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Endoplasmic Reticulum Stress–Induced Cell Death in Dopaminergic Cells: Effect of Resveratrol

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Abstract

Resveratrol, a naturally occurring polyphenol, exhibits antioxidant, antiaging, and anticancer activity. Resveratrol has also been shown to inhibit tumor initiation, promotion, and progression in a variety of cell culture systems. Earlier, we showed that paraquat, a bipyridyl herbicide, triggers endoplasmic reticulum stress, cell dysfunction, and dopaminergic cell death. Due to its antioxidant activity, we assessed the ability of resveratrol to rescue cells from the toxic effects of paraquat. While resveratrol did not have any protective effect at low concentrations, it triggered endoplasmic reticulum (ER) stress-induced cell death at higher concentrations (50–250 μ M). The present study was carried out to determine the mechanism by which resveratrol triggers ER stress and cell death in dopaminergic N27 cells. Our studies demonstrate that resveratrol triggers ER stress and cell dysfunction, caspase activation, p23 cleavage and inhibition of proteasomal activity in dopaminergic N27 cells. While over expression of uncleavable p23 was associated with decreased cell death, down-regulation of p23 protein expression by siRNA resulted in enhancement of ER stress-induced cell death triggered by resveratrol indicating a protective role for the small co-chaperone p23 in dopaminergic cell death.

Keywords

Endoplasmic reticulum; Resveratrol; ER stress; Caspase; Apoptosis; Programmed cell death

Introduction

The endoplasmic reticulum (ER) has several important functions in the cell including post-translational modification, folding, and assembly of newly synthesized secretory proteins; its proper functioning is essential for cell survival. As a membranous compartment associated with such critical roles mentioned above, the ER is extremely sensitive to changes that affect its structure, integrity, and function (Kaufman 1999; Ma and Hendershot 2002; Ron 2002; Sitia and Braakman 2003). Thus, any external or internal factors that impinge on ER structure and function will ultimately cause disruption in protein synthesis, translation, and folding resulting in unfolded or misfolded proteins. The accumulation of unfolded or misfolded proteins results in the failure of the ER to cope with the excess protein load which is termed “ER stress” (Harding et al. 2002; Kopito 2000; Paschen and Frandsen 2001; Rutkowski and Kaufman 2004; Sitia and Braakman 2003). Cells, in turn, activate the “unfolded protein response” (UPR) to avert ER stress. Prolonged ER stress and UPR activation completely overwhelms cellular protective mechanisms ultimately triggering cell death (Bakhshi et al. 2008; Bredesen et al. 2006; Egger et al. 2007; Rao et al. 2001; Rao et al. 2002a, b; Rao et al. 2004a, b; Rao et al. 2006). Using various ER stress inducers including thapsigargin, brefeldin, and tunicamycin, we have identified the roles of several ER stress-induced cell death modulators and effectors (Egger et al. 2007; Rao et al. 2004a, b; Rao et al. 2006).

In a recent study, we reported that dopaminergic N27 cells treated with paraquat (PQ) resulted in ER stress-induced cell death featuring p23 cleavage, caspase processing, activation of ER-associated pro-apoptotic Bcl-2 proteins and inhibition of proteasomal activity (Chinta et al. 2008). Similar phenomena were also observed in cells treated with MPP⁺ or rotenone (unpublished). We hypothesized that PQ and other Parkinson’s disease (PD)-associated toxins may trigger oxidative stress that in turn results in protein modification, misfolding, and impaired protein degradation (Andersen 2000, 2004; Mattson 2006; Rajagopalan and Andersen 2001) finally leading to ER stress-induced cell death. While searching for suitable compounds that could block the ER stress effects triggered by PQ or MPP⁺, we focused our attention on resveratrol, a well known anti-oxidant (Calabrese et al. 2008; de Almeida et al. 2008; Hwang et al. 2008; Rubiolo et al. 2008). To our surprise, while low concentrations of resveratrol were ineffective in rescuing dopaminergic cells from PQ or MPP⁺ toxicity, higher concentrations of resveratrol exacerbated ER stress and caspase-mediated programmed cell death (pcd). The present studies were performed to elucidate the pathways by which resveratrol triggers ER stress-induced cell death in dopaminergic N27 cells.

Experimental Procedures

Cells, Culture Conditions and Cell Extracts

Immortalized rat mesencephalic dopaminergic N27 cells were cultured in RPMI containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cells were derived from dopamine-producing neurons of the rat fetal midbrain via SV40 large T antigen immortalization and exhibit increased levels of tyrosine hydroxylase and dopamine transporter making them a good model system to study effects on the midbrain dopaminergic neurons selectively lost in PD (Adams et al. 1996; Clarkson et al. 1998; Zhou et al. 2000). PC12 rat pheochromocytoma cells were cultured in DMEM (Sigma) supplemented with 5% FBS and 5% horse serum. Human neuroglioma H4 cells (ATCC#HTB-148) were grown in a monolayer in DMEM containing 10% FBS and 1% penicillin/streptomycin. Microglia cells (BV-2) were

cultured in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin. Resveratrol (*trans*-3,4',5-trihydroxystilbene) was purchased from Sigma–Aldrich. A 20-mM stock solution of resveratrol was prepared in 75% ethanol and further diluted in cell culture medium to make working concentrations. Total cell extracts were prepared as previously described (Rao et al. 2001, 2002a, b). Briefly, untreated or resveratrol-treated cells were resuspended in RIPA buffer (50 mM Tris, pH 7.5, 0.5% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl) with 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 Coomassie plus protein assay reagent (Pierce). 100–200 µg of protein from total extracts was used for Western blotting.

Cell-free cytosolic extracts were prepared as previously described (Rao et al. 2001, 2002a, b, 2004a, b). Briefly, the untreated or resveratrol-treated cell pellet was resuspended in hypotonic extraction buffer, transferred to a 2-ml Dounce homogenizer and allowed to swell for 20–30 min on ice. Cells were lysed with 50 gentle strokes with a B-type pestle. The desired extent of lysis (>90%) was monitored under the microscope by trypan blue staining. The cell lysate was centrifuged for 30 min at 16,000×*g* (4°C). The supernatant was collected, protein concentration determined and 100–200 µg protein was used for caspase activity assays and to examine caspase cleavage by Western blotting.

Western Blotting

SDS-polyacrylamide gel electrophoresis (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), a 1:500 dilution of anti-phospho-eIF2 α rabbit monoclonal antibody, anti-eIF2 α mouse monoclonal antibody, anti-caspase-7, or anti-caspase-3 antibodies (all from Cell Signaling Laboratories), a 1:1,000 dilution of anti-p23 monoclonal antibody (BD Biosciences), or a 1:500 dilution of anti-GADD153 antibody (Santa Cruz). To confirm uniform loading of proteins across conditions, immunoblots were reprobed with a 1:100,000 dilution of anti-GAPDH rabbit polyclonal antibody (Research Diagnostics Inc). The films were scanned and the integrated optical density (OD) of the bands was estimated using the ChemiImager 4400 imaging system (Alpha Innotech Corp). The band density was expressed as a percentage ratio of densito-metric optical density of the protein of interest to that of GAPDH.

Plasmids, cDNA Transfection, siRNA Synthesis, and Transfection

Wild-type (WT) Flag-p23 and caspase mutant Flag-p23D142N cDNA were generated as described earlier (Rao et al. 2006). Transient transfections were performed using TransIT-Neural transfection reagent obtained from MIRUS Bio Corporation. Typically, 2×10⁶ cells were seeded into 10-cm dishes and transfected 1 day later with 6 µg of pcDNA3, WT p23 cDNA or p23D142N cDNA using a ratio of 1 µg:3 µl of DNA:TransIT-neural transfection reagent. The transfection efficiency using these conditions was approximately 50–60%. Small interfering RNAs (siRNA) were generated by in vitro transcription using the Silencer siRNA Construction Kit from Ambion as described earlier (Rao et al. 2004a, b, 2006). siRNAs were designed to target two or more regions of p23 based on predicted accessible (loop) and unique (specific) regions. The following siRNA sequences were designed to specifically target the mouse p23 gene: p23 (GenBank: AF153479) regions 419–439 (5' AAGTAGATGGAGCAGATGATG) and 446–466 (5'-AAGACATTTATTTATTTAAAAA). Transfection of siRNA was carried out as described earlier (Rao et al. 2006) using GeneSilencer siRNA transfection reagent (Gelantis) according to the manufacturer's instructions. To estimate the efficiency of the transfections, fluorescently labeled siRNAs targeting the luciferase gene (region 152–173) from the luciferase expressing vector pGL2-control

(Promega) were also used. Forty-eight hours after p23 siRNA transfection, cell extracts were prepared and subjected to SDS-PAGE and Western blot analysis.

In order to study the effect of ER stress on cells following p23 reduction, cells were transfected with p23 siRNA. Twenty-four hours after transfection, cells were exposed to 100 μ M resveratrol for 24 h. Resveratrol-induced cell death was quantified by MTT assay and cell death was determined as the percentage of live cells over the total number of cells.

Caspase Activity Assay

The fluorogenic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (BOC-DEVD-AFC; Enzyme Systems Products) was dissolved in DMSO as a 10-mM stock solution. Cell extracts (50–100 μ g of protein) from untreated or resveratrol-treated cells were incubated with 100 μ M peptide substrate. Q-VD-OPH (QVD, MP Biomedicals) was dissolved in DMSO according to the manufacturer's data sheet and further diluted with cell-culture media. Caspase activity was determined by measuring the release of amino-4-trifluoromethylcoumarin from the synthetic substrate using a continuous recording instrument 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 that is based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. Briefly, cell extracts (25–50 μ g of protein) from untreated or resveratrol-treated cells were incubated with 1 mM substrate at 37°C for 1 h. The free AMC fluorescence was quantified using 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 (Ellerby et al. 1997; Rao et al. 2001, 2002a, b). The cell pellet was resuspended in 1 $\times$ phosphate-buffered saline (PBS)/0.4% Trypan blue and cells were counted using a hemocytometer. Cell death was determined as the percentage of dead cells over the total number of cells. Statistical significance was determined by two-way analysis of variance (ANOVA).

In addition, the MTT assay was also performed according to the manufacturer's protocol (Sigma) for evaluation of cell death (Chinta and Andersen 2006). Briefly, cells were seeded into 24-well plates at a density of 50,000/well. Following treatment with resveratrol, 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 results were quantified by measuring the absorbance 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.

Evaluation of cell death by flow cytometry was performed as described (Bakhshi et al. 2008; Egger et al. 2007). Briefly, media and cells from untreated or resveratrol-treated samples were collected by trypsinization, gently spun down, and stained with 3 μ g/ml GFPAn-V and 5 μ g/ml propidium iodide in Annexin-V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), incubated at room temperature for 15 min, diluted in 200 μ l of Annexin-V binding buffer, and analyzed using a LSR flow cytometer (BD Biosciences). Data were processed with CellQuest Pro (BD Biosciences). This method appears to be more sensitive for measuring dying

and dead cells and may explain the slight difference in the viability of N27 cells at 24 h of resveratrol treatment as measured by this method and MTT assay (Fig. 1A–C).

Statistical Analysis

All experiments were performed at least three times unless otherwise indicated. Cell death assays and enzyme activity measurements were performed in triplicate and repeated independently three times. Results are expressed as mean $\pm$ SD. Data from cell death, enzyme activity assays and band densitometry were statistically analyzed using one-way ANOVA (GraphPad Prism), followed by between-group comparisons using the Newman–Keuls test. A value of $p < 0.05$ was considered statistically significant.

Results

Resveratrol Triggers ER Stress-Induced Cell Death in Dopaminergic Cells

The effect of resveratrol on the viability of dopaminergic N27 cells was examined by treating cells with different concentrations of resveratrol for 48 h (Fig. 1A) or with 100 μ M resveratrol for various time periods (Fig. 1B). As shown in Fig. 1, resveratrol induced both dose and time-dependent decreases in dopaminergic cell numbers as compared with untreated cells. We also tested the effects of resveratrol on other neural/glial cells. PC12, H4 neuro-glioma, and BV2 microglial cells were also susceptible to resveratrol-induced cell death that was typically associated at concentrations greater than 200 μ M (data not shown). Therefore, to determine the mechanism by which resveratrol triggers cell death, further studies were carried out on dopaminergic N27 cells.

To determine whether the decrease in dopaminergic N27 cell viability was attributable to apoptosis, we analyzed cell death by flow cytometry. Cells exposing phosphatidylserine which serves as a marker of apoptosis, can be labeled with a His-GFP-Annexin-V (GFPAn-V) fusion protein (Bakhshi et al. 2008; Egger et al. 2007) and loss of plasma membrane integrity is followed by uptake of propidium iodide (PI; Egger et al. 2007). Analysis of GFPAn-V/PI-stained N27 cells showed that treatment with resveratrol led to the exposure of phosphatidylserine and increased uptake of PI (Fig. 1C). These observations were quantified by flow cytometry of GFPAn-V/PI-stained cells. After 24 h of resveratrol treatment, flow cytometry analysis revealed that approximately 50% of the cells were GFPAn-V positive with 100 μ M resveratrol treatment (Fig. 1C). We believe that this method may be more sensitive for measuring dying and dead cells and may explain the slight difference in the viability of N27 cells at 24 h of resveratrol treatment compared to the cell viability by MTT assay (Fig. 1B).

ER stress is defined as an imbalance between the load of client proteins facing the ER and the organelle's ability to process that load (Rao et al. 2004a, b; Welihinda et al. 1999). This process is marked by the induction of protein expression of several ER stress sensor proteins including the GRP family of chaperone proteins (Liu et al. 1997; Rao et al. 2001, 2002a, b), phosphorylation of eIF2 α (Harding et al. 2002; Rutkowski and Kaufman 2004) and upregulation of proapoptotic GADD 153/CHOP protein expression. As shown in Fig. 2A and B, resveratrol treatment of dopaminergic N27 cells resulted in the induction of protein expression of GRP94, GRP78, and proapoptotic GADD153 and also triggered increased phosphorylation of eIF2 α suggesting that resveratrol triggers an ER stress response in dopaminergic N27 cells.

Salubrinal (Sal), an inhibitor of serine/threonine phosphatase PP1, blocks dephosphorylation of eIF2 α , and thereby inhibits ER stress-induced cell death. Sal also blocks cleavage of ER-specific caspase-12 and decreases misfolded protein aggregates in cells, thus lowering the

chronic ER stress response and increasing cell survival (Boyce et al. 2005; Reijonen et al. 2008; Smith et al. 2005; Sokka et al. 2007). To study the effect of Sal on resveratrol-treated N27 cells, we added Sal (15 μ M) in combination with resveratrol (100 μ M). As shown in Fig. 2C, Sal partially blocked ER stress-induced cell death triggered by resveratrol and significantly increased the survival of dopaminergic N27 cells.

Resveratrol Triggers Caspase-Dependent ER Stress-Induced Cell Death

We and others had earlier demonstrated that ER stress-induced cell death features caspase-7 cleavage and activation (Rao et al. 2001, 2002a, b) resulting in the generation of a large and small subunit (Riedl and Salvesen 2007). We tested whether caspase-7 and caspase-3 are cleaved during ER stress-induced cell death triggered by resveratrol. As shown in Fig. 3A, resveratrol treatment of dopaminergic N27 cells resulted in the processing and cleavage of caspase-7 and caspase-3. Cleavage of caspase-7 and caspase-3 was also associated with the generation of the cleaved form of the proteins.

Caspase activity measurements on cell extracts were also performed using Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-AFC) as a substrate. As shown in Fig. 3B, N27 cells treated with resveratrol activated caspases as demonstrated by increased DEVDase activity. Q-VD-OPH (QVD), a broad spectrum cell-permeable caspase inhibitor (Caserta et al. 2003; Dursun et al. 2006; Yang et al. 2004) significantly suppressed the DEVDase activity triggered by resveratrol. Similarly, treatment of dopaminergic N27 cells with resveratrol in the presence of QVD significantly enhanced cell viability (data not shown).

Expression of p23 in Resveratrol-Treated Dopaminergic Cells and Effect of RNA Interference

We previously demonstrated that p23, an HSP90 co-chaperone protein, plays a role in mediating ER stress-induced cell death; p23 is cleaved to yield a 19-kD product during ER stress-induced cell death and cleavage of p23 is associated with increased cell death (Rao et al. 2006). As shown in Fig. 4A, resveratrol treatment of dopaminergic N27 cells resulted in the processing of p23 and formation of the 19-kD cleaved fragment similar to our earlier observations (Rao et al. 2006). The antibody we employed recognized both full length p23 and the 19-kD cleaved product (Fig. 4A, top panel). In addition, we also generated a neo-epitope antibody that recognized only the cleaved fragment (19 kD) in resveratrol-treated samples and not the parent p23 (Fig. 4A, center panel).

We had also previously demonstrated that p23 is cleaved by caspases at a specific D142 site and that 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). We tested whether expression of caspase mutant p23 (p23D142N) would result in a reduction of dopaminergic N27 cell death triggered by resveratrol. Cells were either left untransfected or transfected with a mock vector, Flag-p23 or Flag-p23D142N expression construct. The efficiency of transfection was monitored by immunoblot analysis using anti-Flag antibody (Chinta et al. 2008; Rao et al. 2006). The protein expression of both WTp23 and p23D142N constructs was similar (data not shown). Following transfection, resveratrol was added to the cells and cell viability was measured 24 h later as described in Methods. As shown in Fig. 4B, while the expression of the mock vector or WTp23 did not result in a significant protection from resveratrol treatment, the caspase mutant p23 significantly suppressed dopaminergic N27 cell death triggered by resveratrol; there was a greater degree of protection in p23D142N transfected cells exposed to resveratrol as compared to mock or WTp23 transfected cells.

To complement the above results and to evaluate the effects of reducing p23 concentration in the cells, we used RNA interference (RNAi) to downregulate the expression of p23. Small

interfering RNAs (siRNAs) were designed to target two regions of the p23 gene based on predicted accessible (loop) and unique (specific) regions. As shown in Fig. 4C, a combination of siRNA targeting nucleotides 419–429 and 446–466 (lane 3) was very effective in reducing the expression of p23 protein (by approximately 85%). The siRNAs did not affect the expression of HSP90 suggesting their specificity. The effect of RNAi on ER stress-induced cell death was also studied by treating dopaminergic N27 cells with resveratrol after transfection with the p23 siRNAs. As shown in Fig. 4D, downregulation of p23 expression by siRNA rendered cells more susceptible to pcd triggered by resveratrol.

Resveratrol Inhibits 20S Proteasomal Activity

To determine whether proteasome activity is altered in dopaminergic N27 cells exposed to resveratrol, 20S proteasome activity was measured in cell extracts isolated from cells exposed to 100- μ M resveratrol for different time periods. Cell extracts were tested for the ability to hydrolyze the peptide substrate LLVY-AMC. As shown in Fig. 5A, resveratrol treatment of dopaminergic N27 cells led to a significant decrease (approximately 50%) in 20S proteasome activity compared to the activity in untreated cells. Inhibition of proteasome activity was also associated with increased expression of ubiquitinated proteins as analyzed by Western blotting (Fig. 5B).

Discussion

Misfolded proteins and associated ER stress are emerging as virtually constant features of neurodegenerative diseases including Parkinson's disease (Bredesen et al. 2006; Forman et al. 2003; Holtz and O'Malley 2003; Kitamura et al. 2002; Rao et al. 2004a, b; Ryu et al. 2002; Takahashi et al. 2003). The exact mechanism by which misfolded proteins trigger dopaminergic neuronal cell death in Parkinson's disease remains unclear. Toxins including MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) 6-OHDA, rotenone and paraquat trigger oxidative stress and death of dopaminergic neurons suggesting that dopaminergic neurons may be particularly vulnerable to oxidative challenges (Andersen 2004). Oxidative stress results in protein modification, increased protein misfolding, and impaired degradation (Andersen 2004; Mattson 2006) ultimately triggering ER stress-associated neuronal cell death (Bredesen et al. 2006; Rao et al. 2004a, b). Thus, it is imperative to discover small molecules that can either block misfolded protein toxicity or protect cells from chronic ER stress and cell death.

Resveratrol, found in various plants like grapes and berries, has been the focus of numerous in vitro and in vivo studies owing to its multifunctional properties including antioxidant and anti-inflammatory activities, anti-platelet aggregation effects, anti-atherogenic properties, growth-inhibiting activities, immunomodulation, and chemoprevention (Calabrese et al. 2008; de Almeida et al. 2008; Hwang et al. 2008; Rubiolo et al. 2008). More recently, work from several laboratories has provided interesting insights into the effects of this compound on the life span of yeasts, worms, and flies (Bauer et al. 2004; de la Lastra and Villegas 2005; Howitz et al. 2003; Wood et al. 2004) implicating the potential of resveratrol as an antiaging agent in treating age-related human diseases.

Recently, we reported that paraquat triggers ER stress-induced cell death in dopaminergic neurons (Chinta et al. 2008). Since paraquat is known to trigger oxidative stress leading to misfolded proteins and ER stress, we turned our attention to resveratrol to see if it could perhaps block PQ-induced toxicity and cell death. Instead of rescuing the cells and to our surprise, resveratrol actually triggered ER stress and dopaminergic cell death at concentrations of 25–100 μ M. The toxicity was not restricted to just dopaminergic N27 cells since other glial- and neural-like cells including PC12, BV-2 microglial cells, and H4 cells were also susceptible to resveratrol toxicity albeit at concentrations greater than 200 μ M. Upon exposure to resveratrol, dopaminergic N27 cells showed marked elevation in the expression of the GRP family of

proteins, phosphorylation of eIF2 α and induction of GADD153 protein expression, all indicating the involvement of ER stress. Inhibition of ER stress-induced cell death by a specific PeIF2 α phosphatase inhibitor salubrinal confirmed the involvement of ER stress in triggering dopaminergic N27 cell death.

Activation of caspases, inhibition of caspase-mediated cell death by a specific caspase inhibitor and cleavage of p23, all indicated involvement of a caspase-dependent cell death pathway triggered by resveratrol. While the significance of resveratrol induced p23 cleavage is still not clear, mutation of the p23D142N site attenuated the cell death process triggered by resveratrol. It is possible that p23 cleavage resulting in the 19-kD product abolishes the ability of p23 to act as an anti-apoptotic protein, thus, rendering dopaminergic cells more susceptible to pcd triggered by resveratrol (Rao et al. 2006). Similarly, the increase in apoptosis seen after downregulating p23 by siRNA supports the conclusion that p23 may play a protective role during ER stress.

Several cellular mechanisms exist by which the balance between ER protein synthesis and degradation is disrupted resulting in ER stress and cell death. Some of these include (a) inhibition of Ca²⁺-ATPase activity resulting in cytosolic Ca²⁺ accumulation (Liu et al. 1998; Yu et al. 1999), (b) disruption of protein disulfide bond formation (Nakamura and Lipton 2008; Uehara et al. 2006), and (c) 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). Our data suggest that inhibition of 20S proteasome activity triggering the accumulation of ubiquitinated proteins may be one of the mechanisms by which resveratrol triggers ER stress-induced dopaminergic cell death.

Recent reports suggest that resveratrol crosses the blood brain barrier and elicits its antioxidant properties thereby reversing oxidative and other forms of cellular stress (Blanchet et al. 2008; Bureau et al. 2008; Jin et al. 2008; Lu et al. 2008; Okawara et al. 2007; Pallas et al. 2008; Rocha-Gonzalez et al. 2008). The neuroprotective activity of resveratrol has been mostly demonstrated in slice cultures or animal models, and the concentration of resveratrol in the majority of these studies ranged from 10 to 100 μ M (Blanchet et al. 2008; Bureau et al. 2008; Okawara et al. 2007; Pallas et al. 2008). Recent studies have also demonstrated the neuroprotective effects of resveratrol in animals exposed to agents that cause degeneration of dopaminergic neurons and trigger PD like symptoms including MPTP and 6-OHDA (Jin et al. 2008; Lu et al. 2008). While resveratrol was partially able to reverse the toxic effects of these drugs, none of the above studies reported the direct effects of resveratrol alone on dopaminergic neurons. One study reported the toxic effects of resveratrol towards neurons at concentrations above 25 μ M in comparison to oxyresveratrol that seemed to be protective at all concentrations studied (Chao et al. 2008).

It is also interesting to note that while daily oral administration of transresveratrol to rats at doses up to 300 mg/kg of body weight for 4 weeks did not result in any apparent adverse effects, resveratrol-associated renal toxicity was observed at higher concentrations (Crowell et al. 2004; Juan et al. 2002). Similarly, there have been only a few controlled human clinical trials to date, and in these trials, resveratrol did not cause any notable adverse effects (Boocock et al. 2007; Walle et al. 2004). However, these studies did not focus on the effects in the brain. It also remains to be determined whether repeated dosing schedules can achieve higher systemic concentrations of resveratrol than those observed in the above study after a single dose and whether such high concentrations may trigger toxicity in the brain.

It is not clear from our data why neural cells and in particular dopaminergic cells are more sensitive to resveratrol toxicity. While our studies do not provide the exact mechanism by which resveratrol elicits its toxic effects, recent reports have indicated that red wine

polyphenols have a propensity to stimulate H₂O₂ production and formation of reactive oxygen species (Duarte et al. 2004) that in some settings may trigger cellular stress. It is possible that cellular stress including oxidative and ER stress-triggered toxicity in neural cells may arise due to a high metabolic rate and lower rate of neural cell turnover compared to cells from other tissues. Neural cells, in particular dopaminergic neurons, are particularly prone to oxidative stress due to their high rate of oxygen metabolism, low levels of antioxidants, and high iron content (Andersen 2000; Andersen 2004; Mattson 2006; Rajagopalan and Andersen 2001). In addition, modification of resveratrol to some unknown metabolites including resveratrol glucuronides and sulfates (Wenzel and Somoza 2005) that may be toxic to dopaminergic neurons may be another mechanism that contributes to neural cell susceptibility to resveratrol toxicity. More experiments are needed to demonstrate unequivocally the relationship of resveratrol and ER stress and further studies in this direction would be helpful in unraveling the exact mechanism by which resveratrol exhibits toxicity towards neural and in particular dopaminergic cells.

Abbreviations

ER, endoplasmic reticulum; pcd, programmed cell death; eIF2 α , eukaryotic initiation factor-2 alpha; GRP, glucose-regulated protein.

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Figure 1.

Resveratrol triggers cell death in dopaminergic N27 cells. N27 cells were either left untreated or treated with resveratrol for 48 h at different concentrations **a** or treated with 100 μM for 12–48 h. **b** Surviving versus apoptotic cells were quantified by the MTT assay as described in the Methods section and results are expressed as percent of untreated surviving cells. Data (mean ± SD) are from three independent experiments performed in triplicate. **c** Representative flow cytometry dot plots from cells stained with GFPAn-V/propidium iodide after 24 h of resveratrol treatment. The average cell counts listed in each dot-plot quadrant are the percent of annexin-V positive cells which represent dying and dead cells. Data are from four independent experiments performed in triplicate

Figure 2.

Resveratrol triggers ER stress-induced cell death in dopaminergic N27 cells. **a** Cell extracts (150 μ g protein) from untreated or resveratrol-treated (100 μ M) dopaminergic cells were analyzed by Western blot for GRP94(94 kDa), GRP78(78 kDa), GADD153(38 kDa), or eIF2 α (38 kDa). The eIF2 α blot was reprobed with antiserum specific for phospho-eIF2 α (40 kDa). Blots were reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of three independent experiments. **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 $\pm$ SD) are from four independent experiments. * $p < 0.05$ relative to the band density of the untreated control sample. **c** Salubrinal protects cells against ER stress-induced apoptosis triggered by resveratrol. Dopaminergic N27 cells were either left untreated or exposed to resveratrol (100 μ M) in the presence or absence of salubrinal (15 μ M). Surviving versus dead cells were analyzed by MTT assay as described in the Methods section and results are expressed as percent of untreated surviving cells. Data (mean $\pm$ SD) are from three independent experiments performed in triplicate. * $p < 0.05$ relative to the activity of the resveratrol treated sample

Figure 3.

Requirement of downstream caspases in ER stress-induced cell death triggered by resveratrol. **a** Cell-free extracts from dopaminergic N27 cells were prepared as described in the Methods section and analyzed by Western blot. Membranes were probed with anti-caspase-3 antibody or anti-caspase-7 antibody. Blots were reprobbed with GAPDH antiserum to assess equality of loading. Each blot is a representative of three independent experiments. **b** N27 cells were treated with resveratrol (100 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) 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 a continuous recording instrument as described in the Methods section. Caspase activity was expressed as percent of control DEVDase activity per milligram protein. Data (mean $\pm$ SD) are from three independent experiments performed in triplicate. * $p < 0.05$ relative to the activity of the resveratrol-treated sample

Figure 4.

Resveratrol triggers cleavage of caspase substrates. **a** Dopaminergic N27 cells were treated with resveratrol (100 μM) for the indicated times. Cell extracts (100 μg of protein) were prepared as described in the Methods section and analyzed by Western blot. Membranes were probed with anti-p23 antibody or neo-specific anti-p19 antibody. Blots were reprobed with GAPDH antiserum to assess equality of loading. Each blot is representative of three independent experiments. **b** p23 caspase mutant (p23D142N) inhibits ER stress-induced cell death triggered by resveratrol. Dopaminergic N27 cells were either left untransfected or were transfected with 6 μg of pcDNA3, wild-type Flag-p23 or Flag-p23D142N construct. After 24 h, cells were treated with 100 μM resveratrol for 24 h. Cells were gently lifted and washed once with PBS at room temperature. Surviving versus dead cells were analyzed by MTT assay as described in the Methods section and data were normalized to untreated non-transfected control set to 100%. Data (mean ± SD) are from three independent experiments performed in triplicate. **c** Transfection of siRNA-targeting p23. Small interfering RNAs (siRNAs) were designed to

target two regions of p23. Dopaminergic N27 cells were either left untransfected (*lane 1*) or co-transfected with a combination of two siRNAs (80 nM) designed to target the p23 gene transcript (nucleotides 419–429 and 446–466; *lane 3*) as described in Methods. To estimate the efficiency of transfection, fluorescently labeled siRNA targeting the luciferase gene was used (*lane 2*). Cells were gently lifted 48 h after siRNA transfection and washed once with PBS at room temperature. Cell lysates were immunoblotted with anti-p23 and anti-HSP90 antibodies. None of the siRNAs inhibited the expression of HSP90. Cell extracts were also probed with anti-GAPDH as a loading control. **d** Effect of RNA interference on ER stress-induced cell death. Dopaminergic N27 cells were co-transfected with a combination of two siRNAs (80 nM) designed to target the p23 gene transcript (nucleotides 419–429 and 446–466) as described in Methods. 24 h after transfection, cells were exposed to resveratrol (100 μ M) for 24 h. Cells were gently lifted and washed once with PBS at room temperature. Surviving versus dead cells were analyzed by MTT assay as described in the Methods section and data were normalized to untreated non-transfected control levels set to 100%. Data (mean $\pm$ SD) are from three independent experiments performed in triplicate

Figure 5.

Resveratrol inhibits 20S proteasomal activity and triggers accumulation of ubiquitinated proteins. **a** Dopaminergic N27 cells were treated with resveratrol (100 μ M) for the indicated times. 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 milligram protein. Data (mean $\pm$ SD) are from three independent experiments performed in triplicate; * p <0.05 relative to untreated control sample. **b** Dopaminergic N27 cells were treated with resveratrol (100 μ M) for the indicated times. Cell extracts (100 μ g protein) were analyzed by immunoblotting with anti-ubiquitin antibody