Neurochemistry International 58 (2011) 512–520

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

The accumulation of neurotoxic proteins, induced by proteasome inhibition, is reverted by trehalose, an enhancer of autophagy, in human neuroblastoma cells

M.J. Casarejos [a], R.M. Solano [a], A. Gómez [a], J. Perucho [a], J.G. de Yébenes [b], M.A. Mena [a,*]

[a] Departamento de Neurobiología-Investigación. CIBERned, Hospital Ramón y Cajal, Carretera de Colmenar, km 9, Madrid 28034, Spain
[b] Departamento de Neurología, CIBERned, Hospital Ramón y Cajal, Madrid 28034, Spain

ARTICLE INFO

Article history:
Received 6 October 2010
Received in revised form 28 December 2010
Accepted 4 January 2011
Available online 11 January 2011

Keywords:
α-Synuclein
Tau
Autophagy-lysosomal pathway
Chaperones
DJ-1
Glutathione
p-Tau
Proteasome activity
Parkinson's disease
Ubiquitination

ABSTRACT

Neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, Huntington's disease and others are due to accumulation of abnormal proteins which fold improperly and impair neuronal function. Accumulation of these proteins could be achieved by several mechanisms including mutation, overproduction or impairment of its degradation. Inhibition of the normal protein degradation is produced by blockade of the ubiquitin proteasome system. We have shown that epoxomicin, a proteasome inhibitor, increases the levels of proteins involved in neurodegenerative disorders such as α-synuclein and hyper phosphorylated tau in NB69 human neuroblastoma cells and that such increase correlates with an enhanced rate of cell death. We then investigated whether the stimulation of autophagy, an alternative mechanism for elimination of abnormal proteins, by treatment with trehalose, counteracts the effects of proteasomal blockade. Trehalose, a disaccharide present in many non-mammalian species, known to enhance autophagy, protects cells against various environmental stresses. Treatment with trehalose produced a dose and time-dependent increase in the number of autophagosomes and markers of autophagy in NB69 cells. Trehalose did not change the number of total neither the number of dividing cells in the culture but it completely prevented the necrosis of NB69 induced by epoxomicin. In addition, the treatment with trehalose reverted the accumulation, induced by epoxomicin, of polyubiquitinated proteins, total and phosphorylated tau, p-GSK-3, and α-synuclein, as well as the α-synuclein intracellular aggregates. The effects of trehalose were not mediated through activation of free radical scavenging compounds, like GSH, or mitochondrial proteins, like DJ1, but trehalose reduced the activation of ERK and chaperone HSP-70 induced by epoxomicin. Inhibition of ERK phosphorylation prevented the epoxomicin-induced cell death. Inhibition of autophagy reverted the neuroprotective effects of trehalose in epoxomicin-induced cell death. These results suggest that trehalose is a powerful modifier of abnormal protein accumulation in neurodegenerative diseases.

1. Introduction

Neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) are characterized by abnormal accumulation of different proteins in neurons and glia (Dohm et al., 2008; Enokido et al., 2010; McMillan and Leverenz, 2010; Nakamura and Lipton, 2010). Amyloid β-peptide and tau accumulate in AD and other dementias, α-synuclein in PD and huntingtin in HD. The mechanisms of accumulation of these abnormal proteins are unknown in most cases, though mutation, overproduction, abnormal processing and abnormal elimination have been proposed.

The processing of abnormal proteins is performed through different complementary systems including the ubiquitin-proteasome system, the chaperone-mediated autophagy and the macroautophagy. Impairment of abnormal protein processing by interfering with proteasomal function produces accumulation of toxic proteins which may interfere with synaptic plasticity and neuronal function. The ubiquitin proteasome system (UPS) is one of the most important cellular mechanisms, together with the autophagy-lysosomal pathway (ALP), for elimination of proteins (Korolchuk et al., 2009; Matsuda and Tanaka, 2010; Rubinsztein, 2007; Yue et al., 2009). The role of the proteasome and autophagy in the clearance of abnormal proteins in neurodegenerative diseases has been demonstrated (Alvarez-Erviti et al., 2010; Bove

Abbreviations: AD, Alzheimer's disease; ALP, autophagy-lysosomal pathway; AMC, 7-amino-4-methylcoumarin; BrdU, bromodeoxyuridine; ERK, extracellular-signal-regulated kinase; GSK-3, glycogen synthase kinase 3; GSH, glutathione; HSP, heat shock proteins; HD, Huntington's disease; LC3, microtuble-associated protein light chain 3; PK-KO, parkin null mice; PD, Parkinson's disease; PGPH, peptidylglutamyl-peptidase-like; UPS, ubiquitin proteasome system.

* Corresponding author. Tel.: +34 91 3368384; fax: +34 91 3369016.
E-mail address: maria.a.mena@hrc.es (M.A. Mena).

0197-0186/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2011.01.008

et al., 2006; Dehay et al., 2010; Maher, 2008; Malkus et al., 2009; Matsuda and Tanaka, 2010; McNaught and Olanow, 2006; Yamamoto et al., 2007). It is of interest to know whether the abnormal proteasomal function could be compensated by enhancing other mechanisms of elimination of protein function such as autophagy.

Epoxomicin is a cell-permeable, natural product that selectively and irreversibly inhibits proteasome activity. Epoxomicin modifies four catalytic sub-units of the 20S proteasome, resulting primarily in inhibition of the chymotrypsin-like activity (Meng et al., 1999). Trehalose is the natural sugar of invertebrates, fungi and many plants. It protects the integrity of the cells against several stress insults (Chen and Haddad, 2004; Jain and Roy, 2009). It is not clear how trehalose mediates many of its protective effects. A chaperone function and an enhancement of protein-folding through direct protein-trehalose interactions have been described (Welch and Brown, 1996; Jain and Roy, 2009). Trehalose inhibits amyloid formation in vitro and prevents aggregation of beta-amyloid in models of Alzheimer's disease (AD) (Arora et al., 2004; Liu et al., 2005; Rodriguez-Navarro et al., 2010). Recently, trehalose was shown to inhibit polyglutamine-mediated aggregation in vitro and in vivo models of Huntington disease (HD) (Sarkar et al., 2007; Tanaka et al., 2004). Furthermore, trehalose accelerates the clearance of mutant huntingtin and α-synuclein and inhibits protein misfolding (Sarkar et al., 2007; Singer and Lindquist, 1998).

There are several mechanisms of compensation for abnormal proteasomal function. We have shown that parkin deficiency increases the resistance of midbrain neurons and glia to mild proteasome inhibition (Casarejos et al., 2009). That resistance was due to increased GSH levels and DJ1 protein levels in parkin null (PK-KO) mice. In addition, the autophagy inhibition or the GSH inhibition blocked the differential response to epoxomicin from WT and PK-KO midbrain neuronal cultures (Casarejos et al., 2009).

NB69 human neuroblastoma cells have been used as an interesting model for the study of neurotoxic and neuroprotective drugs effects on catecholamine neurons (Mena et al., 1992, 1995a, b; Rodriguez-Martin et al., 2001). In this system, we studied the effects of epoxomicin on the accumulation of p-tau and α-synuclein, as well as the compensatory role of autophagy, free radical scavengers, chaperones and mitochondrial proteins such as DJ-1. Furthermore, we have studied if trehalose is able to revert the pathological accumulation and aggregation of p-tau and α-synuclein induced by epoxomicin.

In this study, we report that trehalose induces autophagy and protects from the epoxomicin-induced accumulation of polyubiquitinated proteins, α-synuclein, tau, p-tau, p-GSK-3 proteins and α-synuclein intracellular aggregates in human neuroblastoma cells.

2. Materials and methods

2.1. Cell cultures

Human cells NB69 were grown and maintained in medium containing Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l), 4 mM L-glutamine, 1 mM sodium pyruvate, penicillin/streptomycin/fungizone (100 U/ml) and 15% fetal bovine serum (USA origin) which were purchased from GIBCO-Life

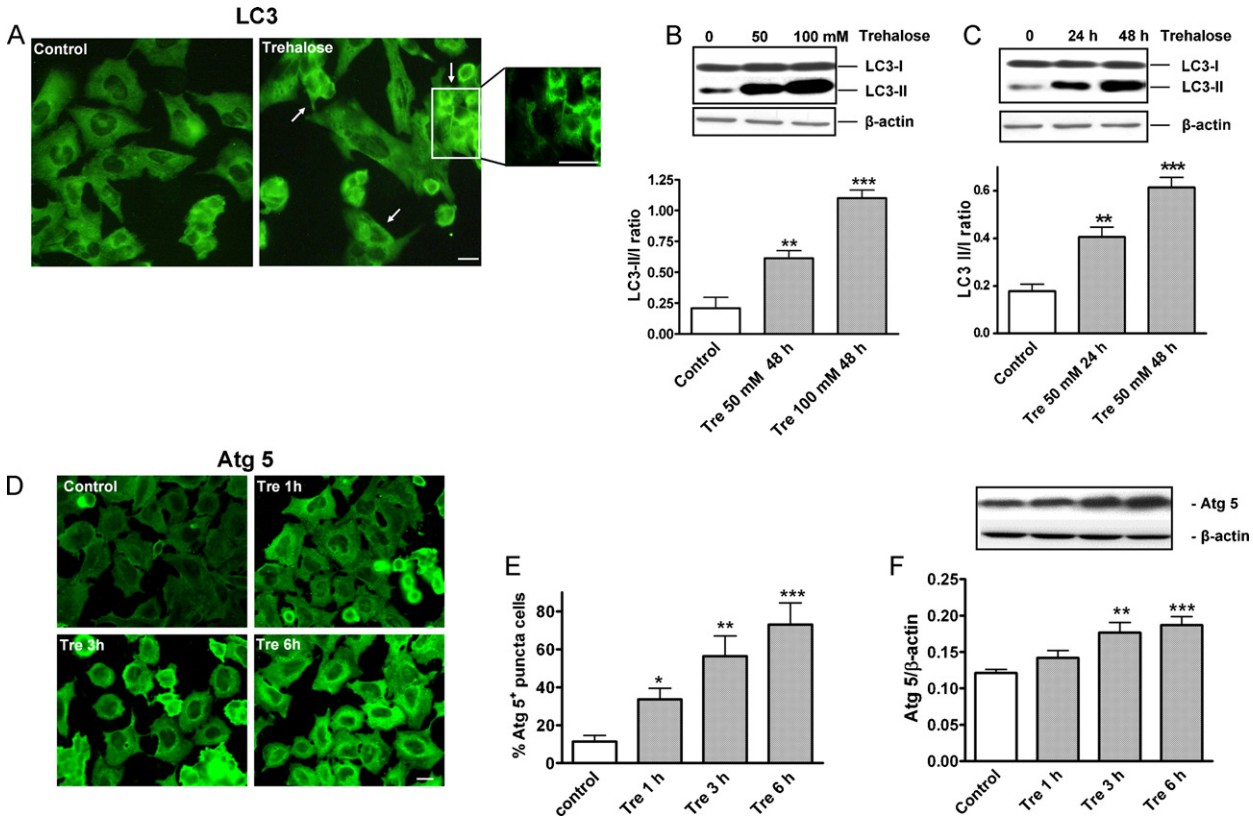

Fig. 1. Dose and time-dependent effects of trehalose on LC3 expression in human neuroblastoma NB69 cells. (A) After 6 days in vitro (DIV) the cells were treated with trehalose (50 mM × 24 h). After treatment, the cells were fixed and detected immunofluorescently with rabbit anti-LC3 antibody (scale bar = 20 μm). A portion of this micrograph has been magnified in the picture on the right. The arrows indicate the presence of LC3 in presumptive autophagosomes. (B) A dose-dependent and (C) time-dependent induction of autophagy by trehalose was determined by measuring the LC3 protein levels using immunoblotting assay with antibody against LC3. The ratio of LC3-II/I was evaluated by densitometric analysis. (D) Atg 5 immunostaining 1, 3 and 6 h after treatment with trehalose, 50 mM (scale bar = 20 μm). (E) Percentage of the dot positive cells (more than 20 dots) and (F) quantification of Atg5 protein in trehalose-treated cells. β-Actin was used as an equal loading of proteins. Values are the mean ± SEM of two independent cultures with four replicates each. Statistical analysis was performed by one-way ANOVA followed by Newman Keuls multiple comparison test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ epoxomicin-treated cultures *vs* controls.

Technologies (Paisley, Scotland, UK). For detection of ubiquitinated proteins, the medium was replaced by a defined medium DMEM/Ham's F12 1:1, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite and 5 μg/ml insulin which were obtained from Sigma (St. Louis, MO) and 100 μM transferrin was supplied by Boehringuer (Mannheim, Germany).

2.2. Chemicals

Epoxomicin, Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC were purchased from Calbiochem (Darmstadt, Germany) and Ac-Arg-Leu-Arg-AMC was purchased from Biomol International LP (Plymouth, PA, USA). 5,5-Dithio-bis-2-nitrobenzoic acid (DTNB), 3-methyladenine (3MA) and reduced (GSH) and oxidized (GSSG) forms of glutathione were from Sigma (Madrid, Spain). U0126 was from Calbiochem (Merck, Darmstadt, Germany). NADPH, the lactate dehydrogenase (LDH) activity kit and GSH reductase (GR) were from Boehringer-Mannheim (Barcelona, Spain). The BCA protein assay kit was from Pierce (Rockford, Ill, USA). All other reagents were of the highest purity commercially available from Merck or Sigma.

2.3. Antibodies

The following antibodies were used: anti-α-synuclein antibody made in mouse was from BD Transduction Laboratories (Franklin Lakes, NJ, USA) and rabbit polyclonal anti-LC3 antibody from MBL Laboratories (Nagoya, Japan). Rabbit polyclonal anti-Atg5 was from Abcam (Cambridge, UK). Anti-mouse IgG fluorescein from Jackson (PA, USA) and anti-rabbit IgG Alexa Fluor from Molecular Probes (Eugene, OR, USA). Mouse monoclonal anti-HSP-70 and goat polyclonal (SQSTM1) anti-p62 were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-DJ1 from Dr. Castaño (Madrid, Spain). Rabbit polyclonal anti p-tau (phospho S199 + S202) from Abcam (Cambridge, UK); mouse monoclonal anti-tau and mouse monoclonal anti-ubiquitin antibodies were from Chemicon (Temecula, CA, USA). Anti-BrdU mouse monoclonal antibody was from Dako (Glostrup, Denmark). Mouse anti-phospho-ERK-1/2 and rabbit anti-ERK-1/2 antibodies from Sigma (Madrid, Spain). Rabbit anti-GSK-3α [pY^{279}]/β [pY^{216}] and mouse anti-GSK3β were from Invitrogen (Carlsbad, CA, USA). Anti-mouse and anti-rabbit-horseradish peroxidase secondary antibodies were from Amersham and rabbit anti-goat-HRP was from Santa Cruz. β-Actin secondary antibody was an anti-mouse phosphatase alkaline conjugated (from Sigma).

2.4. Experimental cell treatments

NB69 cells were grown in 80 cm^2 plastic culture flasks and plated at 1 × 10^5 cells/ml in 15 ml culture medium. The cells were sub-cultured every 7 days and were maintained in a humidified 5% CO_2 atmosphere at 37 °C (Mena et al., 1992). Experiments were performed in 6 or 12-well culture plates for western blot and proteasomal activity, respectively. For the immunocytochemistry detection, the cells were cultured in 13 mm-diameter glass coverslips with a density of 15,000 cells. After 6 DIV (days in vitro) the cultures were pre-treated with trehalose (50 or 100 mM) 15 min before the treatment with epoxomicin (10 nM) for 24 and 48 h.

To test the role of ERK and autophagy in the epoxomicin and trehalose effects, we used the ERK phosphorylation inhibitor, UO126 (10 μM), 30 min before the epoxomicin treatment and the autophagy inhibitor, 3MA (2 and 4 mM), 30 min before the trehalose treatment, respectively.

2.5. Cell survival and proliferation assay

Necrotic cell death was measured according to LDH activity in the culture medium and by trypan blue dye exclusion in cells. LDH activity was measured by using a cytotoxicity detection kit (Casarejos et al., 2005; Solano et al., 2008).

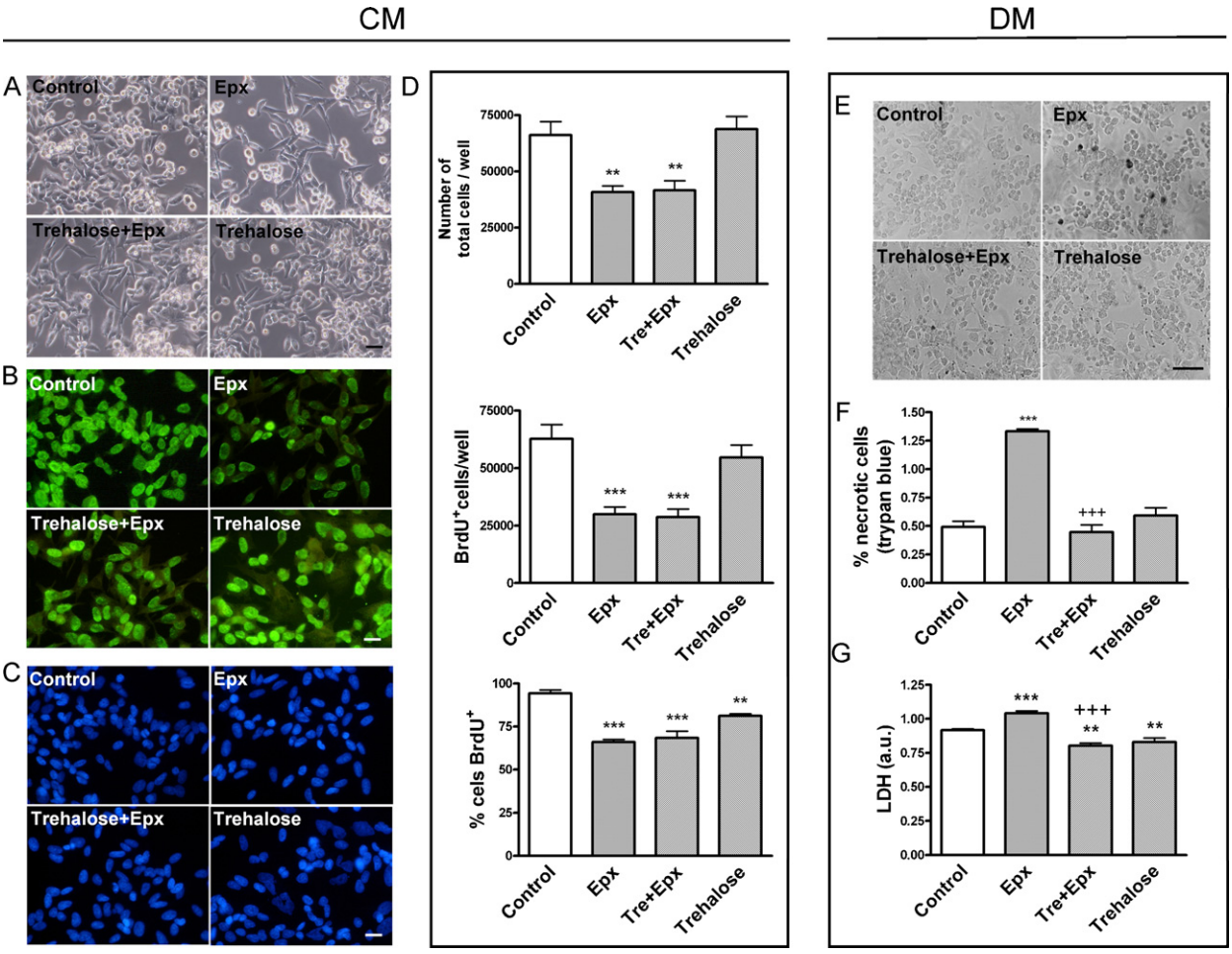

Fig. 2. Effects of epoxomicin (10 nM) and trehalose (50 mM) on cell viability and differentiation in NB69 cells. (A) Phase contrast photomicrographs of NB69 cultures pre-treated with trehalose 15 min before epoxomicin for 24 h. The cells were cultured in completed medium (CM) with serum. (B) Photomicrographs of BrdU$^+$ cells of dividing cells. (C) Photomicrographs of total nuclei stained with bis-benzimide. (Scale bar = 20 μm). (D) Number of total cells and the percentage of BrdU$^+$ cells with respect to the total number. (E) Photomicrographs showing trypan blue dye exclusion in cells treated in defined medium (DM) without serum (scale bar = 20 μm), (F) percentage of necrotic cells and (G) LDH activity in the four experimental groups treated in DM. Values are the mean ± SEM from two independent experiments with 6 replicates each. Statistical analysis was performed by one-way ANOVA followed by Newman Keuls multiple comparison test. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* controls; $^{+++}p < 0.001$ trehalose + epoxomicin *vs* epoxomicin-treated cultures. There is interaction between epoxomicin and trehalose effects on: trypan blue exclusion $F(1,19) = 66.15$ with a p value < 0.0001 and LDH activity $F(1,19) = 13.9$ with a p value < 0.0014. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

To assay cell number and the index of cell proliferation, cell cultures were incubated with 50 μM BrdU 24 h before fixation and, for immunodetection, we used a mouse anti-BrdU antibody (1/20) and anti-mouse Ig-fluorescein antibody. Nuclei were stained by bis-benzimide (Hoechst 33342) and immunostaining was visualized under fluorescent microscopy. The number of immunoreactive cells was counted in predefined parallel strips.

2.6. Immunocytochemistry

After the experimental treatment, the cells were fixed with 4% paraformaldehyde. Then, cells were postfixed and permeabilized in ethanol-acetic acid (19:1) for 10 min at −20 °C and incubated in a blocking solution followed by overnight incubation at 4 °C with the following primary antibodies: the cytoplasmic α-synuclein aggregates, LC3 and Atg5 were determined using monoclonal anti-α-synuclein antibody (1/200), rabbit polyclonal anti-LC3 antibody (1/200), and Rabbit polyclonal anti-Atg5 (1/50), respectively. Fluorescein and Alexa Fluor-conjugated secondary antibodies were used to visualize positive cells under fluorescent microscopy.

2.7. Proteasomal activity measurement

After epoxomicin and trehalose treatments, the cells were washed with PBS, harvested in proteasome lysis buffer and lysed by sonication (VibraCell, level 0.5 for 30 s). The lysates were centrifuged at 12,000 × *g* at 4 °C for 30 min. The protein concentration was assayed from the resulting supernatants by the BCA protein assay kit. Proteasomal activities were quantified by monitoring the accumulation of the fluorescent cleavage product 7-amino-4-methylcoumarin (AMC) from the synthetic proteasomal substrates. In particular, Suc-Leu-Leu-Val-Tyr-AMC, Ac-Arg-Leu-Arg-AMC, and Z-Leu-Leu-Glu-AMC were used to measure chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolizing (PGPH)-like activity of the proteasome, respectively. The assays were carried out with 35 μg of cell lysates and the proteasomal substrates at 37 °C for 60 min incubation. The fluorescence of the released AMC was measured using an automatic multiwell plate reader at excitation/emission wavelengths of 360/465 nm.

2.8. Measurement of GSH

Total glutathione levels were measured by the method of Tietze (1969). Briefly, the cells were washed with PBS, lysed in 200 μl of 0.4 N perchloric acid (PCA) for 30 min at 4 °C. Glutathione content was measured in a P96 automatic reader by the addition of 5,5′ dithio-bis-2-nitrobenzoic acid (DTNB, 0.6 mM), nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH, 0.2 mM) and glutathione reductase (1 U). The reaction was monitored at 412 nm for 6 min.

2.9. Western blot analysis and detection of ubiquitinated proteins

NB69 extracts for Western blot analysis were prepared in ice-cold extraction buffer consisting of 20 mM Tris–HCl (pH 7.4), 10 mM potassium acetate (AcK), 1 mM dithiothreitol (DTT), 0,25% NP-40, 1 mM EDTA, 2 mM EGTA, 1 mM PMSF, protease inhibitors cocktail (SIGMA) and a cocktail of phosphatase inhibitors (100 mM sodium fluoride, 20 mM sodium molybdate and 20 mM β-glicerophosphate). The samples were homogenized, centrifuged at 4 °C and protein content determined by the BCA protein assay kit (Chemicon). Total protein (20–30 μg) was electrophoresed on 10–15% SDS-PAGE gels and transferred to 0.45 μm nitrocellulose membranes (Amersham), as described previously (Casarejos et al., 2005; Solano et al., 2008). After blocking, blots were incubated overnight at 4 °C in 5% non-fat dried milk with primary antibodies: anti-α-synuclein antibody (1/500), the

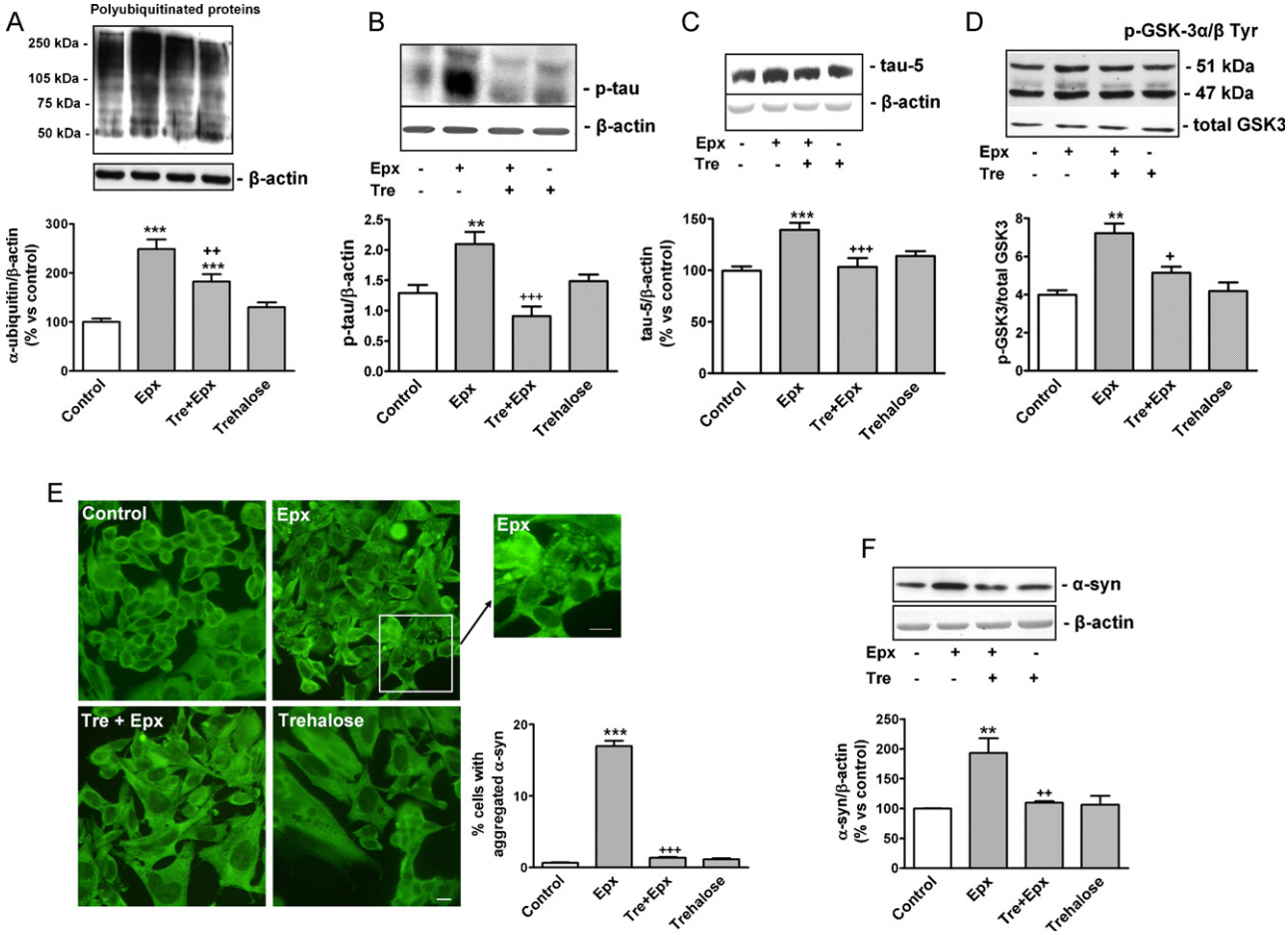

Fig. 3. Trehalose protects from ubiquitination, α-synuclein, p-tau increased levels and GSK3α/β activation induced by epoxomicin. After 6 days in vitro, the cells were pre-treated with trehalose (50 mM) for 15 min followed by addition of epoxomicin (10 nM) or solvent for another 24 h. (A) Accumulation of poly-ubiquitinated proteins and their corresponding densitometric analysis after 24 h of treatment. (B) Western blot and densitometric analysis of p-tau (phospho S199 + S202) and (C) total tau with anti-tau-5. (D) GSK-3α [pY^{279}]/β [pY^{216}]/total GSK3. (E) Immunocytochemistry with anti α-synuclein in the four experimental groups and inset to show the recruitment of α-synuclein to aggresome-like structures after proteasome inhibition (scale bar = 20 μm). (F) Western blot and densitometric analysis of α-synuclein. β-Actin was used as an equal loading of proteins. Values are the mean ± SEM from two independent cultures with 3-4 replicates each. Statistical analysis was performed by one or two-way ANOVA followed by Newman Keuls multiple comparison test and Bonferroni post-test, respectively. $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with controls; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ trehalose + epoxomicin *vs* epoxomicin-treated cultures. There is interaction between epoxomicin and trehalose effects on: poly-ubiquitinated proteins $F(1,18) = 9.11$ with a p value = 0.0074; percentage of cells with aggregated α-synuclein $F(1,13) = 321.65$ and a p value < 0.0001; accumulation of p-tau $F(1,27) = 19.83$ and a p value = 0.0001 and accumulation of α-synuclein $F(1,12) = 9.71$ with a p value = 0.0089.

chaperone anti-Hsp-70 (1/700); anti-p62 (SQSTM-1) (1/500); anti-LC3 (1/500); anti-Atg5 (1/500); anti-tau-5 (1/5000) and anti-p-tau (phospho S199 + S202) (1/1000). Other antibodies and dilutions used in the study were anti-GSK-3α [pY^{279}]/β [pY^{216}] (1/1000), mouse anti-GSK3β (1/1000) and anti-DJ-1 (1/1000).

The levels of pERK protein involved in signaling of survival response pathways, were measured by Western blot according to previously described techniques. We used a mouse anti-phospho-ERK-1/2 and rabbit anti-ERK-1/2 antibodies diluted (1/5000) and (1/10,000), respectively.

To determine changes in ubiquitination, NB69 cultures untreated or treated with epoxomicin (10 nM) and trehalose (50 mM) for 24 h in defined medium without serum, were scraped in 150 μl of lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 50 mM sodium fluoride, 20 mM N-ethyl-maleimide, 100 μM sodium ortovanadate, 1 mM PMSF and protease inhibitors cocktail (SIGMA)] and boiled for 5 min. The lysates were centrifuged at 12,000 × *g* at 4 °C for 30 min. 15 μg of protein were conducted to immunoblot assay with a rabbit polyclonal antibody to ubiquitin. The secondary antibodies (1/1000) followed by ECL detection reagents (Amersham) were used for immunodetection. Immunoblot of β-actin diluted (1/5000) was performed to demonstrate equal protein loading. The blots were quantified by computer-assisted video.

2.10. Statistical analysis

The results were statistically evaluated for significance with one-way ANOVA followed by Newman Keuls multiple comparison test. The interactions between the treatments were analyzed by two-way ANOVA followed by Bonferroni post-test. Differences were considered statistically significant when $p < 0.05$. Analysis of data was performed using the GraphPad PRISM 4 software.

3. Results

3.1. Dose and time-dependent effects of trehalose on the autophagic pathway in human neuroblastoma NB69 cells

Trehalose induces autophagy. After 6 days in vitro, the NB69 cells treated with trehalose, 50 mM, displayed immunoreactive granules to LC3, suggestive of autophagosomes (Fig. 1A). Trehalose, also, increased the expression of activated LC3 II, measured as LC3 II/I and LC3 II/β-actin ratios, in a dose dependent pattern, from 50 to 100 mM (Fig. 1B), and in a time dependent mode, from 24 to 48 h (Fig. 1C). Trehalose induces Atg5 expression after 1, 3 and 6 h of treatment (Fig. 1D–F). Atg5 is an early autophagy marker (Itakura and Mizushima, 2010).

3.2. Trehalose prevents cell death induced by epoxomicin

Epoxomicin, 10 nM × 24 h, an irreversible UPS inhibitor, reduced cell density and BrdU^{+} cells of NB69 cultured in complete medium (Fig. 2A and D) and increased cell death of NB69 cells cultured in defined medium (Fig. 2E, F and G) but co-treatment with trehalose completely reversed these changes. Trehalose, however, failed to modify the epoxomicin-induced reduction of cell division in NB69, as shown by the incorporation of bromodeoxy uridine to these cells (Fig. 2B–D).

3.3. Trehalose prevents the accumulation of polyubiquitinated proteins, α-synuclein, tau and p-tau and p-GSK-3, induced by epoxomicin

Epoxomicin, 10 nM × 24 h, induces the accumulation of polyubiquitinated proteins, and of proteins involved in neurodegenerative disorders such as α-synuclein, tau and p-tau and the kinase involved in the phosphorylation of tau, GSK-3 β (Fig. 3A–F). Pretreatment with trehalose, 50 mM, 15 min before treatment with epoxomicin, prevented these effects in human neuroblastoma cells (Fig. 3A–F).

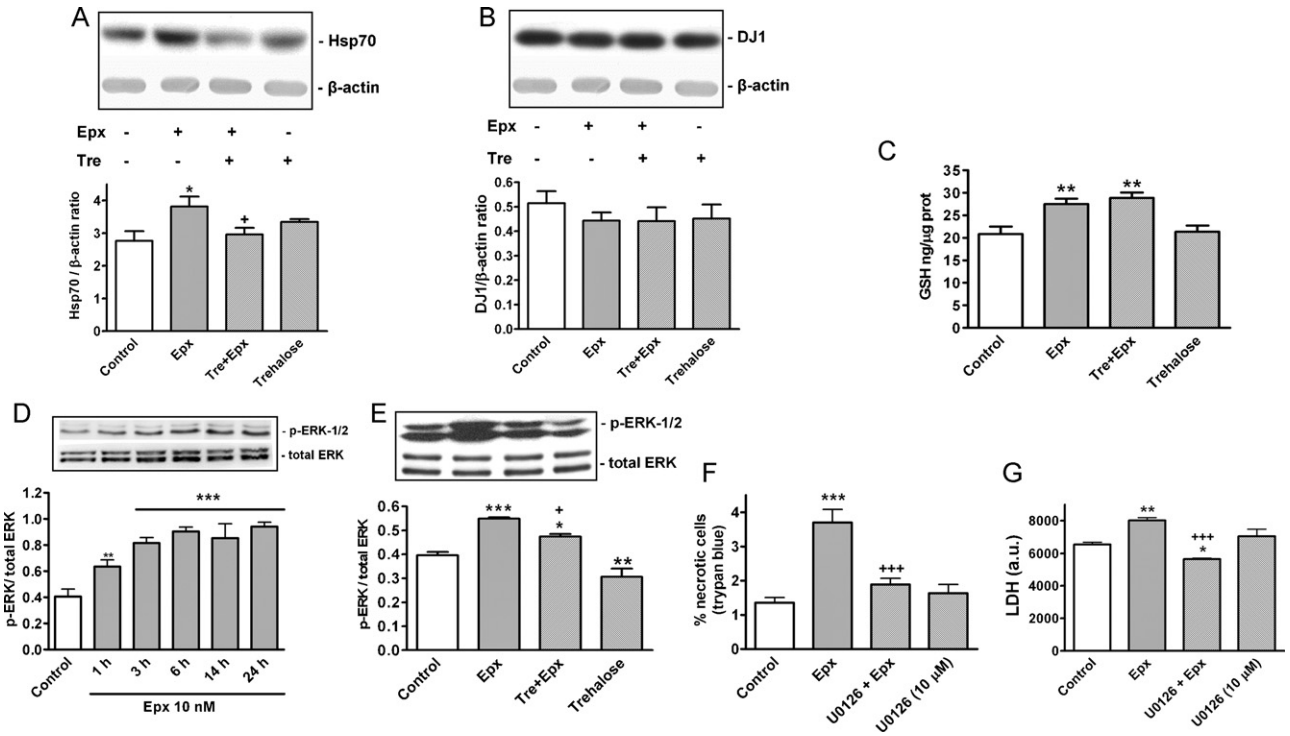

Fig. 4. Effects of epoxomicin and trehalose on chaperone proteins and glutathione homeostasis in NB69 cells. After 6 DIV, NB69 cells were pre-treated with trehalose (50 mM) for 15 min followed by addition of epoxomicin (10 nM) or solvent for another 24 h. (A and B) Western blot of Hsp-70 and DJ-1 expression and their densitometric analysis relative to β-actin. (C) Intracellular glutathione levels. (D) Time course of ERK phosphorylation after epoxomicin treatment. (E) Phosphorylated (active) and total ERK-1/2 were visualized by Western blot analysis and the relative active ERK levels were determined from densitometric scanning. (F) Effects of MEK-ERK pathway inhibition in cell death induced by epoxomicin. Percentage of necrotic cells and (G) LDH activity. Values are the mean ± SEM from two independent experiments with 3–6 replicates each. Statistical analysis was performed by one or two-way ANOVA followed by Newman Keuls multiple comparison test and Bonferroni post-test, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* controls; $^{+}p < 0.05$, $^{+++}p < 0.001$ trehalose + epoxomicin or UO126 + epoxomicin *vs* epoxomicin-treated cultures. There is interaction between epoxomicin and trehalose effects on HSP-70 protein expression $F(1,25) = 22.23$ with a p value < 0.0001. There is interaction between epoxomicin and UO126 effects on percentage of necrotic cells $F(1,20) = 12.51$ with a p value = 0.0021 and on LDH activity $F(1,20) = 30.44$ with a p value < 0.0001.

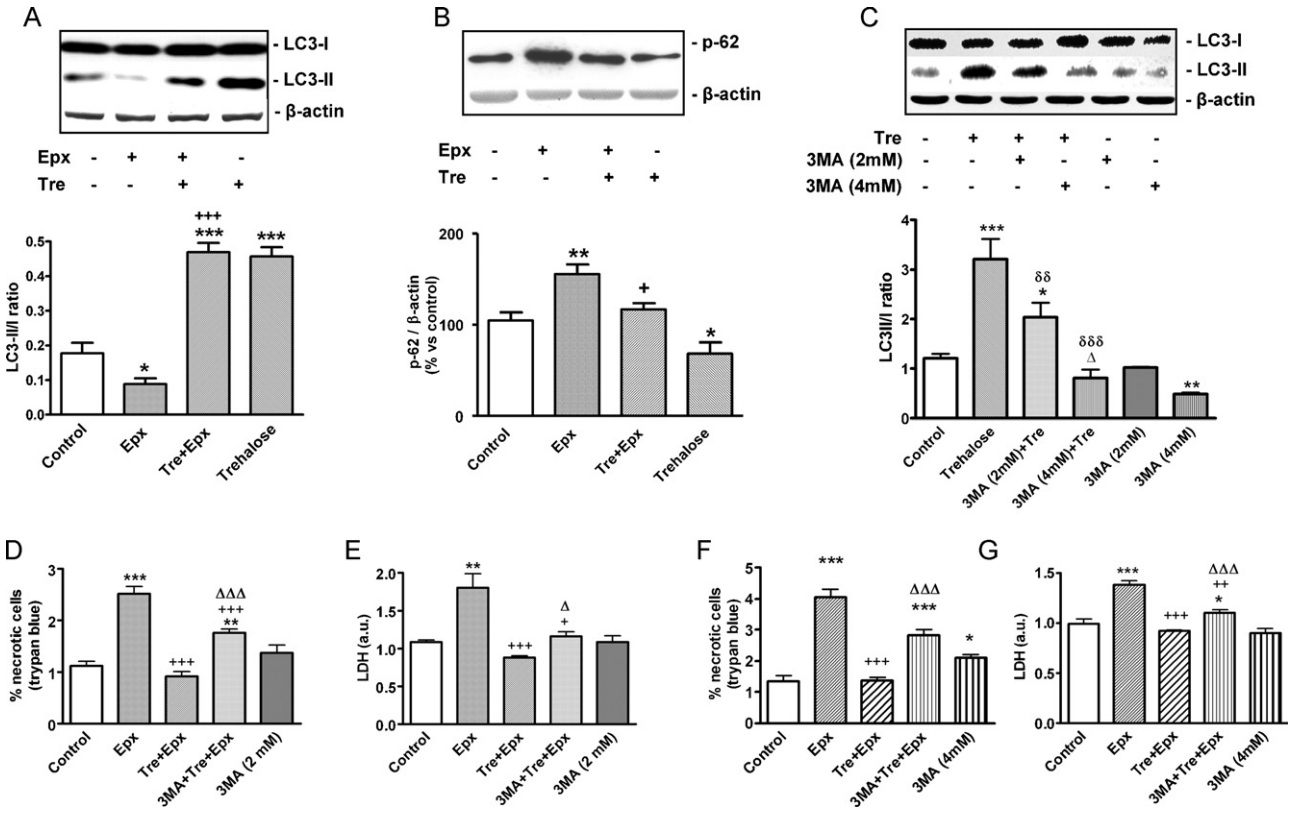

Fig. 5. Trehalose protects from decreased autophagy activity induced by epoxomicin. The NB69 cells were pre-treated with trehalose (50 mM) 15 min before epoxomicin (10 nM) for 24 h. (A) The protein levels of LC3 and (B) p-62 were detected by immunoblotting assay. β-Actin was used as an equal loading of proteins. (C) The effect of autophagy inhibitor, 3-methyladenine (3MA, 2 and 4 mM), on LC3 expression. (D) The effect of 3MA, trehalose and epoxomicin on cell viability. Percentage of necrotic cells (D and F) and LDH levels (E and G). Values are the mean ± SEM from two independent experiments with 3–4 replicates each. Statistical analysis was performed by one or two-way ANOVA followed by Newman Keuls multiple comparison test and Bonferroni post-test, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* controls; $^{+}p < 0.05$, $^{+++}p < 0.001$ trehalose + epoxomicin *vs* epoxomicin-treated cultures; $^{\Delta}p < 0.05$, $^{\Delta\Delta\Delta}p < 0.001$ 3MA + Tre + Epox *vs* trehalose + epoxomicin-treated cultures; $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$ 3MA + Tre *vs* trehalose-treated cultures. There is interaction between epoxomicin and trehalose effects on p-62 protein expression $F(1,11) = 5.04$ and p value = 0.0463.

3.4. Effects of epoxomicin and trehalose on HSP-70, p-ERK, DJ1 and glutathione homeostasis in NB69 cells

Epoxomicin, 10 nM × 24 h, increases the chaperone HSP-70 protein levels; an effect that is prevented by trehalose (Fig. 4A). Epoxomicin and trehalose did not have any effect on the levels of the PD related mitochondrial protein, DJ-1 (Fig. 4B). Epoxomicin increased the free radical scavenger agent GSH, suggesting that blockade of the proteasome system increases the levels of free radicals. Co-treatment with trehalose did not modify these effects, suggesting that the protective effects of trehalose are not related to a reduction of the free radicals (Fig. 4C). Epoxomicin increased the levels of p-ERK after 1, 3, 6, 14 and 24 h of treatment (Fig. 4D). Trehalose reduced p-ERK levels in control and in epoxomicin treated NB69 cells (Fig. 4E). Pre-treatment with U0126, an inhibitor for the MEK-ERK pathway, prevented from epoxomicin-induced necrotic cell death, measured as percentage of trypan blue positive cells and LDH activity, in NB69 cells (Figs. 4F-G).

3.5. Effects of trehalose in epoxomicin induced reduction of the proteasomal function and autophagy markers

The treatment with epoxomicin reduced the levels of the marker for mature autophagosomes, LC3 II/I and increased the marker for precursors of autophagy, such as p-62 (Fig. 5A and B). Treatment with trehalose reverted these changes in epoxomicin-treated NB69 cells (Fig. 5A and B).

In order to demonstrate the direct involvement of autophagy in the protective effect of trehalose, we used the autophagy inhibitor, 3-methyladenine (3MA). Pre-treatment with 3MA, 2 and 4 mM 30 min before the trehalose treatment, blocked the increased LC3 expression induced by trehalose (Fig. 5C) and prevented the neuroprotective effects of trehalose on the cell death-induced by epoxomicin (Figs. 5D–G).

Epoxomicin decreased the proteolytic activity of chymotrypsin-like (Fig. 6A), the trypsin-like (Fig. 6B) and the peptidylglutamyl-peptidase-like (Fig. 6C) components of the proteasome. Trehalose increased the activities of these proteolytic complexes in control but not in epoxomicin-treated NB69 cells (Fig. 6A–C).

4. Discussion

In this study, we have shown that trehalose produces a dose and time-dependent increase in the number of autophagosomes and markers of autophagy in NB69 cells and completely prevents the necrosis of these cells induced by the proteasome inhibitor, epoxomicin. The treatment with trehalose reverted the accumulation, induced by epoxomicin, of polyubiquitinated proteins, total and phosphorylated tau, p-GSK-3, and α-synuclein, as well as the α-synuclein intracellular aggregates. The effects of trehalose were not mediated through activation of free radical scavenging compounds, like GSH, or mitochondrial proteins, like DJ1, but trehalose reduced the activation of p-ERK and chaperone HSP-70 induced by epoxomicin. The inhibition of ERK phosphorylation prevented the epoxomicin-induced cell death and the inhibition of autophagy reverted the neuroprotective effects of trehalose in epoxomicin-induced cell death. Trehalose may act not only as an autophagy inductor in this paradigm.

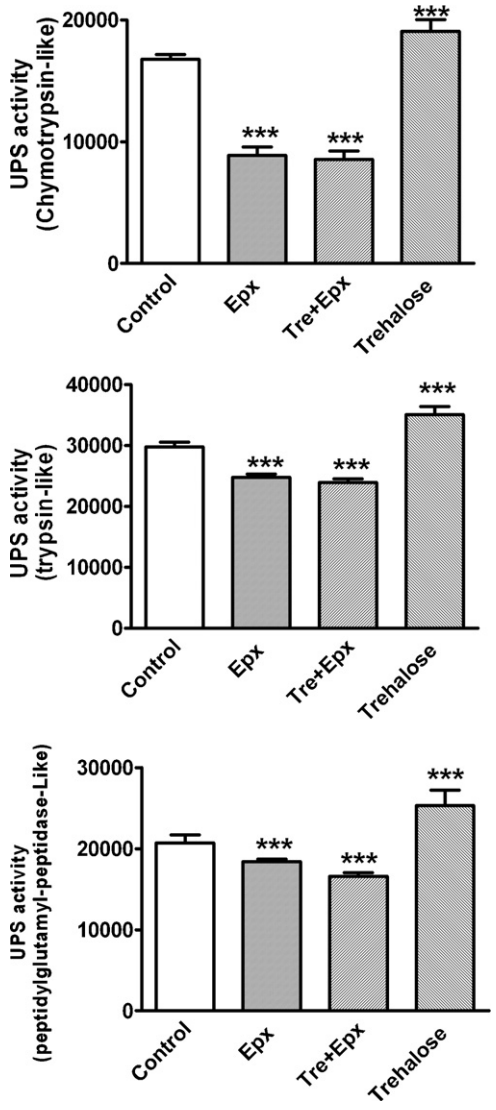

Fig. 6. Trehalose increases UPS activity. The NB69 cells were pre-treated with trehalose (50 mM) 15 min before epoxomicin (10 nM) for 24 h. (A) Chymotrypsin-like, (B) trypsin-like and (C) peptidylglutamyl-peptidase-like proteasome activities in extracts prepared from NB69 cells. Values are the mean ± SEM from two independent experiments with 3–4 replicates each. Statistical analysis was performed by one-way ANOVA followed by Newman Keuls multiple comparison test. ***$p < 0.001$ *vs* controls.

Proteasomal dysfunction has been considered a mechanism of production of neurodegenerative disorders though its relevance in neurodegeneration has been a topic for controversy. Misfolded proteins, including the β-amyloid peptide in AD, PrP protein in prionic disorders, α-synuclein in PD and huntingtin in HD, among others, are considered to play a role in the pathogenesis of these disorders (Forloni et al., 2002; Li et al., 2004; Dohm et al., 2008; Rubio et al., 2009; McMillan and Leverenz, 2010; Rodriguez-Navarro et al., 2010). Abnormal UPS function has been found in the *substantia nigra* in patients with PD (McNaught et al., 2003), though this finding requires confirmation and elucidation of its role; as a cause or a consequence, of the disease (Bove et al., 2006; Yamamoto et al., 2007). In addition, Petrucelli and colleagues (Petrucelli et al., 2002) reported that catecholamine neurons are preferentially susceptible to proteasome inhibition in primary cultures. We found that epoxomicin affects both dopamine and non-dopamine neurons in fetal midbrain cultures, and that the neurotoxic effects were slightly greater in non-dopamine than in dopamine neurons (Casarejos et al., 2009). Our present findings of an increased number of cells with aggregated α-synuclein and increased levels of α-synuclein, a protein involved in PD, by epoxomicin, and its reversal by trehalose, further supports the role of proteasomal dysfunction in PD.

Tau is another protein which accumulates in some neurodegenerative diseases such as AD and the sporadic and hereditary tauopathies. The mechanisms of tau accumulation and hyperphosphorylation after inhibition of the proteasome in neurons are intriguing. The proteasome inhibitor, lactacystin, increases the levels of tau, including phosphorylated tau, in rats (Liu et al., 2009). Additional mechanisms of increased protein accumulation are those related to autophagy dysfunction (Matsuda and Tanaka, 2010; Wong et al., 2008; Yue et al., 2009). We have previously shown that trehalose improves dopamine cell loss and tau pathology in parkin null mice expressing knocked-in human mutant tau (Rodriguez-Navarro et al., 2010). Now, we suggest that stimulation of autophagy, which normalizes the levels of total tau, phosphorylated tau and the tau phosphorylating enzyme GSK-3 β, may be a therapeutic tool in tauopathies related to post-transcriptional abnormalities of tau. All these data support the putative role of autophagy in tauopathies of different causes.

Epoxomicin and other UPS inhibitors induce the accumulation of p53 and ubiquitinated proteins (Meng et al., 1999; Pan et al., 2008). Ubiquitin and LC3-binding protein p62 regulate the formation of protein aggregates which are removed by autophagy (Komatsu et al., 2007). Phosphatidyl-ethanolamine-conjugated LC3 (LC3-II) is localized in the inner and outer membrane of autophagosomes, and the population associated with the inner membrane is degraded after fusion of autophagosomes with lysosomes (Kabeya et al., 2000). P62 mediates diverse signaling pathways including cell stress, survival and inflammation (Moscat et al., 2006; Wooten et al., 2006). Furthermore, p62 handles formation of cytoplasmic ubiquitin-positive inclusions (Komatsu et al., 2007). Our study shows that trehalose counteracts the elevation of p62 levels induced by epoxomicin. UPS inhibition may induce neurodegeneration and it could be reverted by increasing autophagy (Pan et al., 2008).

We have reported that epoxomicin increases the mitochondrial DJ-1 protein levels in parkin null midbrain neurons (Casarejos et al., 2009) suggesting that the inhibition of the proteasomal system induces compensatory activation of mitochondrial function. DJ-1, whose deficiency produces familial PD (Bonifati et al., 2003), modulates transcriptional regulation and anti-oxidative stress. DJ-1 is degraded by the proteasome and, at least, the L166P mutant DJ-1 is degraded very quickly (Miller et al., 2003). The inhibition of the proteasome by epoxomicin could, therefore, impair DJ-1 degradation and the elevation of the levels of this protein could be neuroprotective. DJ-1 up-regulates GSH synthesis during oxidative stress and increases expression of Hsp-70 to protect from α-synuclein toxicity (Zhou and Freed, 2005). In this study, however, we did not find any significant changes in DJ-1 levels by epoxomicin, trehalose or both, suggesting that DJ-1 changes may be restricted to parkin null cells which have an excessive production of free radicals.

The molecular mechanisms underlying the protection by trehalose of epoxomicin-induced toxicity include enhanced expression of heat-shock proteins and reduced activation of p-ERK. Epoxomicin increased the expression of HSP-70, which is prevented by co-treatment with trehalose. Short activation of p-ERK1/2 is implicated in cell survival and long-activation in cell death (Canals et al., 2003; Casarejos et al., 2009; Solano et al., 2008). Here, we show that epoxomicin increased ERK activation in a sustained manner. Trehalose reduced p-ERK1/2 levels in epoxomicin-free and epoxomicin-treated NB69 cells. The involvement of ERK activation in cell death-induced by epoxomicin was

demonstrated using the inhibitor for the MEK-ERK pathway, pre-treatment with 10 μM U0126, prevented the trehalose neuroprotective effects.

Trehalose is a novel mTOR-independent autophagy enhancer which does not readily cross cell membranes, but can be efficiently loaded into mammalian cells via fluid-phase endocytosis and pinocytosis (Wolkers et al., 2001). In mammals, trehalose is split into two molecules of glucose in the small intestine but the effects of trehalose on autophagy are not caused by glucose (Sarkar et al., 2007; Mizushima, 2010). Trehalose, in addition, has anti-aggregation properties and lack of toxicity and it could be a very promising compound for treating a spectrum of protein conformational disorders like prion disorders, HD, AD and PD.

Acknowledgements

Supported in part by grants from the Spanish Ministry of Health, FIS 2007/37, FIS 2010/172, CAM 0202/2006, Lain Entralgo NDG07/4 and CIBER 2006/05/0059. The authors thank Mrs Ma Paz Muñoz and Ms Laura Rodriguez for excellent technical assistance and Mrs. Claire Marsden for editorial help.

References

Alvarez-Erviti, L., Rodriguez-Oroz, M.C., Cooper, J.M., Caballero, C., Ferrer, I., Obeso, J.A., Schapira, A.H., 2010. Chaperone-mediated autophagy markers in Parkinson disease brains. Arch. Neurol. 198, doi:10.1001/archneurol.2010.

Arora, A., Ha, C., Park, C.B., 2004. Inhibition of insulin amyloid formation by small stress molecules. FEBS Lett. 564, 121–125.

Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J.W., Vanacore, N., van Swieten, J.C., Brice, A., Meco, G., van Duijn, C.M., Oostra, B.A., Heutink, P., 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299, 256–259.

Bove, J., Zhou, C., Jackson-Lewis, V., Taylor, J., Chu, Y., Rideout, H.J., Wu, D.C., Kordower, J.H., Petrucelli, L., Przedborski, S., 2006. Proteasome inhibition and Parkinson's disease modeling. Ann. Neurol. 60, 260–264.

Canals, S., Casarejos, M.J., de Bernardo, S., Solano, R.M., Mena, M.A., 2003. Selective and persistent activation of extracellular signal-regulated protein kinase by nitric oxide in glial cells induces neuronal degeneration in glutathione-depleted midbrain cultures. Mol. Cell. Neurosci. 24, 1012–1026.

Casarejos, M.J., Solano, R.M., Menendez, J., Rodriguez-Navarro, J.A., Correa, C., Garcia de Yebenes, J., Mena, M.A., 2005. Differential effects of l-DOPA on monoamine metabolism, cell survival and glutathione production in midbrain neuronal-enriched cultures from parkin knockout and wild-type mice. J. Neurochem. 94, 1005–1014.

Casarejos, M.J., Solano, R.M., Rodriguez-Navarro, J.A., Gomez, A., Perucho, J., Castano, J.G., Garcia de Yebenes, J., Mena, M.A., 2009. Parkin deficiency increases the resistance of midbrain neurons and glia to mild proteasome inhibition: the role of autophagy and glutathione homeostasis. J. Neurochem. 110, 1523–1537.

Chen, Q., Haddad, G.G., 2004. Role of trehalose phosphate synthase and trehalose during hypoxia: from flies to mammals. J. Exp. Biol. 207, 3125–3129.

Dehay, B., Bove, J., Rodriguez-Muela, N., Perier, C., Recasens, A., Boya, P., Vila, M., 2010. Pathogenic lysosomal depletion in Parkinson's disease. J. Neurosci. 30, 12535–12544.

Dohm, C.P., Kermer, P., Bahr, M., 2008. Aggregopathy in neurodegenerative diseases: mechanisms and therapeutic implication. Neurodegener. Dis. 5, 321–338.

Enokido, Y., Tamura, T., Ito, H., Arumughan, A., Komuro, A., Shiwaku, H., Sone, M., Foulle, R., Sawada, H., Ishiguro, H., Ono, T., Murata, M., Kanazawa, I., Tomilin, N., Tagawa, K., Wanker, E.E., Okazawa, H., 2010. Mutant huntingtin impairs Ku70-mediated DNA repair. J. Cell Biol. 189, 425–443.

Forloni, G., Terreni, L., Bertani, I., Fogliarino, S., Invernizzi, R., Assini, A., Ribizzi, G., Negro, A., Calabrese, E., Volonte, M.A., Mariani, C., Franceschi, M., Tabaton, M., Bertoli, A., 2002. Protein misfolding in Alzheimer's and Parkinson's disease: genetics and molecular mechanisms. Neurobiol. Aging 23, 957–976.

Itakura, E., Mizushima, N., 2010. Characterization of autophagosome formation site by a hierarchical analysis of mammalian Atg proteins. Autophagy 6, 764–776.

Jain, N.K., Roy, I., 2009. Effect of trehalose on protein structure. Protein Sci. 18, 24–36.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., Yoshimori, T., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19, 5720–5728.

Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., Ueno, T., Hara, T., Mizushima, N., Iwata, J., Ezaki, J., Murata, S., Hamazaki, J., Nishito, Y., Iemura, S., Natsume, T., Yanagawa, T., Uwayama, J., Warabi, E., Yoshida, H., Ishii, T., Kobayashi, A., Yamamoto, M., Yue, Z., Uchiyama, Y., Kominami, E., Tanaka, K., 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 131, 1149–1163.

Korolchuk, V.I., Menzies, F.M., Rubinsztein, D.C., 2009. A novel link between autophagy and the ubiquitin-proteasome system. Autophagy 5, 862–863.

Liu, R., Barkhordarian, H., Emadi, S., Park, C.B., Sierks, M.R., 2005. Trehalose differentially inhibits aggregation and neurotoxicity of beta-amyloid 40 and 42. Neurobiol. Dis. 20, 74–81.

Li, W., Lesuisse, C., Xu, Y., Troncoso, J., Price, C., D.L. Lee, M.K., 2004. Stabilization of alpha-synuclein protein with aging and familial Parkinson's disease-linked A53T mutation. J. Neurosci. 24, 7400–7409.

Liu, Y.H., Wei, W., Yin, J., Liu, G.P., Wang, Q., Cao, F.Y., Wang, J.Z., 2009. Proteasome inhibition increases tau accumulation independent of phosphorylation. Neurobiol. Aging 30, 1949–1961.

Maher, P., 2008. Proteasome inhibitors prevent oxidative stress-induced nerve cell death by a novel mechanism. Biochem. Pharmacol. 75, 1994–2006.

Malkus, K.A., Tsika, E., Ischiropoulos, H., 2009. Oxidative modifications, mitochondrial dysfunction, and impaired protein degradation in Parkinson's disease: how neurons are lost in the Bermuda triangle. Mol. Neurodegener. 4, 24.

Matsuda, N., Tanaka, K., 2010. Does impairment of the ubiquitin-proteasome system or the autophagy-lysosome pathway predispose individuals to neurodegenerative disorders such as Parkinson's disease? J. Alzheimers Dis. 19, 1–9.

McMillan, P.J., Leverenz, J.B., 2010. From model system to clinical medicine: pathophysiologic links of common proteinopathies. Alzheimers Res. Ther. 2, 26.

McNaught, K.S., Belizaire, R., Isacson, O., Jenner, P., Olanow, C.W., 2003. Altered proteasomal function in sporadic Parkinson's disease. Exp. Neurol. 179, 38–46.

McNaught, K.S., Olanow, C.W., 2006. Proteasome inhibitor-induced model of Parkinson's disease. Ann. Neurol. 60, 243–247.

Mena, M.A., Casarejos, M.J., Bonin, A., Ramos, J.A., Garcia Yebenes, J., 1995a. Effects of dibutyryl cyclic AMP and retinoic acid on the differentiation of dopamine neurons: prevention of cell death by dibutyryl cyclic AMP. J. Neurochem. 65, 2612–2620.

Mena, M.A., Casarejos, M.J., Gimenez-Gallego, G., Garcia de Yebenes, J., 1995b. Fibroblast growth factors: structure-activity on dopamine neurons in vitro. J. Neural Transm. Park Dis. Dement Sect. 9, 1–14.

Mena, M.A., Pardo, B., Casarejos, M.J., Fahn, S., Garcia de Yebenes, J., 1992. Neurotoxicity of levodopa on catecholamine-rich neurons. Mov. Disord. 7, 23–31.

Meng, L., Mohan, R., Kwok, B.H., Elofsson, M., Sin, N., Crews, C.M., 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity. Proc. Natl. Acad. Sci. U.S.A. 96, 10403–10408.

Miller, D.W., Ahmad, R., Hague, S., Baptista, M.J., Canet-Aviles, R., McLendon, C., Carter, D.M., Zhu, P.P., Stadler, J., Chandran, J., Klinefelter, G.R., Blackstone, C., Cookson, M.R., 2003. L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome system. J. Biol. Chem. 278, 36588–36595.

Mizushima, N., 2010. Autophagy. FEBS Lett. 584 (7), 1279.

Moscat, J., Diaz-Meco, M.T., Albert, A., Campuzano, S., 2006. Cell signaling and function organized by PB1 domain interactions. Mol. Cell. 23, 631–640.

Nakamura, T., Lipton, S.A., 2010. S-Nitrosylation of critical protein thiols mediates protein misfolding and mitochondrial dysfunction in neurodegenerative diseases. Antioxid. Redox Signal. 3570 Doi 10. 1089/ars. 2010.

Pan, T., Kondo, S., Zhu, W., Xie, W., Jankovic, J., Le, W., 2008. Neuroprotection of rapamycin in lactacystin-induced neurodegeneration via autophagy enhancement. Neurobiol. Dis. 32, 16–25.

Petrucelli, L., O'Farrell, C., Lockhart, P.J., Baptista, M., Kehoe, K., Vink, L., Choi, P., Wolozin, B., Farrer, M., Hardy, J., Cookson, M.R., 2002. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron 36, 1007–1019.

Rodriguez-Martin, E., Canals, S., Casarejos, M.J., de Bernardo, S., Handler, A., Mena, M.A., 2001. L-DOPA and glia-conditioned medium have additive effects on tyrosine hydroxylase expression in human catecholamine-rich neuroblastoma NB69 cells. J. Neurochem. 78, 535–545.

Rodriguez-Navarro, J.A., Rodriguez, L., Casarejos, M.J., Solano, R.M., Gomez, A., Perucho, J., Cuervo, A.M., Garcia de Yebenes, J., Mena, M.A., 2010. Trehalose ameliorates dopaminergic and tau pathology in parkin deleted/tau overexpressing mice through autophagy activation. Neurobiol. Dis. 39, 423–438.

Rubinsztein, D.C., 2007. Autophagy induction rescues toxicity mediated by proteasome inhibition. Neuron 54, 854–856.

Rubio, I., Rodriguez-Navarro, J., Tomas-Zapico, A., Ruiz, C., Casarejos, M.J., Perucho, J., Gomez, A., Rodal, I., Lucas, J.J., Mena, M.A., de Yebenes, J.G., 2009. Effects of partial suppression of parkin on huntingtin mutant R6/1 mice. Brain Res. 1281, 91–100.

Sarkar, S., Davies, J.E., Huang, Z., Tunnacliffe, A., Rubinsztein, D.C., 2007. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J. Biol. Chem. 282, 5641–5652.

Singer, M.A., Lindquist, S., 1998. Multiple effects of trehalose on protein folding in vitro and in vivo. Mol. Cell. 1, 639–648.

Solano, R.M., Casarejos, M.J., Menendez-Cuervo, J., Rodriguez-Navarro, J.A., Rodriguez-Navarro, J.A., Garcia de Yebenes, J., Mena, M.A., 2008. Glial dysfunction in parkin null mice: effects of aging. J. Neurosci. 28, 598–611.

Tanaka, M., Machida, Y., Niu, S., Ikeda, T., Jana, N.R., Doi, H., Kurosawa, M., Nekooki, M., Nukina, N., 2004. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. Nat. Med. 10, 148–154.

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal. Biochem. 27, 502–522.

Welch, W.J., Brown, C.R., 1996. Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones. 1, 109–115.

Wolkers, W.F., Walker, N.J., Tablin, F., Crowe, J.H., 2001. Human platelets loaded with trehalose survive freeze-drying. Cryobiology 42, 79–87.
Wong, E.S., Tan, J.M., Soong, W.E., Hussein, K., Nukina, N., Dawson, V.L., Dawson, T.M., Cuervo, A.M., Lim, K.L., 2008. Autophagy-mediated clearance of aggresomes is not a universal phenomenon. Hum. Mol. Genet. 17, 2570–2582.
Wooten, M.W., Hu, X., Babu, J.R., Seibenhener, M.L., Geetha, T., Paine, M.G., Wooten, M.C., 2006. Signaling, polyubiquitination trafficking, and inclusions: sequestosome 1/p62s role in neurodegenerative disease. J. Biomed. Biotechnol. 2006, 62079.
Yamamoto, N., Sawada, H., Izumi, Y., Kume, T., Katsuki, H., Shimohama, S., Akaike, A., 2007. Proteasome inhibition induces glutathione synthesis and protects cells from oxidative stress: relevance to Parkinson disease. J. Biol. Chem. 282, 4364–4372.
Yue, Z., Friedman, L., Komatsu, M., Tanaka, K., 2009. The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. Biochim. Biophys. Acta 1793, 1496–1507.
Zhou, W., Freed, C.R., 2005. DJ-1 up-regulates glutathione synthesis during oxidative stress and inhibits A53T alpha-synuclein toxicity. J. Biol. Chem. 280, 43150–43158.