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Inhibition of proteasome activity sensitizes dopamine neurons to protein alterations and oxidative stress

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Summary. Impairment in the capacity of the ubiquitin-proteasome pathway to clear unwanted proteins has been implicated in the cell death that occurs in Parkinson's disease (PD). In support of this concept, defects in proteasomal structure and function, as well as protein aggregates and increased levels of oxidized proteins are found in the substantia nigra of PD patients. We have previously demonstrated that inhibition of proteasome activity in mesencephalic cultures induces degeneration of dopaminergic neurons coupled with the formation of proteinaceous intracellular inclusions. In this study we examined the effect of proteasome inhibition on cultured dopamine neurons when combined with oxidative stress and protein misfolding, in order to better simulate the condition in PD. We demonstrate that two structurally unrelated inhibitors of proteasome activity, lactacystin and carbobenzoxy-L-leucul-L-leucyl-L-leucinal (MG132), cause dose-dependent cell loss that preferentially affects dopaminergic neurons. Conditions that promote protein damage and misfolding such as oxidative stress, heat shock, and canavanine also induce neuronal degeneration with preferential loss of dopamine neurons and cell death is markedly increased when any of these is combined with a proteasome inhibitor. These studies demonstrate a synergistic effect between conditions that promote the formation of damaged proteins and those in which proteasomal function is impaired, and provide further support for the notion that cell loss in PD could be related to a defect in protein handling.

Keywords: Parkinson's disease, proteasome inhibitor, oxidative stress, heat shock, canavanine.

Introduction

Parkinson's disease (PD) is characterized by a relatively selective degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNc) and intracellular proteinaceous inclusions referred to as Lewy bodies. Several

different genetic factors are known to account for a small number of familial cases, but the overwhelming majority of PD patients lack a positive family history and have a sporadic form of the disease. In these cases, there is evidence supporting a role for oxidative stress, excitotoxicity, mitochondrial dysfunction and inflammation in the cascade of events leading to cell death (Jenner et al., 1992; Schapira et al., 1992; Beal, 1998; Hirsch et al., 1998), but how they interact and to what extent any one factor is relevant to neurodegeneration in an individual patient is not known. It is thus becoming clear that there are many different causes of PD. We have recently proposed that defects in the capacity of the ubiquitin-proteasome system (UPS) to clear unwanted and misfolded proteins is a common theme underlying both the familial and sporadic forms of PD (McNaught and Jenner, 2001; McNaught et al., 2001, 2002b).

The UPS is primarily responsible for the degradation and clearance of damaged, mutant, or misfolded proteins in eukaryotic cells (Sherman and Goldberg, 2001). Accumulation of excessive levels of abnormal proteins may interfere with normal cellular function and promote apoptotic cell death (Sherman and Goldberg, 2001). Since neurons do not normally turn over, it is of crucial importance that adequate UPS function be maintained to facilitate neuronal viability. Several studies have suggested that failure of the UPS to clear unwanted proteins may be an important factor in PD. Mutations in the α-synuclein gene which cause the protein to misfold and resist proteasomal degradation have been detected in rare autosomal dominant forms of PD (Polymeropoulos et al., 1997). Various mutations in the gene encoding parkin have been detected in patients with an autosomal recessive juvenile PD. It is now appreciated that parkin is a ubiquitin ligase, and there is evidence indicating that target proteins are increased in a non-ubiquitinated form in the brains of these patients (Shimura et al., 2001; Imai et al., 2001; Zhang et al., 2000; Mizuno et al., 2001). In addition, a mutation in the gene encoding ubiquitin *C*-terminal hydrolase L1 (UCH-L1) has been detected in rare cases of familial PD (Leroy et al., 1998). A defect in this gene could impair de-ubiquitinating activity, and reduce the availability of ubiquitin monomers necessary for the clearance of additional unwanted proteins. Defects in protein handling have also been implicated in sporadic PD. Lewy bodies found in degenerating neurons in PD accumulate a variety of proteins, and levels of oxidized proteins are markedly increased (Jenner and Olanow, 1996; Good et al., 1998; Forno et al., 1986; Farrer et al., 2001). In addition, we and others have shown that there are defects in the structure and function of proteasomes in dopamine neurons of the SNc in PD (McNaught et al., 2002b; Tofaris et al., 2003). These observations suggest that mishandling of misfolded or damaged proteins by proteasomes may contribute to the degeneration of dopaminergic neurons in the different forms of PD (McNaught et al., 2001). In support of this hypothesis, we and others have shown that proteasome inhibition leads to preferential degeneration of cultured dopamine neurons coupled with the formation of ubiquitin/α-synuclein immunoreactive inclusion bodies (Rideout et al., 2001; McNaught et al., 2002a).

The SNc may be particularly vulnerable to suffer neurodegeneration in PD because of its highly oxidative environment and the possibility that oxidative damage may cause proteins to undergo denaturation and chemical modification.

In addition, proteasome activity can be inhibited by oxidative stress (Reinheckel et al., 1998) thereby generating a vicious cycle leading to continued accumulation of abnormal proteins (Ding and Keller, 2001). In the present study, we investigated the effect of proteasome inhibition on dopamine neuronal survival when combined with oxidative stress or factors such as heat shock and canavanine that are known to promote protein misfolding (Kuckelkorn et al., 2000; Tsirigotis et al., 2001) to more closely model the conditions that are operative in PD.

Methods

Materials

Pregnant Sprague-Dewless rats were obtained from Taconic Farms (Germantown, N.Y.). MEM was purchased from GIBCO-Life Technologies (Grand Island, N.Y.), horse serum from Gemini (Calabasas, CA) and NU® serum from Collaborative Biomedical Products (Bedford, MA). Monoclonal antibodies to tyrosine hydroxylase (TH) were purchased from Boehringer Mannheim (Indianapolis, IN). Lactacystin and MG-132 were purchased from Calbiochem (La Jolla, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell cultures

The protocols for handling animals and preparing cell cultures followed the NIH guidelines and were approved by the institutional review committee. Mesencephalic cultures were prepared from embryonic rats on the 14th day of gestation as described previously (Mytilineou et al., 1999). In brief, the mesencephalon was dissected free of meninges and collected in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). The tissue was mechanically dissociated into a single cell suspension and plated in 24 plates pre-coated with L-polyornithine (0.1 mg/ml) at a density of 200,000 cells/cm^2. The medium consisted of MEM supplemented with 2 mM glutamine, 33 mM glucose, 10% horse serum and 10% NU® serum. After 48 h the medium was changed to MEM containing only 5% horse serum.

Immunocytochemistry

Cells were plated on polyornithine coated glass cover slips in 24 well plates. They were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and permeabilized and blocked with 0.3% Triton X-100 and 3% bovine serum albumin (BSA) for 30 min. Cultures were exposed to the TH antibodies (1:1000 dilution) overnight at 4°C. They were then processed with the peroxidase-coupled avidin-biotin kit with 3,3-diaminobenzidine as a chromogen. The number of dopaminergic neurons in cultures was determined by counting the cells positively immunostained for TH. Forty fields (1×1 mm) in two transverse strips across the diameter of the dish were counted using an inverted microscope (Nikon, Melville, NY, U.S.A.) at 20× magnification. This represented 10% of total area of the coverslip.

[^{3}H]-Dopamine and [^{14}C]-GABA uptake

Measurement of dopamine and γ-aminobutyric acid (GABA) uptake was performed as described previously (Mytilineou et al., 1998). Cultures were rinsed with Kreb's phosphate buffer (pH 7.4) and incubated for 30 min at 37°C with the same buffer containing 0.2 mg/ml ascorbic acid, 10 μM GABA, 0.5 μCi/ml [^{3}H]-dopamine (32.6 Ci/mmol; NEN, Boston MA) and 0.05 μCi/ml [^{14}C]-GABA (240 mCi/mmol; NEN, Boston MA). After rinsing, the radioactivity was extracted with 1 ml 95% ethanol, which was added to vials containing scintillation cocktail and the radioactivity measured in a scintillation spectrometer (Packard Tri-Carb 2100). Cultures treated with the neuronal dopamine uptake blocker mazindol (10 μM) and the neuronal GABA uptake blocker diaminobutyric acid (DABA; 1 mM) were used as blanks.

Cell viability assays

MTT assay. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, as described previously (Han et al., 1996). In brief, 50 µl of a 5 mg/ml solution of MTT was added to each cell culture well containing 0.5 ml medium. After 1 hour incubation at 37°C, the medium was carefully removed and the formazan crystals were dissolved in 1 ml isopropyl alcohol by gentle shaking of the plate. Absorbance was determined at 570 nm in a microplate reader (Spectramax 250, Molecular Devices Corporation, Sunnyvale CA).

Lactate dehydrogenase (LDH) assay. A modification of the method described by Bergmeyer et al. (1963) was used to determine LDH activity in the culture medium and the cells. Medium was collected, centrifuged to remove debris and frozen at −80°C until assay. Cells were freeze thawed (×3) in 1.0 ml feeding medium, the medium was collected, centrifuged and the supernatant frozen at −80°C. 50 µl of supernatant and 100 µl of NADH (1.2 mg/ml H_2O stock) were added to 850 µl of buffer and the samples were vortex-mixed. 50 µl of feeding medium was used for blanks. Triplicate aliquots (250 µl) were placed into 96-well plates at room temperature and reaction was initiated by addition of 25 µl of sodium pyruvate (0.36 mg/ml H_2O stock). The rate of disappearance of NADH was measured at 340 nm using a microplate reader.

Treatment protocol

Treatment of the cultures began on the 6th day in vitro with a complete change of the feeding medium and the addition of study interventions as indicated. Two cell permeable inhibitors proteasome inhibitors were tested: the reversible proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), and lactacystin, a more selective and irreversible inhibitor of proteasome (Goldberg, 1997 #802; Meriin, 1998 #803). For heat shock, cultures were placed in a 42°C incubator for 1 hr. Protein damage was also induced by incubation with canavanine (2.5–10 µM), an aminoacid analog the causes protein misfolding (Tsirigotis et al., 2001). For oxidative stress hydrogen peroxide (H_2O_2; 10–40 µM), L-dopa (20–200 µM) or L-buthionine sulfoximine (BSO; 50 µM) were added to the medium for 24 or 48 hours.

Statistical analysis

Values are expressed as means ± SEM. Significance of differences between two groups was determined by two-tailed Student's t test. For multiple comparisons, one-way ANOVA followed by Tukey-Kramer's test was used.

Results

Selective vulnerability of dopamine neurons to proteasome inhibition

The reversible proteasome inhibitor MG-132 caused significant damage to mesencephalic cultures after a 48 h exposure (Fig. 1). Measurement of total cell viability with the MTT assay showed that treatment with 0.5 and 1 µM MG-132 caused a reduction in cell survival to 57% and 32% of control values ($P < 0.001$; Fig. 1A). The toxicity of MG-132 towards dopamine neurons was even greater, with a decrease in the number of TH-positive cells of 15% and 4% of control values after 0.5 and 1 µM MG-132, respectively (Fig. 1A). The most pronounced effect of MG-132 was directed toward the dopamine transporter where significant reductions in the neuronal uptake of [^{3}H]-dopamine were observed at all MG-132 concentrations and there was no measurable uptake after 1 µM MG-132 (Fig. 1B). The neuronal uptake of [^{14}C]-GABA was also reduced by MG-132 treatment, but to a much lesser extent than [^{3}H]-dopamine

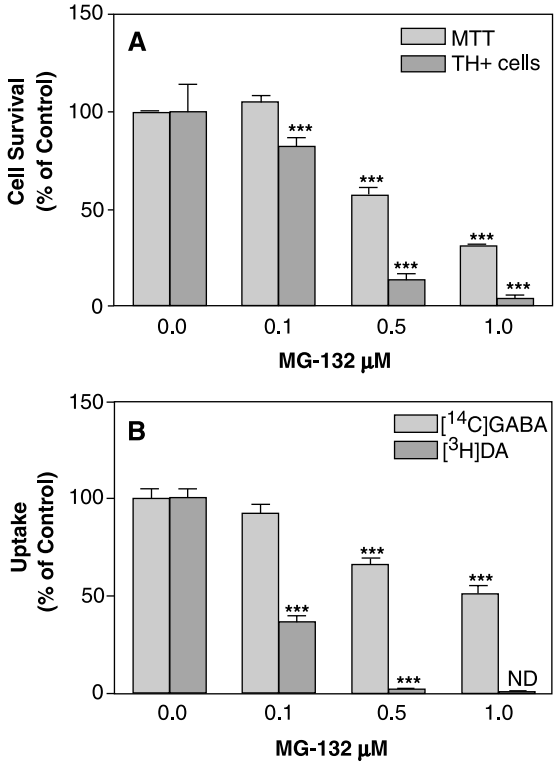

Fig. 1. Proteasome inhibition with MG-132 causes selective damage to dopamine neurons. Mesencephalic cultures were treated with the proteasome inhibitor MG132 (0.1–1 μM) and were analyzed 48 h later for overall cell viability with MTT assay (**A**) dopamine neuron survival with TH staining (**A**) and [^{3}H]dopamine and [^{14}C]GABA uptake (**B**). Bars are means ± SEM expressed as per cent of untreated controls (N = 4–6 cultures per group). ***$p < 0.001$ compared to corresponding controls. One-way ANOVA followed by Tukey test

uptake. These data are in agreement with our previous study where lactacystin, a non-reversible proteasome inhibitor also showed that dopamine neurons are preferentially sensitive to proteasome inhibition (McNaught et al., 2002a). The decrease in [^{3}H]-dopamine uptake was not due to a direct inhibitory effect of MG-132 or lactacystin on the dopamine transporter, because addition of 0.5 and 1 μM MG-132 or 5 and 10 μM lactacystin together with [^{3}H]-dopamine during the uptake assay had no effect (results not shown).

Proteasome inhibition increases neuronal vulnerability to abnormal proteins

To determine the ability of proteasome-impaired cells to handle damaged proteins, we pre-treated mesencephalic cultures with the irreversible proteasome inhibitor lactacystin for 1 hour, and then incubated the cells at 42°C for 1 hour to induce heat shock. Thereafter the temperature was returned to 37°C (normal maintenance) for 24 or 48 hours. Cell damage was determined by measuring the amount of LDH released into the medium during the incubation period. Heat shock alone did not cause measurable damage to control cultures while

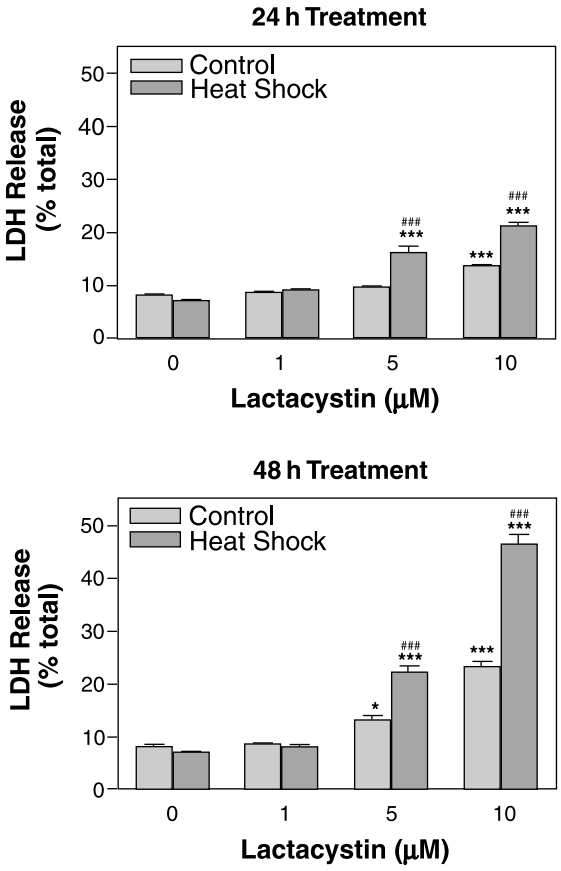

Fig. 2. Heat shock increases the damage caused by proteasome inhibition. Mesencephalic cultures were treated with lactacystin for 1 hour and then exposed to heat shock for an additional 1 hour. Loss of cell viability was determined by measuring LDH released in the medium 24 or 48 hours later. Bars are means ± SEM (N = 5–6 cultures per group) of LDH released in the medium expressed as percent of the total LDH contained in medium plus cells. LDH in the medium of control cultures was 8.1 ± 0.3 and 8.2 ± 0.4 percent of total after 24 and 48 hours respectively. There was no effect of heat shock in control cultures with LDH levels of 7.6 ± 0.3 and 7.0 ± 0.2 percent of total after 24 and 48 hours, respectively. Lactacystin induced a time and concentration dependent increase in cell death; at concentrations of 5 and 10 μM cell death induced by lactacystin was significantly increased when combined with heat shock. *$p < 0.5$; ***$p < 0.001$ compared with respective controls; $^{###}p < 0.001$ compared to corresponding groups not exposed to heat shock. One-way ANOVA followed by Tukey test

lactacystin induced cell death in a time- and concentration-dependent manner (Fig. 2). When lactacystin was combined with heat shock, cytotoxicity was significantly increased. At a lactacystin concentration of 10 μM administered for 48 hours, heat shock increased LDH release from 24% to 47% of total ($P < 0.001$).

Lactacystin markedly inhibited [^{3}H]-dopamine uptake in both a concentration and time dependent manner, with 10 μM inducing a reduction of 60% and

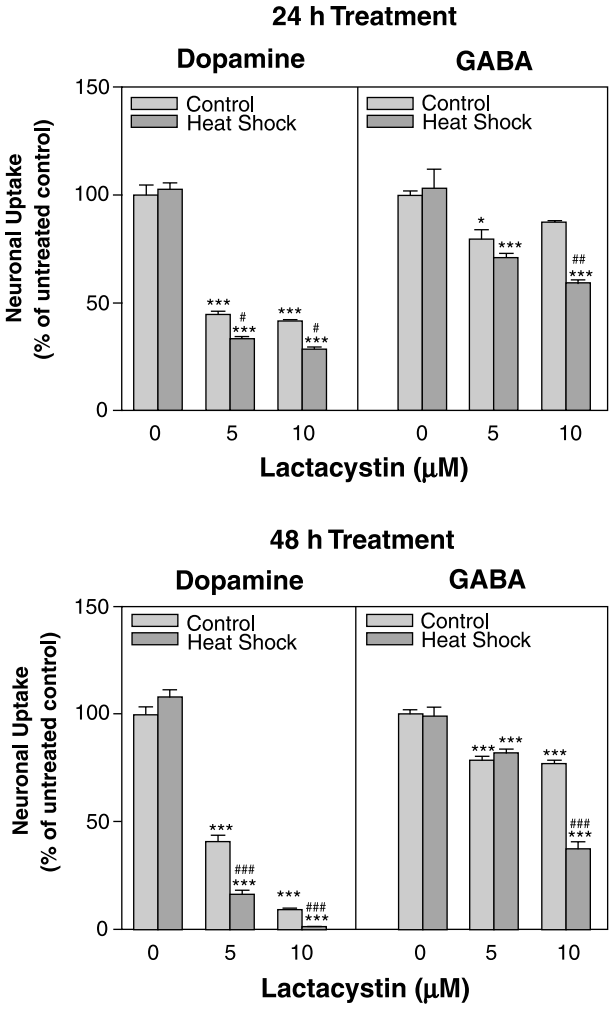

Fig. 3. Heat shock and proteasome inhibition act synergistically to induce degeneration of dopamine neurons. Mesencephalic cultures were treated with lactacystin and heat shock as in Fig. 2 and analyzed for [^{3}H]dopamine and [^{14}C]GABA uptake after 24 and 48 hours. Bars are means ± SEM expressed as per cent of untreated controls. *$p < 0.05$; ***$p < 0.001$ compared to untreated control; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared to the corresponding group not treated with heat shock. One-way ANOVA followed by Tukey test

90% at 24 and 48 hours respectively (Fig. 3). Heat shock by itself had no effect on [^{3}H]-dopamine uptake but increased the damage caused by lactacystin reducing uptake values to 17% and 1% of control with 5 and 10 μM respectively (Fig. 3). Lactacystin had a lesser effect on [^{14}C]-GABA uptake, reducing it by approximately 20%. Heat shock caused a further reduction in [^{14}C]-GABA uptake, but only at the higher lactacystin concentration (Fig. 3).

We examined the effect of proteasome inhibition on the toxicity of canavanine, an arginine analog which is incorporated into proteins and generates

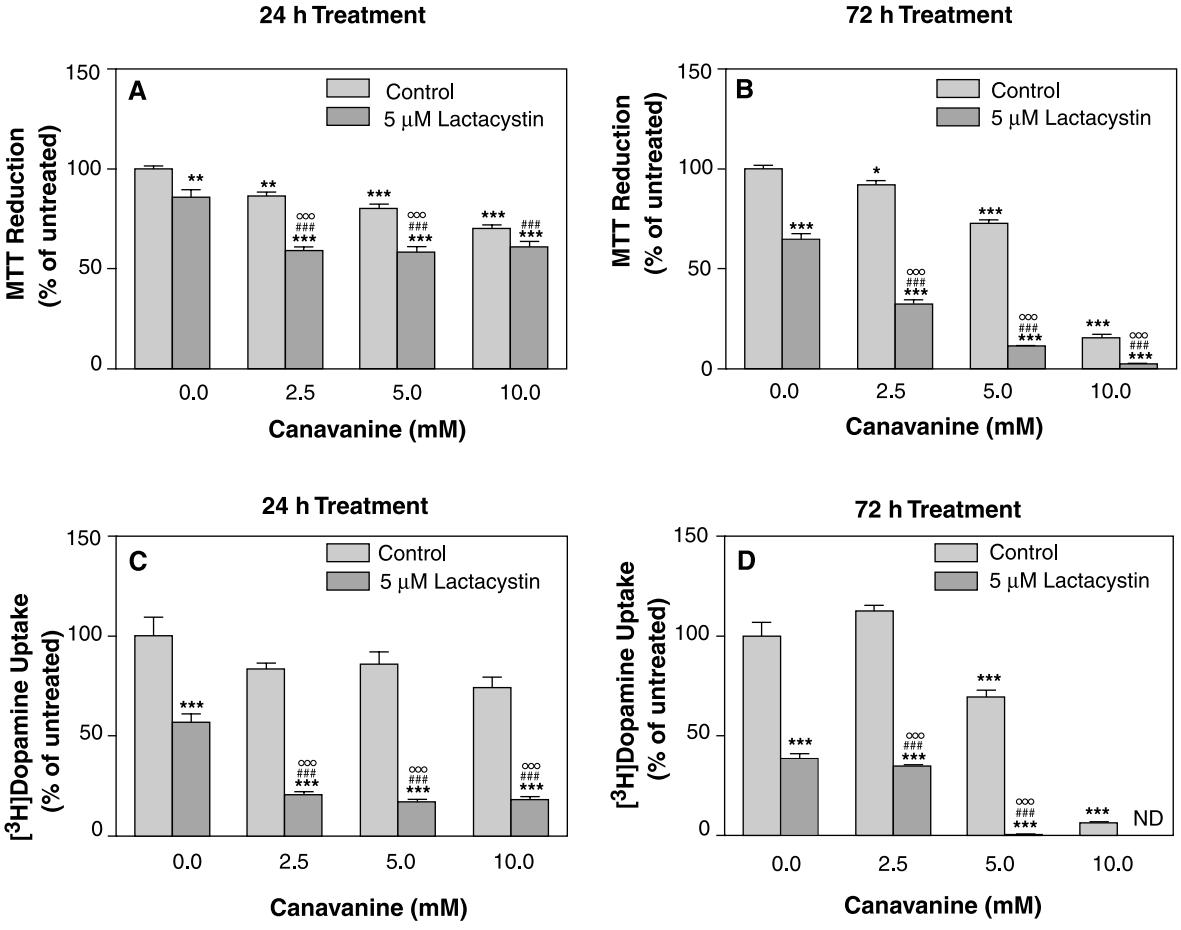

Fig. 4. Proteasome inhibition increases the damage caused by canavanine-induced protein misfolding. Mesencephalic cultures were treated with increasing concentrations of canavanine, in the presence or absence of 5 μM lactacystin, and the cultures were assayed 24 and 72 h later for cell survival with MTT (**A**, **B**) and for [^{3}H]dopamine uptake (**C**, **D**). Bars are means ± SEM presented as % of untreated controls. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to untreated controls. $^{###}p<0.001$ compared to lactacystin alone. $^{\circ\circ\circ}p<0.001$ compared to the corresponding group not treated with lactacystin. One-way ANOVA followed by Tukey test

misfolding (Tsirigotis et al., 2001). Canavanine alone caused a loss of cell viability, which was concentration dependent and increased with prolonged exposure (Fig. 4A and B). Lactacystin alone in a concentration of 5 μM also caused modest cell damage. The combination of canavanine and proteasome inhibition, however, had a synergistic effect particularly after 72 h treatment (Fig. 4B). Similarly, canavanine and lactacystin inhibited [^{3}H]-dopamine uptake, and the combination of canavanine and lactacystin increased the toxicity in a synergistic manner (Fig. 4C and D). Canavanine inhibited [^{3}H] dopamine uptake to a greater degree than [^{14}C] GABA uptake, and the addition of lactacystin increased the differential toxicity (data not shown).

Proteasome inhibition sensitizes mesencephalic cells to oxidative stress

We examined the effect of proteasome inhibition on the survival of mesencephalic cultures exposed to oxidative stress. Incubation with L-dopa and H_2O_2, as well as treatment with the glutathione (GSH) synthesis inhibitor buthionine sulfoxamine (BSO) were used to induce oxidative stress (Mytilineou et al., 1993, 1999). Inhibition of proteasome activity with lactacystin resulted in significantly more damage to mesencephalic cultures treated with L-dopa than when it was applied by itself (Fig. 5A). Similarly, treatment with lactacystin increased H_2O_2-induced toxicity. Exposure of mesencephalic cultures to 40 μM H_2O_2 for 24 hours caused a 12% loss of cell viability as determined by the MTT assay, while lactacystin 5 μM alone caused a 21% cell loss. Combining H_2O_2 with lactacystin resulted in a significantly increased cell loss of 52% ($p < 0.001$, $N = 3$). Similarly, exposure of mesencephalic cultures to 50 μM BSO, a concentration which causes profound depletion in the levels of GSH but does not

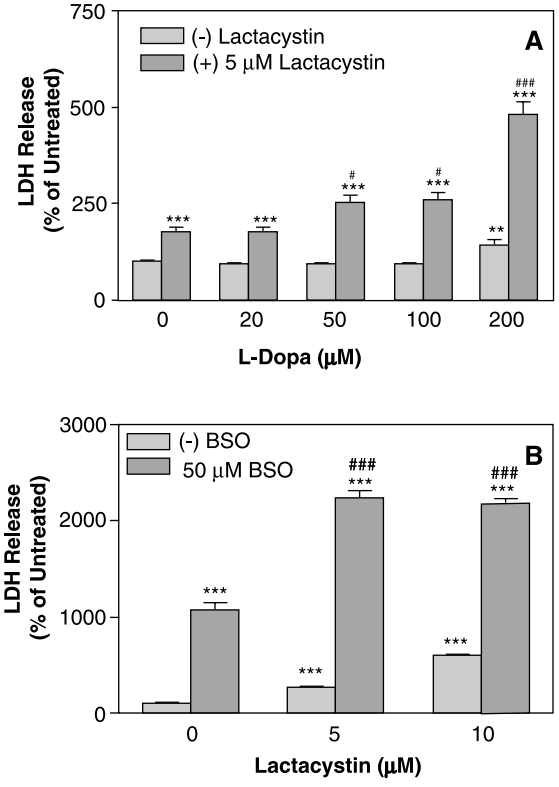

Fig. 5. Oxidative stress increases damage caused by proteasome inhibition. Mesencephalic cultures were treated with L-Dopa (**A**), and BSO (**B**) in the presence or absence of lactacystin. Cell viability was determined 48 hours later by measuring the percent of total LDH released in the medium. Bars are means ± SEM expressed as percent of untreated controls. ($N = 4$ cultures per group for L-Dopa and 8 cultures for BSO experiments). $^{***}p < 0.001$ compared to untreated controls; $^{\#}p < 0.05$ $^{\#\#\#}p < 0.001$ compared to lactacystin alone (**A**) or to the corresponding group not treated with lacacystin (**B**). One-way ANOVA followed by Tukey test

Table 1. Antioxidants, free radical trapping agents and inhibitors of release and metabolism of arachidonic acid do not protect cells from damage caused by proteasome inhibition

Compounds tested	Percentage change in cell viability compared to proteasome inhibition alone			
	MG-132 (μM)		Lactacystin (μM)	
	0.5	1.0	5	10
Ascorbic acid (200 μM)	+5.7	+2.8	–	–
Sodium salicylate (2 mM)	+0.8	−0.2	−4.9	−7.3
MAFP (10 μM)	−4.3	+0.8	−3.2	−12.1
Indomethacin (50 μM)	−2.1	+0.5	−11.4	−11.8
Acetylsalicylic acid (2 mM)	−2.4	−1.9	−0.5	+6.3
Biacalein (5 μM)	+1.6	+4.4	+1.1	−3.5

Mesencephalic cultures were treated with MG-132 or lactacystin at the concentrations indicated for 48 h. various compounds tested were added together with the proteasome inhibitors. The numbers represent the effect of the compounds expressed as % change in the loss of cell viability caused by the proteasome inhibitors alone. Cell viability tested with the MTT assay varied in different experiments from 12.7 to 34.7% of control for 0.5 μM MG-132, from 9.4 to 19.1% of control for 1 μM MG-132, from 79.9 to 81.2% of control for 5 μM lactacystin and from 39.6 to 48.4 for 10 μM lactacystin. No significant change in cell viability resulted from treatment with any of the compounds (ANOVA). The highest concentrations of the compounds that caused no damage to the cells are shown in the Table. Lower concentrations had similar effects

induce degeneration in low cell density cultures (Mytilineou et al., 1999), sensitized the cells to degeneration induced by lactacystin (Fig. 5B).

To determine whether survival following proteasome inhibition could be improved by diminishing oxidative stress, we tested several compounds for potential protective activity (Table 1). These included the antioxidant ascorbic acid, the free radical trapping agent sodium salicylate, the inhibitor of phospholipase A_2 (PLA_2) activity methyl arachidonyl fluorophosphonate (MAFP), the cyclooxygenase (COX) inhibitors indomethacin and acetylsalicylic acid (aspirin) and the lipoxygenase (LOX) inhibitor biacalein. None of these compounds provided significant protection against MG-132 or lactacystin treatment.

Discussion

Proteasomes are the primary system responsible for the degradation and clearance of intracytoplasmic misfolded unwanted proteins in eukaryocytic cells (Sherman and Goldberg, 2001). Proteasome inhibition has been shown to cause degeneration of cultured PC12 cells and mesencephalic dopamine neurons coupled with the formation of ubiquitin/α-synuclein immunoreactive intracellular inclusions reminiscent of the Lewy bodies that characterize PD (Rideout et al., 2001; McNaught et al., 2002a). In the latter study, degeneration preferentially affected dopaminergic neurons. These observations support the notion that improper clearance of cellular proteins can result in protein accumulation, inclusions, and neurodegeneration and may therefore be relevant to PD.

In the present study, we confirm that proteasome inhibition, with either the reversible inhibitor MG132 or the irreversible inhibitor lactacystin, induces degeneration of cultured mesencephalic neurons with a preferential loss of dopaminergic cells. Here, the decrease in dopamine uptake was greater than the decrease in the number of TH positive neurons, suggesting that proteasome inhibition primarily affects the terminals of dopamine neurons. We further show that when proteasome inhibition is combined with conditions that promote protein misfolding, such as heat shock or canavanine exposure, there is more severe cell damage, again with preferential toxicity to dopaminergic neurons. Compromised proteasome activity also increased cell damage associated with oxidative stress caused by L-DOPA, H_2O_2 or BSO. These findings suggest that a combination of abnormal proteins, oxidative stress, and impairment of proteasomal activity, all of which occur in the SNc in PD (Jenner and Olanow, 1996; McNaught et al., 2002b), act synergistically to promote degeneration of dopamine neurons and could collectively play a role in the neurodegenerative process that occurs in PD.

There has been increasing interest in the possibility that impaired clearance of abnormal or unwanted proteins by the UPS may be common to the different forms of PD (McNaught et al., 2001). Mutations in genes that encode for components of the UPS, namely parkin (a ubiquitin ligase) and UCH-L1 (a de-ubiquitinating enzyme) have been identified in small numbers of patients with familial PD (Mizuno et al., 2001). Mutations in α-synuclein are associated with an autosomal dominant familial form of PD (Polymeropoulos, 1998) and appear to cause the protein to form fibrils and resist proteasomal degradation (Shtilerman et al., 2002). More recent studies similarly demonstrate that familial PD can also be associated with gene defects that promote protein misfolding or increased levels of brain proteins (Hague et al., 2001; Singleton et al., 2003). Most cases of PD are not familial, and occur sporadically. Here too, there is evidence that the UPS does not function normally. PD is associated with protein aggregates and increased levels of oxidized proteins in the SNc. Further, there is evidence that proteasomal structure and enzymatic functions in the SNc are abnormal in PD (McNaught and Jenner, 2001; McNaught et al., 2002b).

In the present study we confirm that proteasome inhibitors induce degeneration of mesencephalic cells and that dopamine neurons are preferentially affected. Further, we demonstrate that neurodegeneration induced by proteasome inhibition is significantly enhanced by factors that promote protein misfolding such as heat shock and canavanine and by oxidative stress induced by H_2O_2, glutathione depletion and levodopa. Each of these conditions is likely to induce damage to proteins and increase demands placed on the proteasomal system, thereby potentially accounting for increased neuronal degeneration in the presence of proteasomal dysfunction. Each of these factors is also relevant to PD. The SNc is known to contain increased levels of oxidatively damaged proteins and to be in a state of oxidative stress (Jenner and Olanow, 1998). Specifically, GSH levels are decreased in the SNc in PD (Perry et al., 1982; Riederer et al., 1989) as well as in asymptomatic individuals with incidental Lewy bodies who are thought to have a pre-clinical form of the disease (Sian et al., 1994). L-dopa is commonly used in the treatment of PD, and has the potential to generate peroxide and other

oxidizing species by virtue of its enzymatic metabolism or auto-oxidation (Graham, 1978; Cohen, 1983). In high concentrations, L-dopa is toxic to dopamine neurons in tissue culture (Mytilineou et al., 1993; Mena et al., 1997; Melamed et al., 1998), but has not been shown to induce neuronal degeneration in *in vivo* models (Hefti et al., 1981; Perry et al., 1984; Zeng et al., 2001). Here we show that both GSH depletion and L-dopa can augment neurodegeneration associated with proteasome inhibition. The mechanism whereby this occurs is not yet known, but we assume that it relates to the capacity of oxidative stress to damage proteins and thus increase demands on the already inhibited proteasomal system. In support of this concept, heat shock and canavanine which are known to alter protein conformation and promote misfolding synergistically increase the cell death induced by proteasome inhibitors.

The basis for the selective vulnerability of dopamine neurons in PD and in our model system is not yet clear. However, it can be postulated that the highly oxidative environment of dopamine neurons may lead to the formation of relatively large numbers of misfolded proteins and present the UPS with the need to clear a large number of damaged proteins even under normal circumstances. This region might therefore be particularly susceptible to further oxidative stress which increases the load on the UPS and makes it particularly sensitive to proteasomal damage. Oxidative stress, as found in the nigra in PD, might also directly affect proteasomes and compromise their ability to clear damaged proteins (Bulteau et al., 2001; Keller et al., 2000; Elkon et al., 2001). Interestingly, although oxidative stress and proteasome inhibition synergistically decrease cell survival in mesencephalic cultures, administration of the antioxidant ascorbic acid or the free radical trapping agent sodium salycilate failed to prevent cell loss induced by MG-132 or lactacystin alone. Impairment of proteasome function has been reported to cause release and metabolism of arachidonic acid (Levine, 2000a, b) and our earlier studies show that inhibition of arachidonic acid metabolism prevents damage from oxidative stress caused by GSH depletion (Mytilineou et al., 1999). However, no protection against the loss of viability resulting from lactacystin or MG-132 treatment was provided by inhibition of PLA_2 activity with MAFP, inhibition of COX activity with aspirin and indomethacin, or with the specific LOX inhibitor biacalein. These findings suggest that protein accumulation directly inhibits critical cell functions or activates cell death pathways.

In summary, genetic studies in PD patients have implicated mishandling of unwanted proteins in the etiopathogenesis of this condition. The findings of defects in proteasomal function in the SNc of PD patients coupled with oxidative stress and high levels of oxidized proteins suggest that defects in the UPS are relevant to the sporadic form of the disorder as well. Our present studies demonstrate that proteasomal inhibition induces degeneration of cultured mesencephalic dopamine neurons and that this effect is significantly enhanced by protein misfolding and oxidative stress as also occur in PD. Further investigation into the biology of dopamine neurons should help to unravel the mechanism whereby dopamine neurons are selectively affected by proteasome inhibitors. This may also shed light on the etiopathogenesis of late onset idiopathic Parkinson's disease.

Acknowledgments

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References

Beal MF (1998) Excitotoxicity and nitric oxide in Parkinson's disease pathogenesis. Ann Neurol 44: S110–S114

Bergmeyer HU, Bernt E, Hess B (eds) (1963) Lactate dehydrogenase. Academic Press, New York

Bulteau AL, Lundberg KC, Humphries KM, Sadek HA, Szweda PA, Friguet B, Szweda LI (2001) Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. J Biol Chem 276: 30057–30063

Cohen G (1983) The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. J Neural Transm [Suppl] 19: 89–103

Ding Q, Keller JN (2001) Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. J Neurochem 77: 1010–1017

Elkon H, Melamed E, Offen D (2001) 6-Hyrpxydopamine increases ubiqutin-conjugates and protein degradation: implications for the pathogenesis of Parkinson's disease. Cell Mol Neurobiol 21: 771–781

Farrer M, Chan P, Chen R, Tan L, Lincoln S, Hernandez D, Forno L, Gwinn-Hardy K, Petrucelli L, Hussey J, Singleton A, Tanner C, Hardy J, Langston JW (2001) Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 50: 293–300

Forno LS, Sternberger LA, Sternberger NH, Strefling AM, Swanson K, Eng LF (1986) Reaction of Lewy bodies with antibodies to phosphorylated and non-phosphorylated neurofilaments. Neurosci Lett 64: 253–258

Good PF, Hsu A, Werner P, Perl DP, Olanow CW (1998) Protein nitration in Parkinson's disease. J Neuropathol Exp Neurol 57: 338–342

Graham DG (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 14: 633–643

Hague S, Rogaeva E, Hernandez D, et al. (2003) Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 54: 271–274

Han SK, Mytilineou C, Cohen G (1996) L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. J Neurochem 66: 501–510

Hefti F, Melamed E, Bhawan J, Wurtman RJ (1981) Long-term administration of L-dpa does not damage dopamiergic neurons in the mouse. Neurology 31: 1194–1195

Hirsch EC, Hunot S, Damier P, Faucheux B (1998) Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? Ann Neurol 44: S115–S120

Imai Y, Soda M, Inoue H, et al. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 105: 891–902

Jenner P, Olanow CW (1996) Oxidative stress and the pathogenesis of Parkinson's disease. Neurology 47: S161–S170

Jenner P, Olanow CW (1998) Understanding cell death in Parkinson's disease. Ann Neurol 44: S72–S84

Jenner P, Dexter DT, Sian J, Schapira AH, Marsden CD (1992) Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. Ann Neurol 32 [Suppl]: S82–S87

Keller JN, Huang FF, Dimayuga ER, Maragos WF (2000) Dopamine induces proteasome inhibition in neural PC12 cell line. Free Rad Biol Med 29: 1037–1042

Kuckelkorn U, Knuehl C, Boes-Fabian B, Drung I, Kloetzel PM (2000) The effect of heat shock on 20S/26S proteasomes. Biol Chem 381: 1017–1023

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. Nature 395: 451–452

Levine L (2000a) A combination of lactacystin and 12-O-tetradecanoylphorbol-13-acetate enhances the release of arachidonic acid from cells in cultures: release is reduced in confluent and serum deprived cells. Prostagland Leukot Essent Fatty Acids 63: 363–370

Levine L (2000b) Lactacystin stimulates arachidonic acid metabolism in rat liver cells: effects of cell density on arachidonic acid release, PGI2 production and cyclooxygenase activity. Prostagland Leukot Essent Fatty Acids 63: 371–375

McNaught KS, Jenner P (2001) Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci Lett 297: 191–194

McNaught KS, Olanow CW, Halliwell B, Isacson O, Jenner P (2001) Failure of the ubiquitin-proteasome system in Parkinson's disease. Nat Rev Neurosci 2: 589–594

McNaught KSP, Mytilineou C, JnoBaptiste R, Yabut J, Shashidharan P, Jenner P, Olanow CW (2002a) Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures. J Neurochem 81: 301–306

McNaught KSP, Belizaire R, Jenner P, Olanow CW, Isacson O (2002b) Selective loss of 20S proteasome α-subunits in the substantia nigra pars compacta in Parkinson's disease. Neurosci Lett 326: 155–158

Melamed E, Offen D, Shirvan A, Djaldetti R, Barzilai A, Ziv I (1998) Levodopa toxicity and apoptosis. Ann Neurol 44: S149–S154

Mena MA, Casarejos MJ, Carazo A, Paino CL, Garcia de Yebenes J (1997) Glia protect fetal midbrain dopamine neurons in culture from L-DOPA toxicity through multiple mechanisms. J Neural Transm 104: 317–328

Mizuno Y, Hattori N, Mori H, Suzuki T, Tanaka K (2001) Parkin and Parkinson's disease. Curr Opin Neurol 14: 477–482

Mytilineou C, Han SK, Cohen G (1993) Toxic and protective effects of L-dopa on mesencephalic cell cultures. J Neurochem 61: 1470–1478

Mytilineou C, Leonardi EK, Radcliffe P, Heinonen EH, Han SK, Werner P, Cohen G, Olanow CW (1998) Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. J Pharmacol Exp Ther 284: 700–706

Mytilineou C, Kokotos Leonardi ET, Kramer BC, Jamindar T, Olanow CW (1999) Glial cells mediate toxicity in glutathione-depleted mesencephalic cultures. J Neurochem 73: 112–119

Perry TL, Godin DV, Hansen S (1982) Parkinson's disease: a disorder due to nigral glutathione deficiency? Neurosci Lett 33: 305–310

Perry TL, Yong VW, Ito M, Foulks JG, Wall RA, Godin DV, Clavier RM (1984) Nigrostriatal dopaminergic neurons remain undamaged in rats given high doses of L-DOPA and carbidopa chronically. J Neurochem 43: 990–993

Polymeropoulos MH (1998) Autosomal dominant Parkinson's disease and alpha-synuclein. Ann Neurol 44: S63–S64

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276: 2045–2047

Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, Grune T (1998) Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochem J 335: 637–642

Rideout HJ, Larsen KE, Sulzer D, Stefanis L (2001) Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. J Neurochem 78: 899–908

Riederer P, Sofic E, Rausch WD, et al. (1989) Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. J Neurochem 52: 515–520

Schapira AH, Mann VM, Cooper JM, Krige D, Jenner PJ, Marsden CD (1992) Mitochondrial function in Parkinson's disease. The Royal Kings and Queens Parkinson's Disease Research Group. Ann Neurol 32: S116–S124

Sherman MY, Goldberg AL (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29: 15–32

Shimura H, Schlossmacher MG, Hattori N, et al. (2001) Ubiquitination of a new form of {alpha}-synuclein by Parkin from human brain: implications for Parkinson's disease. Science 293: 263–269

Shtilerman MD, Ding TT, Lansbury PT Jr (2002) Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? Biochemistry 41: 3855–3860

Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, Jenner P, Marsden CD (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann Neurol 36: 348–355

Singleton AB, Farrer M, Johnson J, et al. (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science 302: 841

Tofaris GK, Razzaq A, Ghetti B, Lilley K, Spillantini MG (2003) Ubiquitination of alpha-synuclein in Lewy bodies is a pathological event not associated with impairment of proteasome function. J Biol Chem 278: 44405–44411

Tsirigotis M, Zhang M, Chiu RK, Wouters BG, Gray DA (2001) Sensitivity of mammalian cells expressing mutant ubiquitin to protein-damaging agents. J Biol Chem 276: 46073–46078

Zeng BY, Pearce RK, MacKenzie GM, Jenner P (2001) Chronic high dose L-dopa treatment does not alter the levels of dopamine D-1, D-2 or D-3 receptor in the striatum of normal monkeys: an autoradiographic study. J Neural Transm 108: 925–941

Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci USA 97: 13354–13359

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