHDA. RNA extracts from 6-OHDA (0.1–0.2 m*M*)-treated cells were hybridized with p112, PA28, and RC6 probes on Northern blot (*see* Materials and Methods). The levels of mRNAs were normalized to GAPDH gene expression (presented as % ± SE; slope of p112 = 0.416, $p < 0.0001$, $n = 3$).

model of PD. A possible role of 6-OHDA in the etiology of the disease was even suggested by Andrew et al. (1993) and Jellinger et al. (1995), who collected evidence for 6-OHDA production through the metabolism in an L-DOPA-treated PD patient. 6-OHDA toxicity in various neuronal cultures has already been shown in many studies. Most of them demonstrated that its toxicity was associated with the production of ROS and severe oxidative stress, which eventually lead to apoptotic cell death (Ziv et al., 1994; Offen et al., 1997; Barzilai et al., 2000). It has also been suggested that ROS produced during the normal metabolism of dopamine, the local neurotransmitter in the SNc, might initiate the degenerative process in PD (Graham, 1978; Simantov et al., 1996; Offen et al., 1997). Dopaminergic neurons are also chronically exposed to free radicals generated by accumulated iron, as shown in

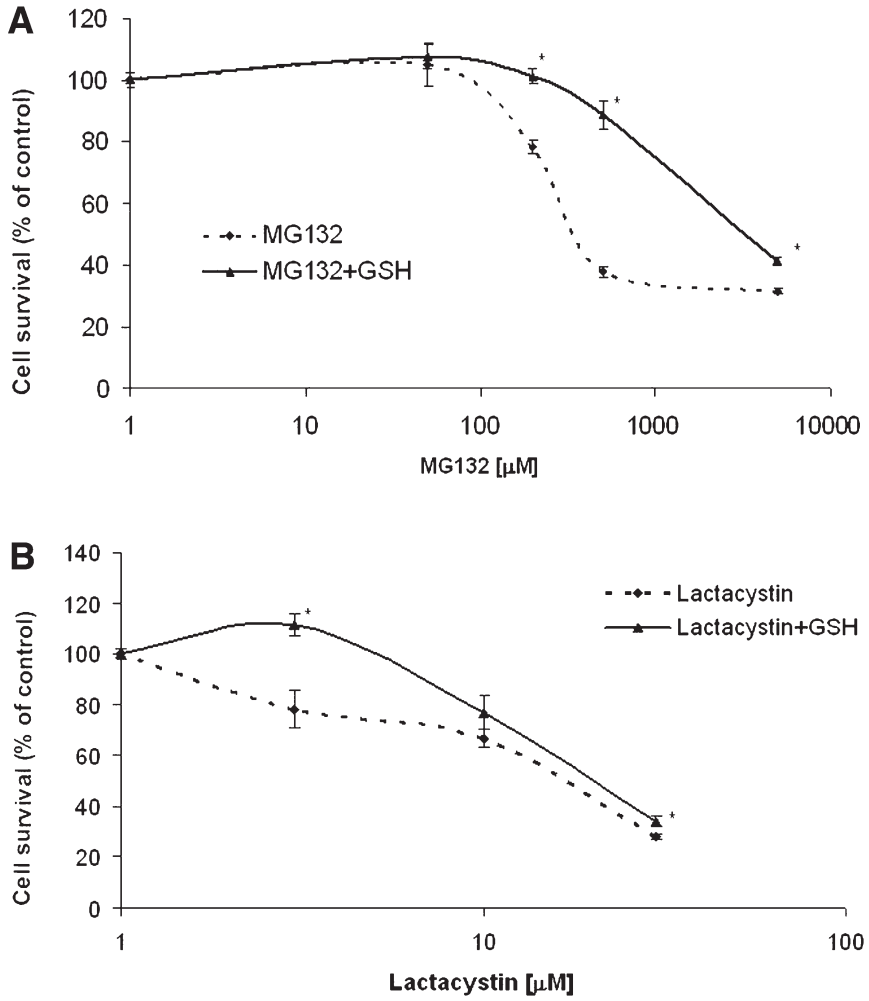

Fig. 7. GSH protects against cell death induced by proteasome activity inhibition. PC12 cells were treated with MG132 (0.05–5 m*M*) **(A)** or lactacystin (0.03–30 μ*M*) **(B)** in the presence of GSH (1 m*M*). Cell survival was measured by neutral red (mean ± S.E., $n = 4$).

animals and in postmortem studies (Youdim et al., 1989). Furthermore, the mitochondrial dysfunction observed in the SNc of PD patients might be the source for the toxic ROS or the result of endogenous oxidative stress (Mizuno et al., 1989, 1995; Hasegawa et al., 1990; Schapira et al., 1990; Swerdlow et al., 1996).

Oxidative stress conditions, characterized by the production of free radicals, cause the extraction of electrons from neighboring molecules and the attack of cellular proteins, lipids, and DNA, leading to the impairment of their functions and to cell damage (Davies, 1987; Dexter et al., 1994). Postmortem studies of brains from sporadic PD patients revealed several chemical fingerprints of these damaging oxidative events, including carbonyl group formation on cellular proteins (Alam et al., 1997a; Floor and Wetzel, 1998), nitrated α-synuclein (Giasson et al., 2000), and higher levels of cholesterol hydroperoxide, malondialdehyde, protein adducts of 4-hydroxy-2-noneal (HNE), and 8-hydroxy-2-deoxyguanosine (Yoritaka et al., 1996; Alam et al., 1997b).

Recently, interest has begun to focus on the possibility that the impairment of UPS clearance of damaged proteins plays a key role in the pathogenesis of neurodegenerative diseases, in general, and in PD, in particular (Jenner and Olanow, 1998; Ding and Keller, 2001; McNaught et al., 2001a;). Normally, damaged proteins are recognized and eliminated by the ubiquitin-proteasome pathway (Alves-Rodrigues et al., 1998). It was shown that ubiquitin-proteasome is recruited for the removal of proteins that had been oxidatively modified by heavy metals such as cadmium chloride ($CdCl_2$), zinc chloride

($ZnCl_2$), and hydrogen peroxide (H_2O_2) (Figueiredo-Pereira and Cohen, 1999). However, excessively damaged proteins or impairment of the ubiquitin-proteasome function might result in the aggregation of ubiquitin conjugations and undigested proteins (Halliwell and Jenner, 1998; McNaught et al., 2001).

We have previously demonstrated that exposure of neuronal cell lines to 6-OHDA increases the levels of free ubiquitin and ubiquitin-conjugated proteins in a dose-dependent manner. We also reported that inhibition of proteasome activity by MG132 enhances the sensitivity of cells to 6-OHDA toxicity. Moreover, administration of the antioxidant NAC to 6-OHDA-treated cells protected them and reduced protein degradation (Elkon et al., 2001).

Our results demonstrate that proteasome activity increases during mild oxidative stress; however, when the oxidative stress insult becomes rather severe, there is a dramatic decline in UPS function. Our data are in agreement with other studies reporting that mild oxidative injury by other pro-oxidants stimulates proteasome activity (Strack et al., 1996; Reinheckel et al., 1998; Ding and Keller, 2001). We can speculate that the rise in proteasome activity is attributable to the effort to compensate for the elevation in the accumulation of damaged proteins in the cell. However, in cases of severe oxidative stress, high levels of damaged proteins might inhibit normal cellular functions, including protein degradation via the proteasome.

It is important to note that the proteasome's enzymatic activities are not correlated to its subunit mRNA expression in response to increased 6-OHDA doses. Its activities in doses higher than 0.1 m*M* 6-OHDA decline, whereas mRNA transcripts remain stable or increase. This variation is probably attributable to sensitivity to severe oxidative insult of the proteins and/or enzymatic activities.

6-Hydroxydopamine (6-OHDA), which induced the elevated expression of p112 in the 19S regulatory subunit, might reflect a compensatory mechanism to clear flooding levels of proteasome-targeted ubiquitinated proteins. It has been shown that the ATP- and ubiquitin-dependent 26S proteasome is four times more vulnerable to oxidative stress than the 20S proteasome (Reinheckel et al., 1998).

Our findings on the changes of proteasome activity in response to 6-OHDA exposure are similar to those of Keller et al. (2000b) on the effect of dopamine in PC12 cells. They showed that dopamine induced a dose- and time-dependent decrease in proteasome activity prior to cell death. Addition of monoamine oxidase inhibitors, which lower the metabolism of dopamine to its metabolites or an inhibitor of dopamine uptake, protects the cells against dopamine toxicity and the associated proteasome impairment. Moreover, application of the proteasome inhibitor lactacystin increased the toxicity of dopamine and the levels of oxidized proteins (Keller et al., 2000b). In contrast, our study focused on the effect of lower levels of 6-OHDA (10–100 μ*M*), which showed increases in proteasome activities. This phenomenon of stimulation of proteasome activities was also reported when various cell lines were exposed to mild oxidative injury induced by various oxidants (Strack et al., 1996; Reinheckel et al., 1998). Interestingly, it was also demonstrated that proteasome activities were impaired during aging of CNS and in non-CNS systems (Hayashi and Goto, 1998; Bulteau et al., 2000; Keller et al., 2000a, 2000c). It was also shown that nitric oxide and related species, as well as the lipid peroxidation product HNE, reduced proteasome activity (Friguet and Szweda, 1997; Grune et al. 1998; Glockzin et al., 1999; Keller et al., 2000b; Shringarpure et al., 2000). Cells expressing mutant α-synuclein, found to be associated with autosomal-dominant FPD, showed increased sensitivity to apoptotic cell death when treated with subtoxic concentrations of an exogenous proteasome inhibitor (Stefanis et al., 2001). Moreover, failure of the proteasome function is strongly associated with the aggregation of proteins and neurodegeneration. Severe oxidative stress causes extensive oxidation and fragmentation, cross-linking, and aggregation of proteins that become resistant to proteolytic digestion. These aggregated, cross-linked, oxidized proteins actually bind to the 20S proteasome and act as irreversible inhibitors (Bence et al., 2001; Davies et al., 2001). Our experiments demonstrate that selective inhibition of the UPS by MG132 and lactacystin caused cell death in 24 h of treatment. However, although cells were not espoused to any exogenous oxidants, pretreatment with antioxidants reduced cell death induced by MG132 and lactacystin. Thus, in the situation of UPS inhibition or failure, the blockade of physiological ROS production during normal aerobic metabolism is enough to ameliorate cell viability. This finding emphasizes the increased vulnerability of dopaminergic neurons in the SNc in conditions where the proteasome activity is decreased (McNaught and Jenner, 2001; McNaught et al. 2002, 2003). The state of oxidative stress of the SNc, probably induced by the metabolism of local neurotransmitters, might lead to UPS impairment. Exposure of cells to additional damage by the endogenous ROS production of normal aerobic metabolism might enhance the toxic effects. This

pathologic vicious circle might contribute to the loss of nigral neurons in PD brains. We believe that the development of potent, brain-penetrating antioxidants or enhancers of the UPS might intervene in this toxic circle and act to slow down disease progression.

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