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Proteasomal inhibition reduces *parkin* mRNA in PC12 and SH-SY5Y cells

Andreas Koch [a,1], Klaus Lehmann-Horn [a,1,2], Justus C. Dächsel [a,1,3], Thomas Gasser [b], Philipp J. Kahle [b], Christoph B. Lücking [a,*]

[a] *Department of Neurology, Klinikum Grosshadern, Ludwig Maximilians University, Marchioninistr. 15, 81377 Munich, Germany*
[b] *Hertie Institute for Clinical Brain Research, Department for Neurodegenerative Diseases, University Clinics Tübingen, Otfried-Müller-Strasse 27, 72076 Tübingen, Germany*

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Abstract

Mutations in the gene encoding the E3 ubiquitin-protein ligase parkin have been shown to be a common genetic cause of familial early-onset Parkinson's disease (PD). In addition to its function in the ubiquitin–proteasome system (UPS), parkin has been ascribed general neuroprotective properties. Stress and mutation induced decreases in parkin solubility leading to compromised cytoprotection have recently been reported. We systematically investigated whether PD-related stresses including MG132 and epoxomicin (proteasomal impairment), tunicamycin (unfolded protein stress), and rotenone (mitochondrial dysfunction) resulted in expressional changes of *parkin* and other E3 ubiquitin ligases (*dorfin*, *SIAH-1*). Rotenone and tunicamycin did not change *parkin* mRNA levels, whereas proteasomal inhibition resulted in a reduction of *parkin* mRNA in PC12 cells as well as in SH-SY5Y cells. Therefore, surprisingly, cells did not react with a compensatory *parkin* upregulation under proteasomal inhibition, although, in parallel, parkin protein shifted to the insoluble fraction, reducing soluble parkin levels in the cytosol. Since the mRNA of the parkin-coregulated gene *PACRG* paralleled the *parkin* mRNA at least partly, we suspect a promoter-driven mechanism. Our study, therefore, shows a link between proteasomal impairment and *parkin* expression levels in cell culture, which is intriguing in the context of the described and debated proteasomal dysfunction in the substantia nigra of PD patients.

Keywords: Parkinson's disease; Ubiquitin–proteasome system (UPS); Proteasomal dysfunction; Parkin; Siah-1; Dorfin

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. Although the majority of cases are considered sporadic, mutations in several genes have been shown to be the disease cause in rare familial forms of PD [1]. Based on the cellular function of the proteins encoded by these genes various mechanisms leading to the dopaminergic degeneration observed in PD are currently discussed (for review see e.g. [2]). Mutations in the gene coding for parkin are associated with autosomal-recessive, early onset PD. As an E3 ubiquitin-protein ligase, parkin is responsible for the ligation of ubiquitin to specific substrate proteins. This leads, among other effects, to targeting of the substrates to the proteasome [3]. An important role for the ubiquitin–proteasome system (UPS) in PD has been supported by the finding of impaired proteasomal activity in the substantia nigra of PD brains [4]. In addition, parkin was shown to possess neuroprotective properties against various neurotoxic insults [5–8]. This might be due to parkin-mediated ubiquitinylation of key components of cellular viability regulating signalling cascades, such as the Jun kinase and NF-κB pathways [9–11].

* Corresponding author. Tel.: +49 89 70950; fax: +49 89 7095 3677.
E-mail address: cluecking@nefo.med.uni-muenchen.de (C.B. Lücking).
[1] These authors equally contributed to this work.
[2] Current address: Department of Neurology, Klinikum rechts der Isar, Technical University Munich, Ismaninger Str. 22, 81675 Munich, Germany.
[3] Current address: Mayo Clinic, Department of Neuroscience, 4500 San Pablo Road, Jacksonville, FL 32224, USA.

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To further elucidate these protective properties we were interested in the regulation of *parkin* under PD-related stress conditions, namely proteasome impairment, unfolded protein stress, and mitochondrial failure. Two other E3 ubiquitin-ligases, dorfin and Siah-1, were included as controls. These enzymes, like parkin, have been shown to ubiquitylate Synphilin-1 [12–14], an interactor of α-Synuclein [15], and were found in Lewy bodies, the pathological hallmark of PD [13, 14].

2. Materials and methods

Non-differentiated PC12 cells (tet-off, Clontech, Karlsruhe, Germany) were cultured as previously described [16]. SH-SY5Y cells (ATCC, Manassas, USA) were cultured according to the manufacturer's instructions. Cells were seeded out at an approximate density of 1.5×10^4 cells/cm^2 (PC12) or 0.5×10^4 cells/cm^2 (SH-SY5Y). Twenty-four hours later, fresh medium containing the stressors was added. Cells were harvested, centrifuged, and pellets immediately frozen at −80 °C at the time points specified below. All experiments were performed at least in triplicates. In a first set of experiments, mRNA expression was analyzed after 3, 6, 9, and 18 h in PC12 cells stressed with 125 μM MG132, 4.7 μM tunicamycin or 50 μM rotenone. These concentrations were established to obtain cell death rates of 20–30% after 24 h, which allowed (a) comparable toxic conditions and (b) satisfactory mRNA amounts and quality. In a second set of experiments, MG132 was used in lower concentrations (60 and 30 μM) as well as in the commonly used concentrations of 10 μM. In addition, another – more specific – proteasome inhibitor, epoxomicin, was introduced, concentrated at 0.5 μM, which is within the range used in the literature (0.1–1.0 μM). Controls received the amount of DMSO which was used to dissolve the respective toxin. For cell death quantification, either FACS analysis (FACSCalibur™-Cytometer, Becton Dickinson Biosciences, Franklin Lakes, USA) after propidium iodide staining (0.5 μg/μl, for MG132 treatment) or analysis of chromatin condensation/nuclear fragmentation after Hoechst staining (0.5 μg/μl, for tunicamycin and rotenone treatment) was performed.

mRNA was isolated with RNeasy MiniKit including an on-column DNA digest (both Qiagen, Hilden, Germany) out of approximately 3 million PC12 cells and 1 million SH-SY5Y cells. For reverse transcription, cDNA synthesis kits AMV (Roche Diagnostic, Mannheim, Germany) and QuantiTect (Quiagen, Hilden, Germany) were used with random primers following the manufacturer's instructions. Quantitative RT-PCR was performed with the Light Cycler Instrument 2.0 using Light Cycler FastStart DNA Master SYBR Green I for hot start application (both Roche Diagnostic, Mannheim, Germany), as described by the manufacturer. The genes analyzed and the primers used are given in Table 1. All primers extended over exon boundaries to exclude amplification of genomic DNA. PCR products showed a single peak in the Light Cycler melting curve. To quantify mRNA expression, we first calculated for each sample the ratio between the gene of interest and a housekeeping gene, HPRT (hypoxanthine phosphoribosyltransferase) in PC12 or GAP-DH (glyceraldehyde-3-phosphate dehydrogenase) in SH-SY5Y. We could therefore control for slight differences in RT-PCR efficiency or template amount. Then, the mean and the 95% confidence interval of these relative expression values were calculated for the three independent reactions. Only expression changes of more than 2-fold between stressed vs. control cells were considered as relevant. Unless otherwise indicated, HPRT and GAP-DH values did not differ for more than 0.5 cycles in treated vs. untreated samples.

For Western Blot analysis, three pellets of 3×10^6 PC12 cells and 1×10^6 SH-SY5Y cells each were pooled, lysed (RIPA buffer: Triton-X-100 1%, sodium deoxycholate 1%, SDS 1%, NaCl 0.15 M, Tris–HCl 50 mM pH 7.2) homogenized and centrifuged. Equal amounts of protein (50 μg) were subjected to denaturing 10% polyacrylamide gel electrophoresis. For analysis of the insoluble fraction, RIPA-buffer containing 8 M urea was added to the pellets. Anti-parkin antibodies (Cell Signalling, Beverly, USA) diluted 1:1000, anti-actin (Sigma, Deisenhofen, Germany) diluted 1:2000 (PC12) or 1:4000 (SH-SY5Y) and secondary antibodies (anti-rabbit and anti-mouse, Dako, Hamburg, Germany) diluted 1:2000 and 1:8000, respectively, were used. For quantification of the Western blot bands, the blots were scanned with a flat bed scanner. Using the software TINA 2.08e by Raytest, the optical density was calculated after standardized manual definition of each band. Then the background was subtracted and the result divided by the surface. The resulting values were used to calculate the ratio between each parkin band and its corresponding actin band, yielding a normalized relative parkin value. We also tried to quantify the PACRG protein by using the only existing commercially available PACRG-antibody (Rockland Immunocytochemicals, Gilbertsville, PA, USA, Code 600-401-474) in dilutions down to 1:500.

To determine the −258T/G promoter polymorphism in SH-SY5Y cells, the region of interest was amplified by PCR (primers: forward 5́-GCATTTGTTT AAGCTCAGGGTCTC-3́ and reverse 5́-CCTGCTGGGAGTCGTAGTTCTAA C-3́). The resulting 441 bp PCR product was analyzed by restriction enzyme analysis with AlwNI (New England Biolabs) and by direct sequencing on an Abi Prism 310 Gene Analyzer (Applied Biosystems).

3. Results

In the first set of experiments, under treatment with tunicamycin or rotenone, *parkin* and *dorfin* mRNA levels did not change more than 2-fold in PC12 cells at the investigated time points (3, 6, 9, 18 h, data not shown). However, under proteasomal inhibition with MG132 (125 μM), we observed a distinct, time-dependent and strong reduction of *parkin* mRNA in PC12 cells, whereas *dorfin* mRNA levels were not regulated. A similar reduction was seen under 60, 30 and 10 μM MG132 (data not shown). Prompted by these observations, we used a more specific proteasome inhibitor (epoxomicin, 0.5 μM) and observed again a *parkin* mRNA reduction (Fig. 1a). We than analyzed later time points (14, 24, 38 and 48 h) which still showed a strong reduction of *parkin* mRNA (Fig. 1b). Finally, we analyzed a second neuronal cell model

Table 1
Genes analyzed and primers used for RT-PCR

Gene	Forward primer	Reverse primer
Rat HPRT	GAC TTT GCT TTC CTT GGT CA	TTC AAA TCC CTG AAG TGC TC
Human GAP-DH	CAG GGC TGC TTT TAA CTC TG	GAG TCC TTC CAC GAT ACC AA
Human parkin	CGT GGA GAA AAG GTC AAG AA	GGG CCT TTG CAA TAC ACA TA
Rat parkin	ACC ACA GAG GAA AAG TCA CG	GGC CTT TGC AGT AGA CAA AA
Human PACRG	GAT GGG CTT TGT GAA ATG AC	TCT CAG CTG ACA CAA CCA GA
Rat PACRG	TGC CTG GAA GGT AGA GAT TG	CTT TGA GCG TGA CAC AGA TG
Human dorfin	GCA GCA GCT GAT GAT ATA AAG	ACA CAG GAA TGC CAA TAA TC
Rat dorfin	TGG GAC TAA CAC AGC CAT AGA	ATT CAG AAT GGA CCC TGC CAT
Human Siah-1	TAT GTG TTA CCG CCC ATT CT	TCA CAT CCA GAA GAC GCA TA
Rat Siah-1	TTG GCG AGT CTT TTC GAG TGT	AAG GCC TGA ACT CAC AGA GCT