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Coupling Endoplasmic Reticulum Stress to the Cell Death Program in Dopaminergic Cells: Effect of Paraquat

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Abstract

Parkinson's disease (PD) features oxidative stress and accumulation of misfolded (unfolded, alternatively folded, or mutant) proteins with associated loss of dopaminergic neurons. Oxidative stress and the accumulated misfolded proteins elicit cellular responses that include an endoplasmic reticulum (ER) stress response that may protect cells against the toxic buildup of misfolded proteins. Chronic ER stress and accumulation of misfolded proteins in excessive amounts, however, overwhelm the cellular 'quality control' system and impair the protective mechanisms designed to promote correct folding and degrade faulty proteins, ultimately leading to organelle dysfunction and neuronal cell death. Paraquat belongs to a class of bipyridyl herbicides and triggers oxidative stress and dopaminergic cell death. Epidemiological studies suggest an increased risk for developing PD following chronic exposure to paraquat. The present study was carried out to determine the role of paraquat in triggering cellular stress particularly ER stress and to elucidate the pathways that couple ER stress to dopaminergic cell death. We demonstrate that paraquat triggers ER stress, cell dysfunction, and dopaminergic cell death. p23, a small co-chaperone protein, is cleaved during ER stress-induced cell death triggered by paraquat and blockage of the caspase cleavage site of p23 was associated with decreased cell death. Paraquat also inhibits proteasomal activity that may further trigger accumulation of misfolded proteins resulting in ER stress. Our results indicate a protective role for p23 in PD-related programmed cell death. The data also underscore the involvement of ER, caspases, and the proteasomal system in ER stress-induced cell death process.

Keywords

Endoplasmic reticulum; Parkinson's disease; Paraquat; ER stress; Caspase; p23; Cell death

Introduction

Parkinson's disease (PD) is a progressive age-associated neurodegenerative disease with preferential loss of dopaminergic neurons in substantia nigra of midbrain. Although, PD has been heavily researched in last two decades, the exact cause of selective dopaminergic neuronal loss is not known. Systemic exposure of animals to a class of bipyridyl herbicides including paraquat (PQ) triggers oxidative stress and dopaminergic cell death suggesting that dopaminergic neurons may be particularly vulnerable to oxidative challenges (Andersen 2000, 2003, 2004; Chinta and Andersen 2006, Chinta et al. 2006; Kaur et al. 2007; McCormack et al. 2005; Peng et al. 2004, 2005; Yang and Tiffany-Castiglioni 2005). The exact mechanism by which oxidative stress triggers dopaminergic cell death is not clear; however, it may involve the accumulation of misfolded proteins. Oxidative stress triggers protein modification, protein misfolding and impaired protein degradation (Andersen 2000, 2004; Danielson and Andersen 2008; Mattson 2006; Rajagopalan and Andersen 2001). The accumulation of misfolded proteins can cause an imbalance between the synthesis of new proteins and the endoplasmic reticulum's (ER's) ability to process newly synthesized proteins, resulting in the failure of the ER to cope with the excess protein load, termed "ER stress" (Harding et al. 2002; Kopito 2000; Paschen and Frandsen 2001; Rutkowski and Kaufman 2004; Sitia and Braakman 2003). Prolonged ER stress can eventually overwhelm the cellular protective mechanisms, ultimately triggering programmed cell death (pcd) (Bredesen et al. 2006; Rao et al. 2001, 2002a, b; Rao and Bredesen 2004; Rao et al. 2004a, b). Misfolded proteins and associated ER stress are emerging as important features of PD (Forman et al. 2003; Hashimoto et al. 2003; Holtz and O'Malley 2003; Kitamura et al. 2002; Lindholm et al. 2006; Ryu et al. 2002; Takahashi et al. 2003; Wang and Takahashi 2007). Toxins, including PQ, trigger the death of dopaminergic neurons in vitro and in vivo and are widely used to model PD (Andersen 2003; Holtz and O'Malley 2003; McCormack et al. 2002; McCormack and Di Monte 2003; McCormack et al. 2005; Peng et al. 2004; Ryu et al. 2002). One of the mechanisms by which PQ triggers dopaminergic cell death may involve chronic ER stress and downstream ER-associated cell death pathways (Conn et al. 2004; Holtz and O'Malley 2003; Ryu et al. 2002; Uehara et al. 2006). We therefore examined the ability of PQ to elicit ER stress in a dopaminergic rat cell line. Our findings suggest that PQ triggers ER stress that in turn triggers caspase-dependent cell death pathways and that these pathways feature p23 cleavage and inhibition of proteasome activity.

Experimental Methods

Cells, Culture Conditions and Cell Extracts

Immortalized rat mesencephalic dopaminergic N27 cells which exhibit increased levels of TH and DAT were derived from dopamine-producing neurons of the rat fetal midbrain via SV40 large T antigen immortalization, and therefore consist of cells comparable to the midbrain dopaminergic neurons selectively lost in PD (Adams et al. 1996; Clarkson et al. 1998; Zhou et al. 2000). N27 cells were cultured in RPMI containing 10% FBS and 1% penicillin/streptomycin. Paraquat (PQ, Methyl Viologen) was obtained from Sigma Chemical Co. Stock solutions made in water were further diluted with cell culture media. ER stress and cell death were induced by treating cells with 500 μ M PQ (unless otherwise indicated). We and others have demonstrated the effect of PQ in triggering cell death at concentrations ranging from 100 μ M-1 mM (Fei et al. 2008; Peng et al. 2004; Yang and Tiffany-Castiglioni 2007, 2008). Q-

VD-OPH (QVD, MP Biomedicals, Aurora, Ohio, USA), a cell-permeable irreversible caspase inhibitor was dissolved in DMSO according to the manufacturer's data sheet and further diluted with cell culture media. Salubrinol (Sal; EMD Biosciences) was dissolved in DMSO and diluted in cell culture media before addition to cells. Cell extracts were prepared as previously described (Rao et al. 2001, 2002a, b). Briefly, untreated or PQ-treated cells were re-suspended in RIPA buffer (50 mM Tris, pH 7.5, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl) with or without protease inhibitors (Complete, Mini, Roche), sonicated for 10 s on ice and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and protein concentration was determined using Bradford reagent (BioRad). 100–200 µg protein from total extracts was used for Western blotting.

Transfection

Transient transfections were performed using TransIT T-Neural transfection reagent obtained from MIRUS Bio Corporation (Madison, WI). Wild-type (WT) p23 and mutant p23D142N cDNA were generated as described earlier (Rao et al. 2006). Briefly, N-terminal Flag-p23 was subcloned into a pcDNA3 expression vector (Invitrogen) by PCR-amplification of p23. Using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), the caspase cleavage site mutant D142N was generated (Rao et al. 2006). Sequences of the constructs were confirmed by DNA sequencing and Western blot analyses were performed to verify protein expression. Typically, 2×10^6 or 4×10^6 cells were seeded in 10- or 15-cm dishes, respectively, and transfected 1 day later with 6 µg of pcDNA3, WT p23, or p23D142N using a ratio of 1 µg:3 µl of DNA: TransIT T-neural transfection reagent. The transfection efficiency using these conditions was approximately 50–60%.

Western Blotting

SDS-PAGE and Western blot analyses were performed as described earlier (Rao et al. 2001; 2002a, b; 2004a, b). Membranes were probed with either a 1:500 dilution of anti-KDEL monoclonal antibody (Stressgen), 1:500 dilution of anti-phospho-eIF2α rabbit monoclonal antibody, anti-eIF2α mouse monoclonal antibody, rat-specific anti-cleaved PARP, anti-caspase-7, or anti-caspase-3 antibodies (all from Cell Signaling Laboratories), a 1:1000 dilution of anti-p23 monoclonal antibody (BD Biosciences), a 1:500 dilution of anti-GADD153 antibody (Santa Cruz), or a 1:50,000 dilution of anti-GAPDH rabbit polyclonal anti-body (Research Diagnostics Inc). To confirm uniform loading of proteins across conditions, immunoblots were stripped and reprobed with GAPDH (1:50,000). Blots were quantified by band densitometry of scanned films using NIH Image 1.61 software (National Institutes of Health, Bethesda, MD, USA). The band density was expressed as a percentage ratio of the densitometric optical density of the protein of interest to that of GAPDH.

Caspase Activity Assay

The fluorogenic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin was purchased from Enzyme Systems Products and dissolved in DMSO as a 10 mM stock solution. Cell extracts (50–100 µg of protein) from untreated or PQ-treated cells were incubated with 100 µM peptide substrate. Caspase activity was determined by measuring the release of amino-4-trifluoromethylcoumarin from the synthetic substrate using continuous recording instruments, as described earlier (Rao et al. 2002a, b). Caspase activity was analyzed using a SpectraMAX 340 plate reader (Molecular Devices) at excitation and emission wavelengths of 444 and 538 nm, respectively.

Proteasome Activity

Assessment of 20S proteasome activity was carried out using Chemicon's 20S proteasome activity assay kit based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC)

following cleavage of the labeled substrate LLVY-AMC. Briefly, cell extracts (25–50 μ g of protein) from untreated or PQ-treated cells were incubated with 1 mM substrate at 37°C for 1 h. Fluorescence was measured in a Spectramax plate reader at excitation and emission wavelengths of 380 and 460 nm, respectively.

Evaluation of Cell Death

Assessment of cell death was carried out by pelleting floating and adherent cells (after trypsinization) as previously described (Chinta et al. 2006). Briefly, cells were seeded in 24-well plates at a density of 50,000 per well. Following treatment with PQ, viable cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, 50 μ l of 5 mg/ml MTT was added to the cells (500 μ l) and incubated at 37°C for 2 h. The medium was discarded, the dark blue formazan crystalline product was dissolved in dimethyl sulfoxide and the absorbance was analyzed in a Spectramax plate reader (Molecular Devices) at 570 nm. Cell death was determined as the percentage of live cells over the total number of cells.

Statistical Analysis

All experiments were performed four to five times. Cell death and enzyme activity assays were performed in triplicate and repeated independently four to five times. Results are presented as mean $\pm$ SD. Data from cell death, enzyme activity assays, and band densitometry were statistically analyzed using one-way ANOVA (GraphPad Prism software; San Diego, CA, USA), followed by between-group comparisons using the Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Paraquat Triggers ER Stress-induced Cell Death in Dopaminergic Cells

Understanding the mechanisms by which environmental agents such as PQ elicit dopaminergic cell toxicity is critical in identifying pathways involved in PD pathogenesis and potential therapeutic targets. We therefore sought to determine whether PQ induces ER stress and triggers ER stress-induced cell death in dopaminergic N27 cells in culture.

A high level of GRP78 and GRP94 protein expression is indicative of ER stress (Li et al. 1992; Liu et al. 1997; Rao et al. 2001, 2002a, b; 2004; 2006). In addition, phosphorylation of the α subunit of the eukaryotic initiation factor-2 (eIF2 α) is a well documented mechanism of down-regulating protein synthesis triggered by agents that induce ER stress and protein misfolding (Harding et al. 2002; Rutkowski and Kaufman 2004). Similarly, GADD153/CHOP, a 30-kD protein that triggers growth arrest and DNA damage, is ubiquitously expressed at very low levels under basal conditions but is robustly expressed by perturbations that induce ER stress in a wide variety of cells (Harding et al. 2002; Rutkowski and Kaufman 2004; Yamaguchi and Wang 2004). As shown in Fig. 1a, PQ treatment of dopaminergic N27 cells resulted in the induction of GRP78 and GRP94 expression and triggered increased phosphorylation of eIF2 α and induction of GADD153 expression. Coinciding with the increased expression of ER stress proteins, PQ treatment also resulted in a time (Fig. 1a) and concentration-dependent cell death of dopaminergic N27 cells (Fig. 1c).

Salubrinal (Sal) is an inhibitor of serine/threonine phosphatase PP1 and inhibits the phosphatase activity responsible for dephosphorylating Po4eIF2 α that in turn blocks ER stress-induced cell death (Boyce et al. 2005). Sal selectively modulates the translational control branch of the unfolded protein response by inducing eIF2 α phosphorylation (Boyce et al. 2005; Reijonen et al. 2008; Smith et al. 2005; Sokka et al. 2007). To study the effect of Sal on PQ-treated N27 cells, we added Sal in combination with PQ and as shown in Fig. 2a, Sal

blocked ER stress-induced cell death triggered by PQ and increased survival significantly of the dopaminergic N27 cells. Sal also blocked the processing of caspase-7 that gets activated by ER stress signals confirming its anti-apoptotic effect (Fig. 2b) (Boyce et al. 2005; Rao et al. 2001).

Cleavage of PARP and p23 and Effect of Uncleavable p23

PARP, a 116-kD nuclear poly (ADP-ribose) polymerase that is involved in DNA repair is cleaved by caspases in vitro and is one of the main cleavage targets of caspase-3 in vivo (Lazebnik et al. 1994). As shown in Fig. 3, ER stress-induced cell death triggered by PQ resulted in cleavage of PARP to yield an 85-kD cleaved fragment as described earlier (Rao et al. 2002a, b). We had earlier demonstrated that the small co-chaperone protein p23 is cleaved during ER stress-induced cell death; this cleavage requires active caspase-3 and/or caspase-7 (Rao et al. 2006). We asked whether p23 is cleaved in the dopaminergic N27 cells after exposure to PQ. As shown in Fig. 3, p23 is detectable in these cells and furthermore is cleaved to yield the 19-kD product during ER stress-induced cell death.

We had previously demonstrated that p23 is cleaved by caspases at a specific D142 site and mutation of the caspase-susceptible p23D142N site not only blocks caspase cleavage of p23 and formation of the 19-kD product (p23 Δ C18), but also attenuates ER stress-induced cell death triggered by various ER stress inducers (Rao et al. 2006). Shown in Fig. 4 is a representative blot demonstrating the cleavage of p23 by caspase-7 and the resistance of p23D142N to cleavage by caspases. Cellular extracts isolated from dopaminergic N27 cells transfected with either WTP23 or p23D142N expression construct were incubated with recombinant purified caspase-7. While WTP23 is cleaved by caspase-7 to yield the 19-kD product (lane 2), mutation of the caspase-susceptible p23D142N site prevented the protein from being cleaved by caspase-7 (lane 4) (Rao et al. 2006). We now tested whether expression of mutant p23D142N would result in a reduction of dopaminergic N27 cell death triggered by PQ. Cells were either left untransfected or transfected with a mock vector, WTFlag-p23 or Flag-p23D142N expression constructs. The efficiency of transfection was monitored by immunoblot analysis of the proteins using an anti-Flag antibody. As shown in Fig. 4b (inset), the protein expression of both WTP23 and p23D142N expression constructs was very similar. PQ was added to cells and cell viability measured 48 h following transfection as described in the section "Experimental Methods". As shown in Fig. 4b, while the expression of the mock vector or WTP23 did not result in a significant protection from PQ treatment, the p23D142N mutant significantly suppressed dopaminergic N27 cell death triggered by PQ. There was a greater degree of protection in p23D142N transfected cells exposed to PQ as compared to mock or WTP23 transfected cells.

Caspase Cleavage and Activation Triggered by Paraquat

We had earlier demonstrated that ER stress-induced cell death featured caspase cleavage and activation (Rao et al. 2001, 2002a, b). We asked whether a subset of downstream caspases is cleaved in ER stress-induced cell death triggered by PQ. As shown in Fig. 5a, treatment with PQ resulted in the processing of caspases-3 and -7 similar to our earlier observations (Peng et al. 2005). Caspase activity measurements on cell extracts were performed using Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin as substrate. As shown in Fig. 5b, N27 cells treated with PQ activated caspases as demonstrated by increased DEVDase activity. Q-VD-OPH (QVD), a broad spectrum cell-permeable caspase inhibitor with potent antiapoptotic properties (Caserta et al. 2003; Dursun et al. 2006; Yang et al. 2004) significantly suppressed the DEVDase activity triggered by PQ. Since QVD inhibits caspase activity, it prompted us to examine its effect on cell death. Treatment of dopaminergic N27 cells with PQ in the presence of QVD significantly enhanced the cell viability (Fig. 5c).

Paraquat Inhibits 20S Proteasomal Activity

Recent reports have demonstrated that (a) inhibition of proteasomal activity (Bush et al. 1997; Fribley and Wang 2006; Ishii et al. 2007), (b) accumulation of cytosolic Ca^{2+} (Liu et al. 1998; Yu et al. 1999), and (c) disruption of protein disulfide bond formation (Nakamura and Lipton 2008; Uehara et al. 2006) can all trigger an ER stress response. To determine whether proteasome activity is altered in dopaminergic N27 cells exposed to PQ, 20S proteasome activity was measured in cell extracts following drug treatment. Cell extracts were tested for the ability to hydrolyze the peptide substrate LLVY-AMC. As shown in Fig. 6, PQ treatment of dopaminergic N27 cells led to a significant decrease (approximately 60–70%) in 20S proteasome activity compared to the activity in untreated cells. Inhibition of proteasome activity triggers the formation and accumulation of ubiquitinated protein aggregates (Lam et al. 2002). N27 cells were treated with 0.5 mM paraquat for up to 48 h and the levels of ubiquitinated proteins were checked by Western blotting. While the expression of ubiquitinated proteins in untreated cells was low, paraquat treatment significantly increased the expression of ubiquitinated proteins in extracts isolated from dopaminergic N27 cells (Fig. 6).

Discussion

PD is characterized pathologically by the presence of misfolded protein deposits and inclusions in the neuronal cytoplasm (Lewy bodies) and loss of dopaminergic neurons, primarily in the substantia nigra pars compacta (Chinta and Andersen 2006; Chinta et al. 2006; Conway et al. 1998, 2000; Kruger et al. 1998; Polymeropoulos et al. 1997; Spillantini et al. 1997). The ER appears to be a focal point for protein alterations that result in neuronal dysfunction and death in PD. The accumulation of unfolded/misfolded proteins results in the failure of the ER to cope with the excess protein load, termed “ER stress” (Harding et al. 2002; Kopito 2000; Paschen et al. 2001; Rutkowski and Kaufman 2004; Sitia and Braakman 2003). Prolonged ER stress is coupled to specific caspase-dependent and independent death pathways as well as demonstrating cross-talk with the intrinsic and extrinsic apoptotic pathways (Bredesen et al. 2006; Riedl and Salvesen 2007).

In the more common sporadic forms of PD, the initiating causes of the neurodegenerative cascade are less well-defined, but probably involve increased levels of oxidative stress and impaired energy metabolism (Chinta and Andersen 2005; Chinta et al. 2006; McCormack et al. 2005). Such alterations have been shown to disrupt neuronal calcium homeostasis and may therefore feed into the same neurodegenerative cascade initiated by mutations in PD-associated proteins. Toxins including PQ, 6-hydroxydopamine (6-OHDA), and MPP^+ trigger the death of dopaminergic neurons in vitro and in vivo; and are widely used to model PD (Chinta and Andersen 2005; Chinta et al. 2006; Holtz and O'Malley 2003; McCormack et al. 2005; Ryu et al. 2002). Our data confirm that dopaminergic N27 cells are sensitive to PQ treatment. Elevation in the expression of the GRP family of proteins, phosphorylation of eIF2 α , induction of GADD153 protein expression, and inhibition of ER stress-induced cell death by salubrinal, all indicate the involvement of ER stress in triggering dopaminergic neuronal death. These results are in agreement with earlier studies demonstrating transcriptional upregulation of ER stress and unfolded protein response (UPR) specific proapoptotic genes following exposure to MPP^+ and 6-OHDA (Holtz and O'Malley 2003; Ryu et al. 2002).

Activation of caspases, inhibition of caspase-mediated cell death by a specific caspase inhibitor and cleavage of caspase substrates including PARP and p23, all indicate involvement of a caspase-dependent cell death pathway triggered by PQ. p23 is a highly conserved and widely expressed small co-chaperone protein that is typically associated with HSP90 protein and its chaperone activity prevents aggregation of denatured proteins (Bose et al. 1996; Felts and Toft 2003; Johnson et al. 1994; Johnson and Toft 1994; Zhu and Tytgat 2004). We had earlier reported that p23 undergoes a caspase-dependent cleavage during ER stress-induced apoptosis

triggered by various agents (Rao et al. 2006), suggesting that cleavage of p23 may be an indication of cells undergoing some form of cellular stress. In the present report we demonstrate that p23 is cleaved by caspase to yield a 19-kD product during ER stress-induced cell death triggered by PQ. Mutation of the p23D142N site not only blocked the cleavage by caspases and formation of the 19-kD product, but also attenuated the PQ-triggered cell death process, suggesting that ER stress-induced cell death triggered by various agents including PQ may proceed through a caspase-mediated cleavage of p23. Since cleavage of p23 is associated with increased cell death, it is possible that the 19-kD cleaved product displays pro-apoptotic activity analogous to cleaved protein fragments of Bcl-2 and Bid (Cheng et al. 1997; Li et al. 1998).

Inhibition of Ca^{2+} -ATPase activity resulting in cytosolic Ca^{2+} accumulation (Liu et al. 1998; Yu et al. 1999), disruption of protein disulfide bond formation (Nakamura and Lipton 2008; Uehara et al. 2006), and inhibition of the cellular proteasome activity triggering the accumulation of proteins destined for degradation (Bush et al. 1997; Fribley and Wang 2006; Ishii et al. 2007) are some of the mechanisms by which balance between ER protein synthesis and degradation is disrupted resulting in ER stress and cell death. Our data suggest that inhibition of 20S proteasome activity triggering the accumulation of ubiquitinated proteins may be one of the mechanisms by which PQ triggers ER stress-induced dopaminergic cell death. Thus, our results suggest the involvement of ER stress proteins, caspase-dependent cell death pathways, p23 cleavage, and inhibition of proteasomal activity in activating ER stress-induced cell death in dopaminergic N27 cells triggered by PQ. These results are also compatible with the notion of a link between PD, ER stress, and dopaminergic cell death.

Abbreviations

ER	Endoplasmic reticulum
Pcd	Programmed cell death
eIF2 α	Eukaryotic initiation factor-2 alpha
GRP	Glucose regulated protein
PD	Parkinson's disease (PD)

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Fig. 1.

PQ triggers ER stress and cell death in dopaminergic N27 cells. **(a)** N27 cells were either left untreated or treated with PQ (500 μM) for 12–48 h. Cells were gently lifted and washed once with phosphate-buffered saline. Cell extracts (100 μg protein) were analyzed by Western blot analysis for GRP78, GRP94, phospho-eIF2α, eIF2α, or GADD153. The eIF2α blot was probed with antiserum specific for phospho-eIF2α. Blots were re probed with GAPDH antiserum to assess equality of loading. Surviving versus apoptotic cells were quantified as described in the section “Experimental Methods”. Each blot is representative of four independent experiments. Positions of molecular mass markers (in kDa) are indicated at left. **(b)** The band density (integrated density value) is expressed graphically as a percentage ratio of densitometric optical density of the protein of interest to that of GAPDH with denotations of significance obtained from statistical analyses of pooled raw data. Data (mean ± SD) are from four independent experiments. **P* < 0.05 relative to the band density of the untreated control sample. **(c)** N27 cells were treated with different concentrations of PQ for 48 h. Surviving versus dead cells were analyzed by MTT assay as described in the section “Experimental Methods” and results are expressed as percent of untreated surviving cells. Data (mean ± SD) are from five independent experiments performed in triplicate

Fig. 2. Salubrinal protects cells against ER stress-induced apoptosis triggered by PQ. **(a)** N27 cells were either left untreated or exposed to PQ (500 μ M) in the presence or absence of Salubrinal (20 μ M). Surviving versus dead cells were analyzed by MTT assay as described in the section “Experimental Methods”, and results are expressed as percent of untreated surviving cells. Data (mean $\pm$ SD) are from four independent experiments performed in triplicate. **(b)** Sal treatment reduced the PQ-induced processing of caspase-7 from its zymogen (35 kDa) to active (20 kDa) form in N27 cells treated for 48 h. Blots were reprobed with GAPDH antiserum to assess equality of loading. Blot shown is representative of three independent experiments. Positions of molecular mass markers (in kDa) are indicated at left

Fig. 3.

PQ triggers cleavage of caspase substrates. N27 cells were treated with PQ (500 μ M) for the indicated times. Cell extracts (100 μ g of protein) were prepared as described in the section "Experimental Methods" and analyzed by Western blot analysis. Membranes were probed with anti-PARP antibody, rat specific anticleaved PARP antibody, or anti-p23 antibody. The PARP blot was probed with antiserum specific for cleaved PARP. Blots were reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of four independent experiments. Positions of molecular mass markers (in kDa) are indicated at left. The band density (integrated density value) is expressed graphically as a percentage ratio of densitometric optical density of cleaved PARP to that of GAPDH. Data (mean $\pm$ SD) are from four independent experiments. * P < 0.05 relative to the band density of the untreated control sample

Fig. 4.

p23 caspase mutant inhibits ER stress-induced cell death triggered by PQ. (a) Cell extracts were made from N27 cells either untransfected or transfected with wild-type Flag-p23 or Flag-p23D142N. Samples (150 μ g protein) were incubated with 50 ng/ml active caspase-7 at 37°C for 1 h. *Lane 1*, cell extract isolated from cells transfected with wild-type Flag-p23, *Lane 2*, cell extract isolated from cells transfected with wild-type Flag-p23 and incubated with active caspase-7, *Lane 3*, cell extract isolated from cells transfected with Flag-p23D142N construct, *Lane 4*, cell extract isolated from cells transfected with Flag-p23D142N construct and incubated with active caspase-7. Cell extracts (100 μ g protein) were analyzed by immunoblotting with anti-FLAG antibody to detect p23 and its cleavage product (19 kD). Blots

were stripped and reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of four independent experiments. Positions of molecular mass markers (in kDa) are indicated at left. **(b)** Uncleavable p23 (p23D142N) inhibits ER stress-induced cell death. N27 cells were either left untransfected or transfected with 6 μ g of pcDNA3, pcDNAFlag-p23, or Flag-p23D142N constructs. After 24 h, cells were either left untreated or exposed to PQ (500 μ M). Surviving versus dead cells were analyzed by MTT assay as described in the section “Experimental Methods” and data normalized to untreated non-transfected control set to 100%. Data (mean $\pm$ SD) are from four independent experiments. The expression levels of the transfected constructs were monitored by immunoblot analysis (inset) of both WTP23 and p23D142N. Blots were stripped and reprobed with GAPDH antiserum to assess equality of loading. Positions of molecular mass markers (in kDa) are indicated at left

Fig. 5.

Requirement of downstream caspases in ER stress-induced cell death triggered by PQ. **(a)** N27 cells were treated with PQ (500 μ M) for the indicated times. Cell extracts were prepared as described in the section "Experimental Methods" and analyzed by Western blot analysis. Membranes were probed with anti-caspase-3 antibody or anti-caspase-7 antibody. Blots were stripped and reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of four independent experiments. Positions of molecular mass markers (in kDa) are indicated at left. **(b)** N27 cells were treated with PQ (500 μ M) for 48 h in the presence or absence of the cell-permeable caspase specific inhibitor Q-VD-OPH (25 μ M). Cell extracts (50 μ g protein) from untreated or PQ-treated cells were incubated with 100 μ M benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD-AFC) peptide substrate. Caspase activity was determined by measuring the release of amino-4-trifluoromethylcoumarin from the synthetic substrate using continuous recording instruments as described in the section "Experimental Methods". Caspase activity was expressed as percent of control DEVDase activity per mg protein. Data (mean $\pm$ SD) are from four independent experiments performed in triplicate. $*P < 0.01$ relative to the activity of the PQ treated sample. **(c)** N27 cells were either left untreated or exposed to PQ (500 μ M) in the presence or absence of the cell-permeable caspase specific inhibitor Q-VD-OPH (25 μ M). Surviving versus dead cells were analyzed by MTT assay as described in the section "Experimental Methods" and results are expressed as percent of untreated surviving cells. Data (mean $\pm$ SD) are from four independent experiments performed in triplicate. $*P < 0.05$ relative to PQ treated sample

Fig. 6.

PQ inhibits 20S proteasomal activity. N27 cells were treated with PQ (500 μ M) for 48 h. Cell extracts (25 μ g protein) from untreated or PQ-treated cells were incubated with 10 μ M of the fluorophore LLVY-AMC. 20S proteasomal activity was determined by measuring the release of 7-amino-4-methylcoumarin (AMC) following cleavage of the labeled substrate. Fluorescence was measured in a Spectramax plate reader at excitation and emission wavelengths of 380 and 460 nm, respectively. Proteasomal activity was expressed as percent of relative fluorescence units of AMC from untreated cells (RFU units) per mg protein. Data (mean $\pm$ SD) are from four independent experiments performed in triplicate. $*P < 0.01$ relative to untreated control sample. Cell extracts (100 μ g protein) were analyzed by immunoblotting with anti-ubiquitin antibody. Blots were reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of three independent experiments. Positions of molecular mass markers (in kDa) are indicated at left. The band density (integrated density value) is expressed graphically as a percentage ratio of densitometric optical density of the ubiquitinated proteins to that of GAPDH. Data (mean $\pm$ SD) are from three independent experiments. $*P < 0.01$ relative to the band density of the untreated sample