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Proteasome inhibition and aggregation in Parkinson's disease: a comparative study in untransfected and transfected cells

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Abstract

Dysfunction of the ubiquitin-proteasome system (UPS) has been implicated in Parkinson's disease (PD) and other neurodegenerative disorders. We have investigated the effect of UPS inhibition on the metabolism of α-synuclein (SYN) and parkin, two proteins genetically and histopathologically associated to PD. Pharmacological inhibition of proteasome induced accumulation of both parkin and SYN in transfected PC12 cells. We found that this effect was caused by increased protein synthesis rather than impairment of protein degradation, suggesting that inhibition of the UPS might lead to non-specific up-regulation of cytomegalovirus (CMV)-driven transcription. To investigate whether endogenous parkin and SYN can be substrate of the UPS, untransfected PC12 cells and primary mesencephalic neurones were exposed to proteasome inhibitors, and parkin and SYN expression was evaluated at both protein and mRNA level. Under these conditions, we found that proteasome inhibitors did not affect the level of endogenous parkin and SYN. However, we confirmed that dopaminergic neurones were selectively vulnerable to the toxicity of proteasome inhibitors. Our results indicate that studies involving the use of proteasome inhibitors, particularly those in which proteins are expressed from a heterologous promoter, are subjected to potential artefacts that need to be considered for the interpretation of the role of UPS in PD pathogenesis.

Keywords: mesencephalic cultures, parkin, Parkinson's disease, protein misfolding, synuclein, ubiquitin-proteasome.

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Parkinson's disease (PD) is one of the most severe and widespread age-related neurodegenerative disorders, affecting almost 1% of the population aged over 65 years. PD is characterized clinically by resting tremor, rigidity and bradykinesia, resulting from the progressive and selective loss of dopaminergic neurones in the substantia nigra pars compacta, and histopathologically by the intracytoplasmic accumulation of amyloid-like inclusions, known as Lewy bodies (LB), in the spared dopaminergic cells.

Most of PD cases occur sporadically and the specific aetiology of the disease is still unknown. In the last few years, however, evidence has built up in support of a substantial genetic component (Dawson and Dawson 2003). It has been found that α-synuclein (SYN); a presynaptic protein of unknown function is implicated in PD pathogenesis. Two different missense mutations in the SYN gene (A30P and A53T) cause rare familial forms of PD (Polymeropoulos *et al*. 1997; Krüger *et al*. 1998). Based on this evidence SYN was identified as the major component of LB (Spillantini *et al*. 1997). On the other hand, a recessive form of juvenile PD was associated with mutations in the gene encoding parkin (Kitada *et al*. 1998). Unlike the extremely rare incidence of mutations in the SYN gene, it has been found that about 50% of early onset PD patients carry mutations in the gene encoding parkin (Abbas *et al*. 1999). The discovery of these genetic defects have led to the development of useful experimental models, and to significant progress in our understanding of the molecular

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Abbreviations used: Act D, actinomycin D; ALLN, *N*-acetyl-Leu-Leu-Norleu-al; CHX, cycloheximide; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LB, Lewy bodies; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; PC12, pheocromocytoma cells; PD, Parkinson's disease; SYN, α-synuclein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UPS, ubiquitin-proteasome system.

mechanism underlying this devastating neurological disorder (Dawson and Dawson 2003). The self-aggregation capacity of SYN (Conway *et al*. 1998) and the ubiquitin ligase activity attributed to parkin (Shimura *et al*. 2000; Zhang *et al*. 2000) suggested that protein aggregation and dysfunction of the ubiquitin/proteasome degradation system (UPS) might play a causal role in the development of sporadic and familial PD. UPS is a quality control mechanism of the cell, which is responsible for the recognition and degradation of misfolded and damaged proteins. Ageing or stress conditions, as well as pathogenic mutations that are likely to favour protein misfolding, can lead to abnormal accumulation of proteins committed to degradation by the UPS with a consequent saturation of the control machinery. This can generate a feedback effect, which can increase the rate of accumulation of misfolded and potentially cytotoxic proteins. Several pieces of evidence, most of which are based on the analysis of transfected cells, support the hypothesis that accumulation of SYN in PD is a consequence of an alteration of UPS (Bennett *et al*. 1999; Paxinou *et al*. 2001). However, the results obtained with these cellular models may be influenced by the non-physiological conditions of transgenic protein expression, such as overloading of the cellular biosynthetic machinery. In the present study we compared the effects of the pharmacological inhibition of UPS on parkin and SYN expressed in stably transfected PC12 cells, to those exerted by proteasome inhibitors on endogenous parkin and SYN expressed in untransfected PC12 and primary mesencephalic cells.

Materials and methods

PC12 cells

Generation of stably transfected PC12 cell lines expressing human SYN and parkin under the control of the cytomegalovirus (CMV) promoter has been described previously (Forloni *et al*. 2002). PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), and penicillin/streptomycin, and were maintained in an atmosphere of 5% CO_2, 95% air, at 37°C.

Primary tissue cultures

Ventral mesencephalon was removed from outbred Sprague–Dawley rat embryos (E14) according to the procedure described by Pardo *et al*. (1997). The cells were plated (5×10^5 cells/mL) on dishes (Iwaki) precoated with poly D-lysine (50 μg/mL; Sigma, St Louis, MO, USA) in Dulbecco's modified minimal essential medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 15% FBS (HyClone, Logan, UT, USA) and glutamine (2 mM) Cultures were kept at 37°C in a humidified 5% CO_2 atmosphere.

Fluorimetric assay of proteasome activity

Cells were lysed in 10 mM Tris pH 7.4, 0.5 mM DL-dithiothreitol, 5 mM adenosine 5′-triphosphate, 5 mM $MgCl_2$, in the presence of 0.5 mM fluorigenic peptides (suc-LLVT-MCA, boc-LAA-MCA and z-LLG-MCA), and incubated at room temperature for 1 h. The proteasome activity was quantified by evaluating the fluorescence of the peptide substrate at the wavelengths of 380 nm and 460 nm (Luminescence Spectrometer LS50B, Perkin-Elmer).

Western blot

Cells were lysed in 10 mM Tris pH 7.4, 0.5% SDS, incubated at 95°C for 10 min, and protein concentration was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Cell lysates corresponding to 20–50 μg of total protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and blots of the gels were incubated with the primary antibodies. Anti-α-synuclein monoclonal antibody (Transduction Laboratories, BD Biosciences, Palo Alto, CA, USA) and anti-parkin rabbit polyclonal antibodies (Cell Signaling, Beverly, MA, USA) were diluted 1 : 2500 and 1 : 5000, respectively; anti-ubiquitin monoclonal antibody (Chemicon, Temecula, CA, USA) was used at 1 : 7500; and anti-actin monoclonal antibody C4 (Chemicon) was diluted 1 : 10 000. Films exposed by enhanced chemiluminescence were digitalized with an Agfa Arcus2 scanner, and the band intensities were quantitated by NIH Image (National Institutes of Health, USA).

Northern blot and RT-PCR

Total RNA was prepared using the RNA Wiz kit (Ambion, Austin, TX, USA), and Northern blot analysis was performed using the Gene Images CDP-Star Chemiluminescent Detection System (Amersham, Piscataway, NJ, USA). Full-length human SYN and parkin cDNAs were used as a probe. RT-PCR was performed using the GeneAmp RNA PCR core kit (Applied Biosystems, Foster City, CA, USA) according to the manufacture's direction. After a 3-min denaturation step at 95°C, samples were subjected to repeated cycles of denaturation at 94°C for 60 s, primer annealing at 58°C for 30 s and extension at 72°C for 45 s. To stop the reaction within the linear range, previously determined, PCR amplification of β-actin was terminated after 22 cycles, while α-synuclein and parkin amplifications were stopped after 30 cycles. One microlitre of PCR reaction was analyzed by capillary electrophoresis on DNA chip with the BioAnalyser (Mueller *et al*. 2000). Primer sequences were: 5′-GATCATGTTTGAGACCTT-3′ for β-actin forward and 5′-ATCTTGATCTTCATGGTG-3′ for β-actin reverse; 5′-TGCTGTGGATATTGTTGTGG-3′ for α-synuclein forward and 5′-AGGTGCGTAGTCTCATGCTC-3′ for α-synuclein reverse (Rideout *et al*. 2001); 5′-ACACCCAACCTCAGACAAGG-3′ for parkin forward and 5′-ATCAGGGAGTTGGGACAGC- 3′ for parkin reverse (Nakahara *et al*. 2001). The length of amplified cDNA regions was 624 bp for β-actin, 214 bp for α-synuclein, and 252 bp for parkin.

Cell viability, and dopamine and GABA assays

Cell viability was assessed by measuring the level of cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan (Manthorpe *et al*. 1986). Cells were incubated for 3 h at 37°C with 0.4 mg/mL MTT, dissolved in 0.04 M HCl in 2-propanol, and analyzed spectrophotometrically at 540 nm with an automatic microplate reader. To assay the dopamine level, culture medium was removed and 200 μL of ice-cold Kreb's solution was added to each well. After 15 min, the Kreb's solution was removed by aspiration and discarded. $HClO_4$ (200 μL; 0.1 M) was added to each well and cells were homogenized by sonication.

The homogenate was centrifuged at 4500 × *g* at 4°C. Twenty microlitres of supernatant was injected directly into the high performance liquid chromatography apparatus equipped with a reverse phase analytical column (Supelcosil LC18-DB; Supelco, Bellefonte, PA, USA) and electrochemical detector (model 5200; ESA Bedford, MA, USA) and dopamine determined as previously described (Invernizzi *et al*. 1992). GABA was derivatized by mixing 30 μL of supernatant with 10 μL of *o*-phthaldialdehyde-sulfite reagent before separation by high performance liquid chromatography (Forloni *et al*. 2000).

Immunofluoroscence

Cells grown on poly D-Lysine-coated chamber slides (Nunc) were washed with PBS, fixed for 1 h at 4°C with 4% paraformaldehyde and 5% sucrose in PBS, and permeabilized for 1 min at room temperature with 0.125% Triton-X in PBS. After washing with PBS, cells were blocked with 2% FBS in PBS and then incubated with either mouse monoclonal anti-tyrosine hydroxylase (Chemicon) or rabbit polyclonal anti α-synuclein (Chemicon) antibodies diluted 1 : 50 and 1 : 100, respectively, in blocking solution for 1 h at room temperature. Cells were then incubated with Alexa 488-conjugated goat anti-mouse IgG or Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR, USA) diluted 1 : 300 in blocking solution. The microscope slides were mounted with 30% glycerol in PBS and viewed on Olympus FY500 laser confocal scanning system.

Results

Transfected PC12 cells expressing human parkin or SYN were exposed to MG132, N-acetyl-Leu-Leu-Norleu-al (ALLN), or lactacystin, three proteasome inhibitors with different mechanism of action, and parkin and SYN content was determined. The efficacy of the treatment was demonstrated by the time-dependent accumulation of high molecular mass, ubiquitinated proteins (Figs 1a and 2a), and by the fluorometric evaluation of proteasome activity (residual proteolytic activity after 24 h of treatment with MG132 was 6.9 ± 1.7% see Materials and methods). The western blot shown in Fig. 1 A demonstrates a time-dependent increase of parkin in PC12 cells treated with MG132. This effect was first noticeable after 4 h of exposure to the proteasome inhibitor, leading to an approximately eightfold increase after 24 h of treatment (Fig. 1b). We noticed the appearance of high molecular mass proteins that were reactive with the anti-parkin antibody starting after 4 h of treatment (Fig. 1a, lanes 3–5), suggesting that a portion of parkin molecules was ubiquitinated. In PC12 cells expressing human SYN the signal corresponding to the transgenic protein was less intense but became evident after 8 h of exposure to MG132, and increased approximately 30-fold after 24 h (Fig. 1a,b). Similar effects were obtained with ALLN (150 μM, data not shown), or lactacystin (Fig. 2a,c), although the effect of the latter was less dramatic. Our initial interpretation that accumulation of parkin and SYN was due to impairment of proteasomal degradation was subsequently

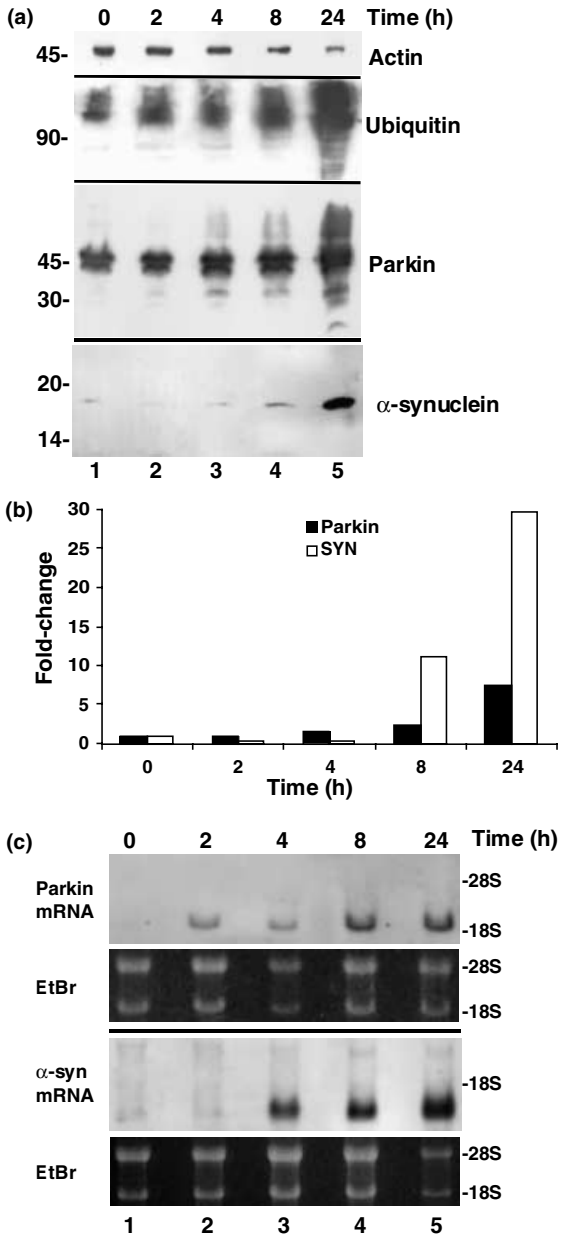

Fig. 1 Treatment of transfected PC12 cells with MG132 increases parkin and α-synuclein protein and mRNA. (a) PC12 cells that were stably transfected to express either human parkin or SYN were exposed to MG132 (10 μM) for the indicated times. Cells were then lysed, and actin, ubiquitin, parkin and SYN analyzed by western blotting. Actin and ubiquitin are shown for parkin-transfected cells, but similar results were obtained for SYN-transfected cells (not shown). Molecular mass markers are in kDa. (b) The amount of parkin and SYN was quantitated by densitometric analysis of the western blots shown in (a) and normalized on the level of actin. (c) Total RNA was extracted from PC12 cells treated with MG132 as in (a), and parkin and α-synuclein mRNA levels were analyzed by northern blotting (upper panels). The EtBr-stained gel in the lower panels demonstrates approximately equal loading of all lanes. Size markers are 18S and 28S rRNA. The experiment shown is representative of two similar ones.

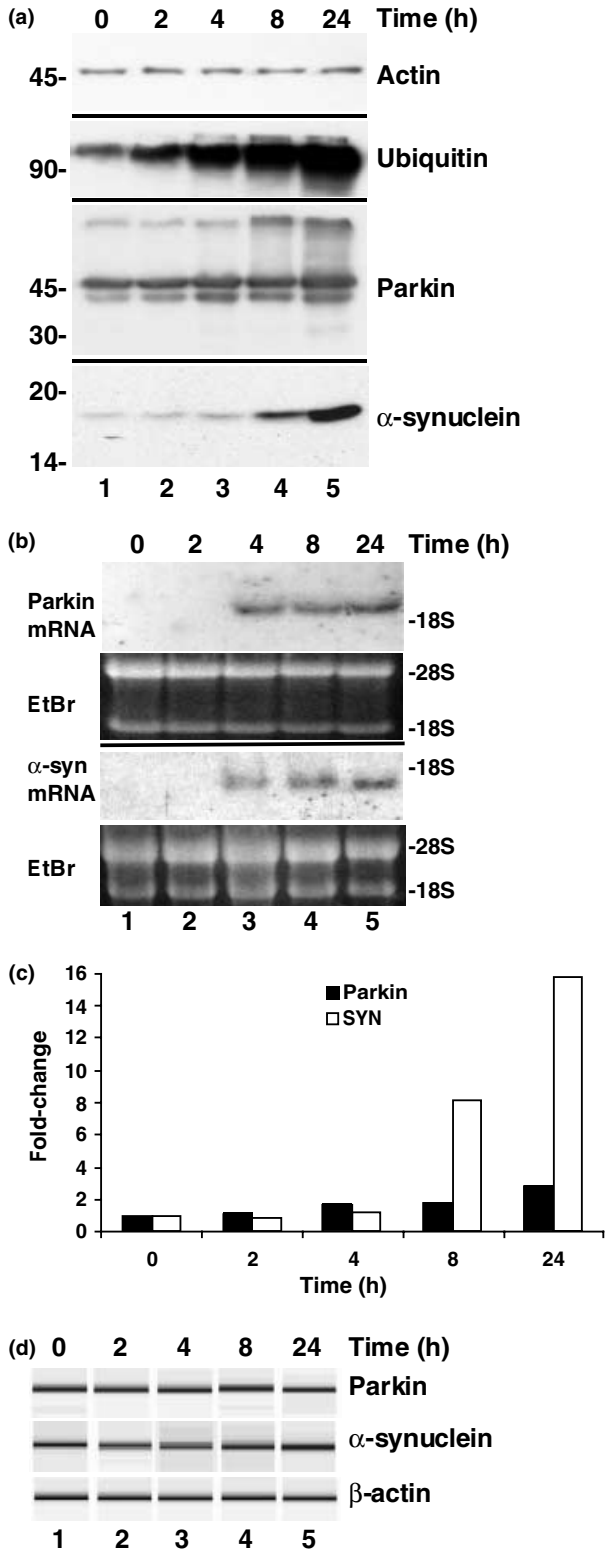

Fig. 2 Effect of lactacystin on parkin and α-synuclein protein and mRNA in transfected and untransfected PC12 cells. (a) PC12 cells that were stably transfected to express either human parkin or SYN were exposed to lactacystin (10 μM) for the indicated times. Cells were then lysed, and actin, ubiquitin, parkin and SYN analyzed by western blot. Actin and ubiquitin are shown for parkin-transfected cells, but similar results were obtained for SYN-transfected cells (not shown). Molecular mass markers are in kDa. (c) The amount of parkin and SYN was quantitated by densitometric analysis of the western blots shown in (a) and normalized on the level of actin. (b) Total RNA was extracted from PC12 cells treated with lactacystin as in (a), and parkin and α-synuclein mRNA levels were analyzed by Northern blotting (upper panels). The EtBr-stained gel in the lower panels demonstrates approximately equal loading of all lanes. Size markers are 18S and 28S rRNA. The experiment shown is representative of two similar ones. (d) Total RNA was extracted from untransfected PC12 cells treated with lactacystin (10 μM), and parkin and α-synuclein mRNA levels were determined by RT-PCR and Bioanalyser. β-Actin mRNA was used as an internal standard.

confuted by the data illustrated in Figs 1(c) and 2(b). In fact, northern blot analysis of RNA extracted from PC12 cells treated with either MG132 or lactacystin showed a dramatic increase of both parkin and SYN mRNAs. In cells treated with MG132, the increase in parking mRNA was first apparent at 2 h (Fig. 1c, top panel, lane 2), and the increase of SYN mRNA after 4 h of treatment (Fig. 1c, third panel, lane 3). Similar results were obtained when cells were exposed to the proteasome inhibitor lactacystin, with the difference that the increase in parkin mRNA was first detected after 4 h of treatment (Fig. 2b). Thus, the accumulation of parkin and SYN induced by proteasome inhibitors appeared to be the consequence of an increase in protein synthesis rather than impairment of protein degradation. To test this hypothesis, PC12 cells expressing human SYN were incubated with proteasome inhibitors either in the absence or presence of actinomycin D (Act D) or cycloheximide (CHX), which inhibit, respectively, mRNA synthesis and translation. As shown in Fig. 3, both Act D and CHX abolished the increase of SYN induced by MG132 (compare lanes 3 and 4 to lane 2).

To evaluate the consequences of proteasome inhibition on the metabolism of parkin and SYN in a more physiological contest, we examined the levels of the endogenous proteins in untransfected PC12 cells. As shown in Fig. 4(a,b), although treatment with MG 132 induces accumulation of high molecular weight, ubiquinated proteins, the amount of endogenous parkin in PC12 cells determined by quantitative western blot analysis was not significantly affected by MG132, suggesting that the proteasome inhibitor did not affect protein degradation or synthesis when parkin was expressed from its natural promoter. Similar results were obtained when cells were treated with ALLN or lactacystin (not shown). In contrast to the western blot analyses in Figs 1 and 2, only one band corresponding to parkin was evident; there was no obvious explanation for this discrepancy. However the presence of double bands recognized by parkin

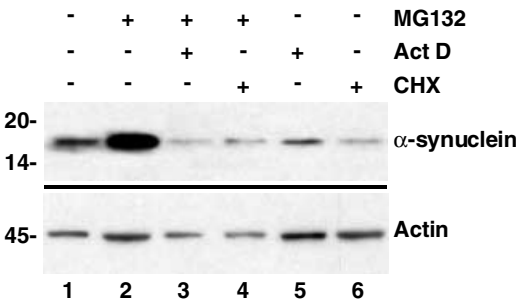

Fig. 3 Inhibitors of protein synthesis abolish the increases of α-synuclein induced by MG132 in transfected PC12 cells. PC12 cells stably transfected to express human SYN were exposed to the vehicle (lane 1), or to MG132 (10 μM) in the absence (lane 2) or presence of Act D (0.05 μg/mL, lane 3), or CHX (0.1 μg/mL, lane 4). After 24 h of treatment, cells were lysed, and analyzed by western blotting using anti-α-synuclein and anti-actin antibodies.

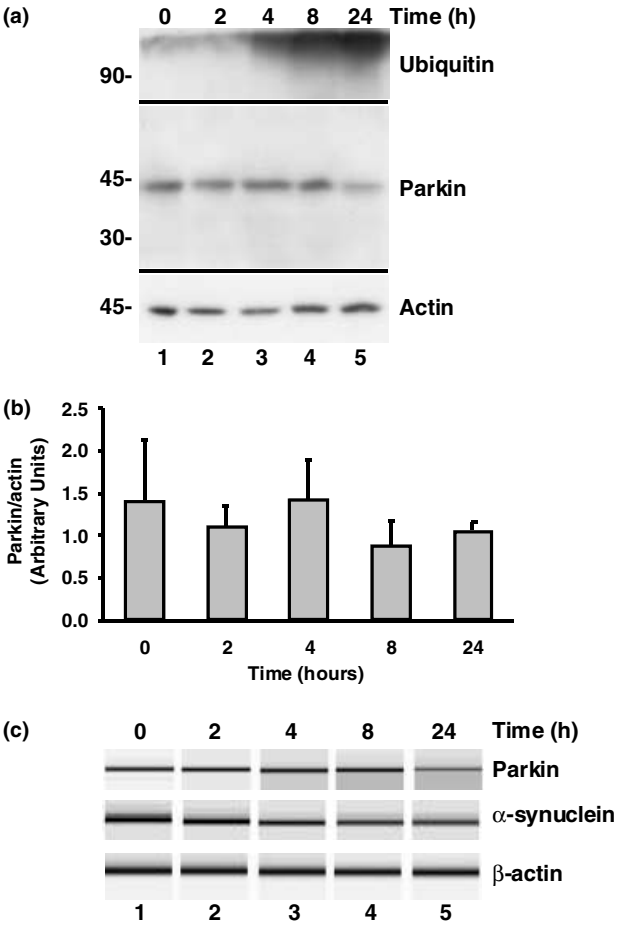

Fig. 4 Proteasome inhibitors do not cause accumulation of endogenous parkin or α-synuclein in PC12 cells. (a) PC12 cells were exposed to MG132 (10 μM) for the indicated times, lysed, and analyzed by western blotting using anti-ubiquitin, anti-parkin and anti-actin antibody. (b) The amount of parkin was quantitated by densitometric analysis of western blots like the one shown in (a) and normalized on the level of actin. Each bar represents the mean ± SEM of three replicates from three independent experiments. (c) Total RNA was extracted from PC12 cells treated with MG132 as in (a), and parkin and α-synuclein mRNA levels were determined by RT-PCR and Bioanalyser, and normalized to the level of β-actin mRNA.

antibody appeared restricted to the PC12-transfected cells, as in PC12 cells or in primary mesencephalic cells parkin was identified in a single band (Fig. 5). The evaluation of parkin expression by RT-PCR showed that the mRNA level was unchanged up to 8 h of treatment, and was slightly reduced at 24 h of treatment with MG132 (Fig. 4c), or remained unchanged up to 24 h of treatment with lactacystin (Fig. 2d). Endogenous SYN was undetectable by western blot either before or after treatment of PC12 cells with proteasome inhibitors (data not shown). SYN mRNA could be detected by RT-PCR, but its level of expression was not significantly changed by lactacystin or MG132 (Figs 2d and 4c, respectively).

The effect of UPS inhibition on the metabolism of SYN and parkin was also investigated in primary mesencephalic cultures, which include the nigro-striatal system (Pardo *et al*. 1997). As shown in Fig. 5(a), MG132 induced accumulation of high molecular mass, ubiquitinated proteins, demonstrating the efficacy of the treatment (top panel). However, proteasome inhibition for up to 24 h had no effect on the level of either parkin or SYN. Similar results were obtained with other proteasome inhibitors, including ALLN (100 μM), and lactacystin (2–10 μM) (data not shown). RT-PCR analysis failed to reveal changes in parkin and SYN mRNA levels in cells treated with MG132 (Fig. 5b). In conclusion, exposure of untransfected PC12 or primary mesencephalic cells to proteasome inhibitors did not induce accumulation of endogenous parkin and SYN.

Notably, in the same conditions we did not find evidence of intracellular accumulation of parkin or SYN by immunofluorescence confocal microscopy (data not shown and Fig. 6). The toxicity of proteasome inhibitors was evaluated in mesencephalic cells exposed to MG132 (Fig. 7a) and lactacystin (Fig. 7b), the general cell viability was determined by colorimetric method (MTT) while the content of dopamine was used to evaluate the effect on dopaminergic cells. At lower concentration (2 μM) both MG 132 or lactacystin significantly reduced the content of dopamine without affecting the MTT signal, at 5 μM the reduction of dopamine content was about 60% while the general viability was slightly decreased (30%). At 10 μM the toxicity of both drugs involved all mesencephalic neurones. The selective sensitivity of dopaminergic cells to a low dose of lactacystin was also demonstrated with the determination of GABA content, which is highly represented in the mesencephalic cells (Fig. 7b); GABA was significantly reduced only at 10 μM. Our data confirmed that proteasome inhibitors were

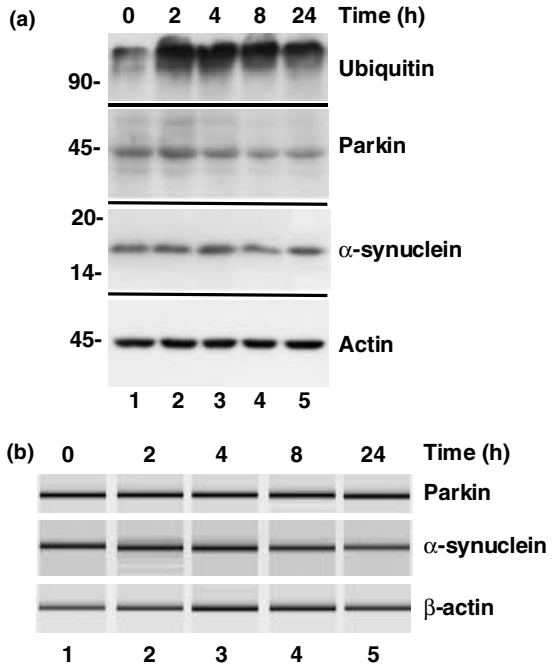

Fig. 5 Proteasome inhibitors do not cause accumulation of parkin and α-synuclein in primary mesencephalic cells. (a) Mesencephalic cells cultured from E14 rats were exposed to MG132 (5 μM) for the indicated times, lysed, and analyzed by western blotting using anti-ubiquitin, anti-parkin, anti-α-synuclein, and anti-actin antibodies. This experiment is representative of three similar ones. (b) Total RNA was extracted from mesencephalic cells treated with MG132 as in (a), and parkin and α-synuclein mRNA levels were determined by RT-PCR and Bioanalyser. β-Actin mRNA was used as an internal standard.

selectively toxic to dopaminergic neurones (McNaught *et al*. 2002b). This selective toxicity was not associated with the presence of inclusion bodies within the cells. As shown in Fig. 6, in the mesencephalic cultures exposed for 24 h to lactacystine or MG132, the few spared dopaminergic cells, identified by tyrosine hydroxylase immunoreactivity, did not exhibit SYN inclusions.

Discussion

The effect of proteasome inhibition on the metabolism of SYN and parkin has been evaluated in stably transfected and untransfected PC12 cells, as well as in primary mesencephalic neurones. In transfected cells, where expression of the exogenous proteins was under the control of the CMV promoter, pharmacological inhibition of UPS induced a dramatic accumulation of both proteins that was not confirmed in untransfected PC12 or primary mesencephalic cells. Although we cannot rule out the possibility that inhibition of the proteasome could account in part for the increase of parkin and SYN, our results strongly indicate that accumulation of these proteins after treatment of transfected cells with proteasome inhibitors was largely due to transcriptional activation of the CMV promoter. Accordingly, we found that inhibitors of protein synthesis at both the transcriptional and translational levels entirely abolished the increase of SYN and parkin induced by proteasome inhibitors. The ubiquitination of the parkin in transfected cells shown by Junn *et al*. (2002) was partially confirmed in our conditions. Thus, we cannot exclude that the increase of

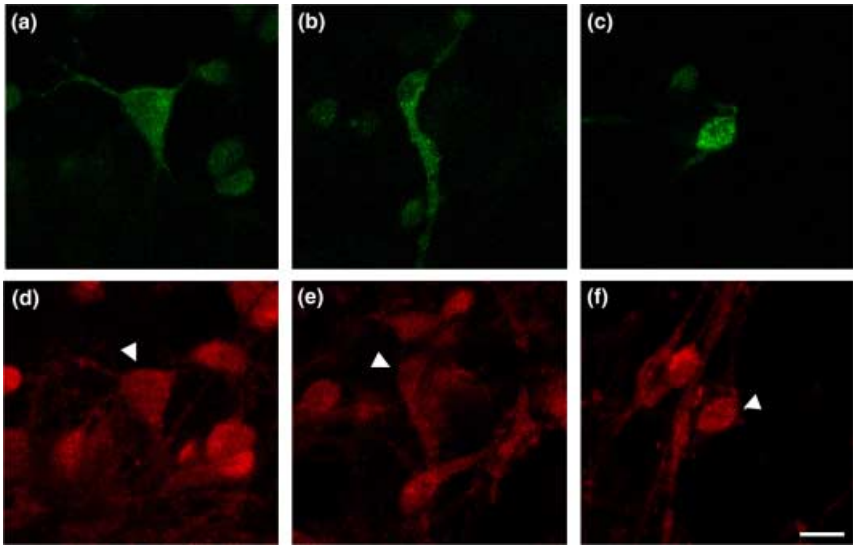

Fig. 6 Proteasome inhibitors do not induce aggregation of α-synuclein in mesencephalic cells. Mesencephalic cells from E14 rats were exposed to the vehicle (a,d), lactacystin 5 μM (b,e), or MG132 5 μM (c,f). After 24 h of treatment, cells were immunostained with anti-tyrosine hydroxylase monoclonal antibody to visualize dopaminergic neurones, and with anti-α-synuclein rabbit polyclonal antiserum, followed by Alexa 488 (green)-conjugated anti-mouse and Alexa 546 (red)-conjugated anti-rabbit secondary antibodies. Cells were viewed with green (a–c), or red (d–f) excitation/emission settings to detect dopaminergic neurones and α-synuclein, respectively. The scale bar, applicable to all panels, is 10 μm.

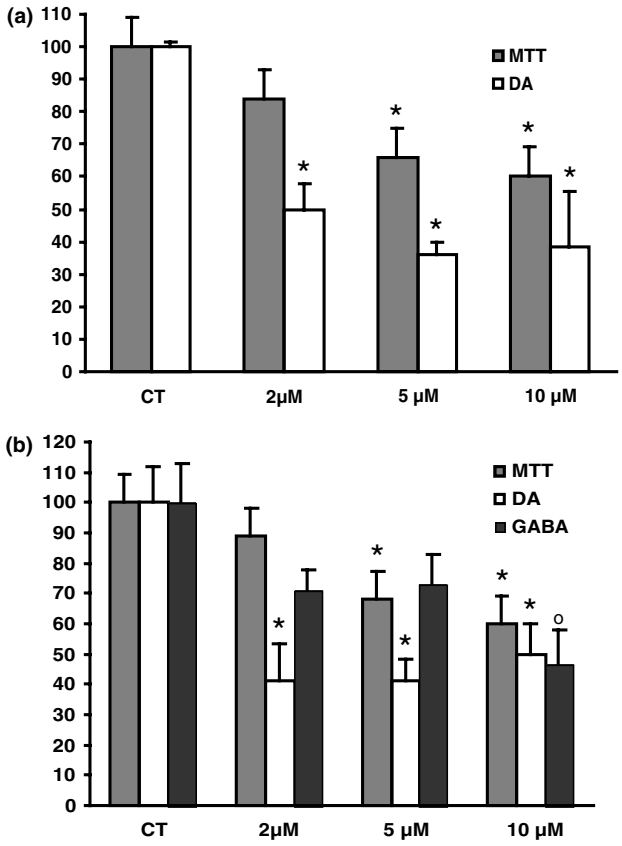

Fig. 7 Quantitative evaluation of the toxic effect of proteasome inhibitors on primary mesencephalic cells. Mesencephalic cells from E14 rats were exposed to either MG132 (a) or lactacystin (b) at the indicated concentrations. Cell survival (grey bars), and dopamine (white bars) and GABA (black bars) levels were quantitated after 24 h, and expressed as a percentage of values for cells treated with the vehicle. Each bar represents the mean ± SEM of 3–20 replicates from 3 to 4 independent experiments; °$p < 0.05$, *$p < 0.01$ vs. control group by Tukey–Kramer test.

parkin was partially due to reduction in degradation, however, the ubiquitination of the protein is not only a degradation signal but could be associated to other cellular functions (Di Fiore *et al*. 2003). Additionally, in untransfected and primary cells, where inhibition of the proteasome did not increase transcription of endogenous SYN and parkin, no accumulation of these proteins was observed. A similar effect of proteasome inhibitors was reported on the transcriptional regulation of the prion protein when this was expressed from the CMV promoter (Drisaldi *et al*. 2003), indicating that proteasome inhibitors can increase CMV-driven transcription independently of the cDNA insert.

Several hypotheses can be envisioned to explain the effect of proteasome inhibitors on the CMV promoter. Inhibition of the UPS might affect the turnover of transcription or translation factors, or activate specific pathways regulating transcription from the CMV promoter. The proteasome inhibitors used in our study activated transcription from the CMV promoter within 2–4 h, and led to a dramatic increase of mRNA and protein levels after 24 h. This potential artefact needs to be considered when the consequences of UPS impairment are evaluated in transfected cells using a CMV-derived expression system. Accumulation of misfolded proteins is suggested to be a common pathogenic mechanism of different neurodegenerative disorders (Forloni *et al*. 2002). The presence of cytoplasmic LB inclusions in spared dopaminergic neurones is the main histopathologic hallmark of PD, however, it remains to be determined whether LB-containing neurones are the ones that withstand neurodegeneration or those committed to die for the presence of the aggregates. As missense mutations in the gene encoding SYN were associated with rare familial forms of early onset PD (Polymeropoulos *et al*. 1997; Krüger *et al*. 1998) and the protein was identified as the major component of LB (Spillantini *et al*. 1997), numerous studies were performed to address the possible role of SYN aggregates in PD pathogenesis (Lotharius and Brundin 2002). These studies have shown that SYN can have neurotoxic as well as neuroprotective activities (da Costa *et al*. 2000; Forloni *et al*. 2000). The notion that protein aggregation is causally linked to PD neurodegeneration is also suggested by the other disease-linked mutations that relate to two proteins directly involved in UPS, parkin and UCHL1. Parkin has been recently identified as an E3 ubiquitin-ligating enzyme (Shimura *et al*. 2000) and it has also been found in LB (Schlossmacher *et al*. 2002). However, in light of our results, many of the findings obtained in transfected cells expressing parkin or SYN under the CMV promoter need to be reconsidered (Junn *et al*. 2002). In these cases, the direct relationship between impairment of proteasome degradation and accumulation of the pathogenic proteins might not be so obvious. In fact, we find that inhibition of the proteasome does not induce accumulation of SYN or parkin in untransfected cells, indicating that the UPS might be not involved in the degradation of either protein under physiological conditions. Similar conclusions were drawn by Ancolio *et al*. (2000) by exposing neuronal cell line to UPS inhibitors. Accordingly, we failed to detect intracellular inclusions of either parkin or SYN in untransfected PC12 or mesencephalic cells exposed to proteasome inhibitors. Nonetheless, we found that treatment with proteasome inhibitors induced selective death of dopaminergic neurones, supporting the contention that this neurotoxic effect is not directly associated with accumulation of SYN or parkin, but is more likely to be the consequence of other events induced by UPS inhibition. The effect on viability induced by UPS is associated with an increase of carbonyls and other parameters of oxidative damage (Lee *et al*. 2001). It has been recently shown that proteasomal dysfunction might mediate the neurotoxicity of 4-hydroxy-2,3-*trans*-noenal (HNE), an end-product of lipid peroxidation (Hyun *et al*. 2002). These

results are in agreement with the selective depletion of the nigro-striatal system in rats treated with proteasome inhibitors, apparently in this case the dopaminergic damage was associated with SYN aggregation (McNaught *et al*. 2002a). Taken together, these findings suggest that the cardinal event of PD pathogenesis is the impairment of UPS that could be associated with SYN accumulation. In SYN-mutated familial PD the aggregation of SYN might induce failure of UPS and consequent death of dopaminergic cell. On the contrary, in familial PD associated with recessive missense mutations of parkin, the mutation might be sufficient to impair the UPS and initiate the neurodegenerative process also in the absence of SYN aggregation (Hayashi *et al*. 2000). Apparently our results support this view, and, in any case, they indicate that the influence of proteasome inhibitors on the metabolism of SYN and parkin in transfected cells need to be carefully analyzed.

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