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Insulin-like Growth Factor-I Mediates Neuroprotection in Proteasome Inhibition-Induced Cytotoxicity in SH-SY5Y Cells

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Abstract

The proteasome is an enzyme complex responsible for targeted intracellular proteolysis. Alterations in proteasome-mediated protein clearance have been implicated in the pathogenesis of aging, Alzheimer's disease (AD) and Parkinson's disease (PD). In such diseases, proteasome inhibition may contribute to formation of abnormal protein aggregates, which in turn activate intracellular unfolded protein responses that cause oxidative stress and apoptosis. In this study, we investigated the protective effect of Insulin-like Growth Factor-I (IGF-1) for neural SH-SY5Y cells treated with the proteasomal inhibitor, Epoxomicin. In SH-SY5Y cells, Epoxomicin treatment results in accumulation of intracellular ubiquitinated proteins and cytochrome c release from damaged mitochondria, leading to cell death, in Epoxomicin time- and dose-dependent manner. In cells treated with small amounts of IGF-1, the same dosages of Epoxomicin reduced both mitochondrial damage (cytochrome c release) and reduced caspase-3 activation and PARP cleavage, both of which are markers of apoptosis. Notably, however, IGF-1-treated SH-SY5Y cells still contained ubiquitinated protein aggregates. This result indicates that IGF-1 blocks the downstream apoptotic consequences of Epoxomicin treatment leading to decreased proteasome function. Clues as to the mechanism for this protective effect come from (a) increased AKT phosphorylation observed in IGF-1-protected cells, vs. cells exposed to Epoxomicin without IGF-1, and (b) reduction of IGF-1 protection by pretreatment of the cells with LY294002 (an

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inhibitor of PI3-kinase). Together these findings suggest that activation of PI3/AKT pathways by IGF-1 is involved in IGF-1 neuroprotection against apoptosis following proteasome inhibition.

Keywords

Apoptosis; Epoxomicin; IGF-1; SH-SY5Y cells; Ubiquitin; Proteasome inhibition

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder that results from the death of dopaminergic neurons in the substantia nigra. Increasing evidence indicates that ubiquitin-proteasome system (UPS) dysfunction leading to accumulation of aberrant or misfolded proteins may activate intracellular stress responses that damage mitochondria, leading in turn to oxidative and nitrosative stress. At present, this sequence of events represents the principal molecular events leading to sporadic and familial forms of PD (Moore et al., 2005). In patients with nonfamilial (sporadic) PD, the 26S/20S proteasomes of neurons in the Substantia Nigra pars compacta (SNc) show both structural and functional deficits (McNaught & Olanow, 2003). More recent work has shown that systemic exposure of rats to synthetic and naturally occurring proteasome inhibitors results in a syndrome that closely parallels many cardinal features of PD, including progressive Parkinsonism, selective neurodegeneration of the nigrostriatal pathway, and the formation of Lewy Body-like intracytoplasmic inclusions, containing α -synuclein and ubiquitin (McNaught et al., 2004). However, difficulty in reproducing this *in vivo* model has also been reported (Kordower et al., 2006). Thus, although these latter results point to the 26S/20S proteasomes as central players in PD development, they also highlight the need for a careful study of neurotoxic mechanisms activated in cultured neurons treated with proteasome inhibitors.

Most cases of PD occur sporadically and are of unknown etiology. However, over the last decade several genetic mutations were found to be responsible for several different forms of inherited PD. These include mutations in the α -synuclein, UchL-1, and parkin genes (Polymeropoulos, 1997; Kitada, 1998; Leroy, 1998). Both of these mutations decrease the proteolytic function of the ubiquitin-proteasome system (PS), resulting in impaired protein turnover in substantia nigra dopaminergic neurons. Subsequent work demonstrated that similar impairment of the proteasomes in these cells occurs in sporadic forms of PD (McNaught et al., 2002).

Pharmacological inhibition of the proteasome is sufficient to cause cell death in primary neuron cultures (Boutillier et al., 1999; Canu et al., 2000; Keller et al., 2000; Qui et al., 2000) and neuronal cell lines (Lopes et al., 1997; Keller et al., 2000b), suggesting that proteasome inhibition may play a central role in diseases involving neuronal loss (Ding and Keller 2001). Proteasome inhibition causes mitochondrial dysfunction, decreased glutathione (GSH) levels, and increased generation of free radicals in cultured mesencephalic dopaminergic neurons (Kikuchi et al., 2003). This effect of proteasomal inhibition is likely to be self-perpetuating, because proteasome activity is inhibited under conditions of oxidative stress (Reinheckel et al., 1998; Okada et al., 1999). Defects in the UPS participate in the pathogenesis of several neurodegenerative disorders (McNaught et al., 2001, 2002); thus, the UPS is an attractive target for therapeutic intervention in PD.

Insulin-like growth factor-1 (IGF-1) is a polypeptide hormone that is essential for the development of the nervous system (Zackenfels et al., 1995). Receptors for IGF-1 are distributed over most neuroanatomical regions of the adult brain (Kar et al., 1993). IGF-1

regulates cell growth, differentiation, and survival in cultured neuronal cell lines, and it is among the most potent anti-apoptotic growth factors present in eukaryotic cells (D'Ercole et al., 1996). IGF-1 protects cells against different kinds of stressors (Lackey et al., 2000). For example, IGF-1 prevents oxidative apoptosis that otherwise would follow treatment of cultured neurons with nitric oxide, hydrogen peroxide (Tamatani et al., 1998a; Heck et al., 1999), peroxynitrite (Saeki, 2002), high glucose concentrations (Cheng and Feldman, 1998; Vestling et al., 2001; Gustafsson et al., 2004), salsolinol (a dopamine-derived tetrahydroisoquinoline) (Shavali et al., 2003) and okadaic acid (Xing et al., 2005). In Alzheimer's disease, IGF-1 regulates the clearance of A β from the brain through carrier-mediated transport (Carro et al., 2002), and it protects cultured neurons against amyloid β -protein-induced death (Dore et al., 1997; Wei et al., 2002).

Protection of several cell types from apoptosis caused by anisomycin, tumor necrosis factor- α (Okubo et al., 1998) and corticosterone (Nitta et al., 2004) has been linked to activation of the PI3/AKT pathway. In the present studies, therefore, we asked whether IGF-1 protects SH-SY5Y neuroblastoma cells against apoptosis induced by an irreversible proteasome inhibitor Epoxomicin. We chose to use SH-SY5Y cells as model dopaminergic neurons because they express tyrosine hydroxylase (TH) and secrete dopamine. The data show that proteasome inhibition by Epoxomicin treatment of these cells causes apoptosis, even at extremely low concentrations. Apoptosis induced by Epoxomicin is prevented, if the cells are pretreated with IGF-1. In these cells, the PI3/AKT is likely activated in an IGF-1 time-dependent fashion. It seems likely that IGF-1 or other agents that activate the PI3/AKT pathway can reduce or limit neurotoxicity following abnormal accumulation of proteins due to proteasome inhibition.

Materials and Methods

Reagents

Minimum essential medium (MEM) and F-12 nutrient mixture (HAM) were purchased from Gibco BRL (Gaithersburg, MD), and fetal bovine serum was from ATCC (Manassas, VA). Recombinant human IGF-1, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Epoxomicin were obtained from Sigma (St. Louis, MO), and 2', 7'-dichlorodihydrofluorescein diacetate (DCF-DA) from Invitrogen (Eugene, OR).

Cell Culture

Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 5% CO₂ at 37° C in MEM:Ham's F12 (1:1) supplemented with 10% fetal bovine serum, penicillin (100units/ml), and streptomycin (100 μ g/ml). Cells were sub-cultured weekly. All experiments were performed between passages 25–35 and ~ 80% cell confluence. The cells were seeded onto 60 mm tissue culture dishes or into multi-well plates. The cell culture medium was replaced every 2 to 3 days.

Proteasome Inhibition

Epoxomicin is a natural product isolated from an Actinomycetes species (Meng et al., 1999). This agent is a cell-permeable, potent, highly specific, and an irreversible inhibitor of the chymotrypsin-like (CT-L), trypsin-like (T-L), and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities of 26S/20S proteasome (Meng et al., 1999). It inhibits by modifying the proteasomal catalytic subunits LMP-7, MECL1, and Z. This drug does not affect the activities of non-proteasomal cellular proteases such as trypsin, cathepsin B, or chymotrypsin (Meng et al. 1999; Princiotta et al., 2001). SH-SY5Y cells were treated with Epoxomicin at different concentrations and for different lengths of time, as noted in each figure and legend. Epoxomicin was dissolved in dimethyl sulfoxide (DMSO). To avoid

misrepresentation and maintain reproducibility, an equivalent amount of vehicle (DMSO) was added to all controls.

Cell Viability

Morphological changes produced by the addition of Epoxomicin, IGF-1 and LY294002 to the cell cultures were followed throughout the experiments by phase-contrast microscopy. Changes in cell viability were tracked, and final cell viability data collected after each treatment regimen, by measuring the conversion of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide] to formazan. The cells were treated with 0.25 mg/ml MTT in fresh media for 2h at 37° C. The medium was removed and the formazan precipitate was solubilized with DMSO at 37° C for 30 minutes. By transferring 100ul aliquots to 96-well plates, optical densities were measured using a microplate reader at 540 nm absorption.

Proteasome Activity Assay

Proteasome activity was quantitatively assessed using the 20S proteasome assay fluorescent kit, according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). Briefly, SH-SY5Y cells were grown in a 96-well plate to a density of $0.6\text{--}0.7 \times 10^6$ cells/well, and then treated with 0, 25, 50, 100 and 200nM concentrations of Epoxomicin for 24 hours. The plate was then centrifuged at $500 \times g$ for five minutes, the culture medium aspirated, and the cells washed with 200 μ l of the 20 S proteasome assay buffer. Next, 100 μ l of the lysis buffer was added to each well, and the plate placed in an orbital shaker for 30 minutes at room temperature. The plate was then centrifuged at $1,000 \times g$ for 10 minutes, and 90 μ l of the supernatant from each well was transferred to corresponding wells in a black 96-well plate. Thereafter, 10 μ l of assay buffer and 10 μ l of the substrate (SUC-LLVY-AMC) were added to each well, and the plate was incubated at 37°C for one hour. Finally, the plate was read using a SpectraMax MS (Molecular Devices, CA) at 360nm (excitation) and 480nm (emission). The results are reported as mean $\pm$ SEM.

Signaling Pathway and Western Blot Analysis

Changes in amounts of cell signaling intermediates were measured by Western blotting. Protein homogenates were prepared as follows: cells were lysed in ice-cold lysis buffer (50mM Tris-HCL (pH7.5), 100mM NaCL, 0.1% Triton X-100, 0.1% SDS 1mM EDTA) containing protease and phosphatase inhibitor cocktail (1:100, Sigma, (P8340)). Clear lysates were obtained by centrifugation at 4°C for 20 minutes at 13000 rpm in a refrigerated microcentrifuge. Protein concentrations were determined using the Micro-BCA assay (Pierce, Rockford, IL) according to the manufacturer's instruction. Equal amounts (20 g) of the protein samples were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membranes, blocked either for 1hour at room temperature or overnight at 4°C with Tris buffer saline containing 0.05% Tween 20 (TBST, pH 7.4) and 5% (w/v) nonfat dried milk. In some instances, proteins samples were run on precast gels (10%) purchased from Biorad. After several washes with TBST, membranes were incubated with specific primary antibodies for 1 hour at room temperature or overnight at 4°C, after which the membranes were washed again and incubated for another 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized via chemiluminescence (ECL), (GE Healthcare), according to the manufacture's instructions. Band densities were analyzed by densitometry using the NIH image software. The antibodies used in these experiments were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) - Ubiquitin (sc-9133), Cell Signaling (Beverly, MA) - p-AKT (9271) and Caspase-3 (9661), Biovision- Cytochrome c (3026) and PARP (3001), and Sigma (St. Louis, MO) - β -Actin (A5441).

Oxidative Stress, Mitochondrial Membrane Potential and Apoptosis

Generation of Reactive Oxygen Species (ROS) was estimated with the fluorescent probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCF-DA), as previously described by Maffi et al. (2008). Cells were grown to 70–80% confluency in 4-well Nunc cover glass chambers, and then were treated either with DMSO alone (control) or 100nM Epoxomicin, for 24 hours. Thirty minutes prior to terminating the experiment, DCF was added at a final concentration of 10 μ M. Cells were then washed once with PBS and immediately imaged using a Leica DMI 6000 epifluorescence microscope equipped with a 20 $\times$ objective and a FITC filter set. Exposure times for image capture were attenuated to minimize photobleaching and phototoxicity to the cells. For reproducibility and comparison purposes, all experimental conditions as well as microscope settings were kept identical among all the experiments.

To measure mitochondrial membrane potentials, cells were plated on glass chambers and treated with vehicle, Epoxomicin and IGF-1, alone or in combination, and labeled with a 20nM final concentration of tetramethylrhodamine methylester (TMRM) for 15 minutes. Multiple random images of each treatment group were captured using an Olympus FV1000 confocal microscope equipped with a 543nm laser and 63 $\times$ oil objective. Image processing and data analysis was done with an NIH Image-J software plugin designed specifically for mitochondrial membrane potential studies (Nernst plugin developed and provided by Optical Imaging Core, UTHSC, San Antonio). All images were generated in the Optical Imaging Core Facility at the MRD, ERAHC.

To detect and quantitate early apoptotic markers such as cytochrome c release, cytosolic proteins were prepared as described by Bianchi et al (2003) and Lu et al (2003). Briefly, cultured cells subjected to experimental treatments were collected and washed twice with PBS. The cell pellets were then resuspended in extraction buffer (220mM mannitol, 58mM sucrose, 50mM PIPES-KOH (pH7.4), 50mM KCL, 5mM EDTA, 2mM DTT, plus protease inhibitor cocktail). After 30–45 minutes incubation on ice, the cells were homogenized by 20 strokes with a glass dounce homogenizer. The homogenate was then centrifuged at 10,000 $\times$ g for 15 minutes. The supernatants were collected and subjected to protein quantitation, followed by Western blot analysis using an anti-cytochrome c antibody, as noted above.

Data analysis

Data were analyzed using one-way analysis of variance and Tukey's comparison test, using either the Instat biostatistic program (GraphPad software) or Sigma Plot. Data are presented as the mean $\pm$ SEM of at least three independent experiments. In the figures, asterisks indicate the degree of significance when compared with the controls, such as * $P < 0.05$ and ** $P < 0.01$, respectively.

Results

Proteasome inhibition induces accumulation of ubiquitinated proteins, followed by apoptosis

SH-SY5Y cells were treated with low stepwise concentrations of Epoxomicin for 24 hrs. As noted by others for cells treated with high (μ M) concentrations of Epoxomicin (Ding and Keller 2001, Koch et al., 2009), the treated cells contained increased amounts of ubiquitinated proteins (data not shown). In our first experiments with SH-SY5Y cells treated with epoximicin, however, we noted that proteasome inhibition and cell death occurred at nM concentrations of the drug (as mentioned in Feuillet et al., 2005). For all experiments in the current study, therefore, we used low doses of Epoxomicin. Figure 1A shows that treatment of cultured SH-SY5Y cells with 10–200nM Epoximicin decreases cell survival in

a dose dependent manner, as evidenced by MTT assay. Figure 1B demonstrates that proteasome activity, measured at the end of the 24h incubation, falls dramatically with increasing concentrations of Epoxomicin. For instance, as little as 25nM of Epoxomicin inhibited proteasome activity in these cells to less than 50% of control values. After treatment of SH-SY5Y cells at different Epoxomicin doses for either 4 and 24 hours, Western blotting analysis using an anti-ubiquitin antibody showed that higher doses of Epoxomicin in the nM range (over 100nM) caused marked accumulation of ubiquitinated protein (Fig. 1C). Figure 1C also shows that longer periods of exposure to Epoxomicin at various concentrations resulted in increased accumulation of ubiquitinated proteins. Hence, for almost all successive experiments, we used 100nM Epoxomicin to determine the time course effect on protein ubiquitination. Fig. 1D shows that accumulation of ubiquitinated proteins occurs as early as 5 minutes after addition of Epoxomicin to the cultures, with a maximal effect plateau at 2–4 hours of exposure. When cells were treated for longer periods of time (8 or 24 hours), further accumulation of high molecular weight ubiquitinated proteins, and a concurrent loss of some low molecular proteins, occurs (Fig. 1D).

Proteasome inhibition leading to abnormal protein aggregation has been reported to activate intracellular stress responses that lead to mitochondrial damage and oxidative stress, detectable as cytochrome c release in neuronal cells, loss in mitochondrial membrane potential and increased free radical generation (Qiu et al, 2000, Ding and Keller, 2001). Cytochrome c release is an early marker for apoptosis. We therefore asked whether proteasome inhibition under our low Epoxomicin-short time course conditions causes the release of cytochrome c. A representative western blot and corresponding densitometry graph of three independent experiments in Fig. 2A shows that cytochrome c release is detectable in SH-SY5Y cells treated with 100nM Epoxomicin, beginning as early as 15 min and increasing to 8 hours of incubation. Figure 2B along with the densitometry plot shows that cleavage activation of the pro-apoptotic enzymes caspase-3 and poly (ADP-ribose) polymerase (PARP), both of which are markers of late apoptotic events, from their respective native forms are also a hallmark of late apoptotic signaling events, is detectable by 12 hours after initiation of treatment with Epoxomicin, leveling off at 18–24 hours of exposure. The Western blot in Fig. 2C shows that cells treated with 100nM Epoxomicin for 24 hours contain significantly increased amounts of both caspase-3 active fragments (20/17-kDa) as well as PARP cleavage products. The densitometry graph in 2C is plotted and shows an almost 4–5 fold increased levels for relative intensities of the caspase-3 20 kDa fragment and the PARP 85 kDa fragment normalized to β -actin values. In Fig. 2D the same cells (100nM Epoxomicin at 24 hours) show significantly elevated amounts of intracellular ROS, reflecting increased oxidative stress in the cells.

IGF-1 protects against Epoxomicin toxicity, via pathways that involve PI3/AKT phosphorylation

We next asked whether we could prevent or reverse Epoxomicin-induced cell death by pretreating SH-SY5Y cells with the neuroprotective mitogen, IGF-1. Fig. 3A shows that treatment of cells with 20nM IGF-1 for 30 minutes prior to Epoxomicin exposure promotes cell survival. This is evident from morphological assessments, as shown in representative images (Fig. 3A) of cells treated with 100nM Epoxomicin alone or pretreated with 20nM IGF prior to 100nM Epoxomicin. The corresponding MTT assay in Fig. 3B demonstrates that 50nM and 100nM Epoxomicin treatments (each for 24hrs) decrease cell viability to 63.2% and 32.6%, respectively, while pretreatment with IGF-1, in both cases, raises cell viability to 92.9% and 54.5%, respectively. The data here also suggest that IGF-1 is more effective as a protective agent with lower concentrations of the proteasome inhibitor. Notably, this protective effect of IGF-1 vanishes if the cells are pretreated with the PI3/AKT inhibitor LY294002 for 30 minutes, prior to IGF-1 and Epoxomicin exposure. The rescue

effect of IGF-1 is more dramatic at the lower dose (50nM) of Epoxomicin when compared to the higher dose of 100nM.

IGF-1 pretreatment suppresses proteasome inhibition-induced cell death

In order to confirm that IGF-1 inhibits apoptosis caused by Epoxomicin, SH-SY5Y cells were cultured for 18 hours in the presence or absence of IGF-1, followed by Epoxomicin treatment for 24 hours. IGF-1 alone has a short half-life, however, presence of certain binding proteins may prolong its half life. Thus, 18 hour pretreatment with IGF-1 ensures that all the IGF-1 added is used up in the signaling pathways. Cytosolic protein lysates were then prepared and analyzed by Western blotting and tested for cytochrome c release, described in the "Methods" section. The Western blots and densitometry plots in Fig. 4A show that IGF-1 significantly reduces cytochrome c release following Epoxomicin treatment in these cells, though the degree of protection is noticeably reduced at high (100nM) concentration of the inhibitor. Since cytochrome C release is a test of mitochondrial stability and function, we also measured the mitochondrial membrane potential, or $\Delta\psi_m$ (Fig 4B). In cells treated with 100nM Epoxomicin treatment for 24 hours, there was a significant loss in membrane potential, from 108 to 80 $\Delta\psi_m$. In cells pretreated with IGF-1 prior to Epoxomicin exposure, membrane potential values remained normal (Fig. 4B). To confirm that this cell survival mechanism involves prevention of apoptosis, lysates were prepared from cells cultured with or without a low dose of 20nM IGF-1 for 18 hours, followed by treatment with 100nM Epoxomicin for 24 hours. The Western blots in Fig. 4C show that pretreatment of the cells with IGF-1 prior to Epoxomicin reduces caspase-3 and PARP cleavage.

IGF-1 increases cell survival via PI3/AKT pathway

Having established the effect of IGF-1 as a neuroprotective agent in our model system, we set out to delineate the underlying mechanism of its action. Our key rationale for pre-treating with IGF-1 was that neuroprotective factors in the brain are likely to act via signaling pathways, rather than via direct effects, on participants in the natural stresses that arise in neural cells, e.g. via the proteasomes themselves (if the problem is proteasome inhibition during a disease process). The PI3-kinase/AKT pathway is one of the main pathways stimulated by IGF-1 signaling, and is known to play an important role in neuronal cell growth and survival (Xia et al., 1995). Hence, we next investigated the effect of IGF-1 on this signaling pathway and as a potential therapeutic agent against neurotoxicity caused by proteasome inhibition. To validate the role of AKT phosphorylation -Serine 473 (p-AKT) in IGF-1 signaling, lysates were then prepared and subjected to Western blotting analysis. Figure 5A shows that while the addition of IGF-1 to the cells causes a dramatic increase in the expression of p-AKT, this effect is abolished in the presence of the PI3K inhibitor (LY294002) which blocks AKT phosphorylation. As Fig. 5B and accompanying densitometry plot of three experiments illustrates, IGF-1 stimulates AKT/protein kinase B phosphorylation, and this effect is observed for up to 24 hours. On further examination of the densitometry graph, we also note that although the peak levels of Akt phosphorylation Serine 473 (P-AKT) occur between a broad window of 30 minutes up to 2hrs, although a significant effect on AKT phosphorylation remains well above control levels until 24 hours.

Interestingly, our results indicate that although IGF-1 induces a reduction in basal protein ubiquitination levels in SH-SY5Y cells (Fig 6A), it did not affect Epoxomicin-induced protein ubiquitination, despite its protective effect for the cells. This result implies that abnormal protein aggregates are not themselves the causes of apoptosis in Epoxomicin-treated cells; rather, that the intracellular responses to such aggregates are. Cells were treated with 100nM Epoxomicin for 24 hours and show an insignificant change in AKT phosphorylation when compared to controls, which is induced significantly by IGF-1, as

seen in Figs. 6A (bottom panel). We often observe detect a double band for p-AKT, especially with IGF-1 treatments. The corresponding densitometry analysis of Fig. 6A shows a significant decrease of IGF-induced AKT phosphorylation in the presence of Epoximicin when compared with IGF treated cells alone. However, a significant increase in p-AKT levels still exists when cells are treated with IGF-1 prior to Epoximicin exposure (6B). Taken together, the data suggests that activation of PI3K/AKT signaling represents a potential mechanism for regulation of neuronal protection in SH-SY5Y cells against proteasome inhibition.

Discussion

The ubiquitin-proteasome system is an essential component of intracellular quality control. The activities of its components, and their coordination, decline with age, and they are also impaired in neurodegenerative syndromes, e.g. Alzheimer's and Parkinson's disease (Lopez Salom et al., 2000 and Keller et al., 2000). It is therefore crucial that we develop therapies that may reduce or eliminate cytotoxicity and death associated with neurodegenerative diseases involving abnormal accumulation of ubiquitinated proteins.

In the present study, SH-SY5Y human neuroblastoma cells, which are a well-characterized *in vitro* model of Parkinson's disease, were used to investigate mechanisms for neuroprotection in cells whose proteasomes have been inhibited. Our aim was to delineate the mechanism of proteasome inhibition-mediated cell death, and to determine how and to what extent IGF-1 exerts its protective effect as a signaling molecule. In previous work by others (with SH-SY5Y and other cell lines), using higher concentrations of Epoximicin to elicit proteasome inhibition (Koch et al, 2009), cell death in the treated cells has been shown to be due to oxidative stress (Ding and Keller, 2001, Papa and Rockwell, 2008). Our present findings suggest that even very low concentrations of Epoximicin cause cell death (Fig 1 and 2), although this is not likely to be due directly to increased accumulation of ubiquitinated proteins; instead, events initiated by this accumulation activate pathways that trigger apoptosis, as marked by cytochrome c release from the mitochondria (Fig 2), activation of caspase-3, stimulation of PARP cleavage, as well as a significant loss in cell viability (Fig. 3). In this study, pretreatment with IGF-1 caused an increase in overall cell survival by preventing apoptosis. Our data are consistent with recent work showing that IGF-1 overexpressing mice can eliminate oxidized protein from the cortical brain regions (Crowe et al., 2009). Together, the data suggests that IGF-1 is likely to protect neurons from apoptosis induced by proteasome inhibition. Here it is also important to emphasize that the extent of IGF-1 protection diminishes with increasing concentrations of the proteasome inhibitor, as evidenced by loss in net cell viability. IGF-1 pretreatment accompanied with 100nM Epoximicin exposure did not prevent cytochrome C release, despite an almost complete recovery in mitochondrial membrane potential and suppression of PARP and Caspase 3 cleavage, thus suggesting a continuation of mitochondrial/cellular dysfunction.

IGF-1 is a critical survival factor for neurons (Saeki et al., 2002). It has been reported to protect neuronal cells from both oxidative stress and apoptosis induced by various stimuli (Cheng and Feldman, 1998; Vestling et al., 2001; Shavali et al., 2003; Xing et al., 2005). To determine how IGF-1 protects neuronal cells under one type of stressful stimulus (proteasome inhibition), we initially asked whether its protective effect under these circumstances might involve PI3/AKT signaling. AKT has received widespread attention as an important anti-apoptotic protein through (Kenchappa et al., 2004). In addition, recent work has shown that PI3/AKT activation occurs in neuroprotection against various insults (Encinas, et al., 1999; Wei et al., 2002; Halvorsen et al., 2002; Jiang and Yu, 2005; Hsuan et al., 2006). It has been shown that AKT activation can either interact directly with apoptotic signaling pathways or with transcription factors to promote cell survival (Datta et al., 1999).

In data shown here, exposure of SH-SY5Y cells to IGF-1 induced phosphorylation of AKT. On the other hand, inhibition of the PI3K pathway by LY294002 blocked this AKT phosphorylation and considerably reduced all IGF-1-mediated neuroprotection. Thus, one likely function of IGF-1 activates PI3/AKT pathways to prevent intracellular stress responses that otherwise would cause apoptosis via Epoxomicin-induced oxidative stress.

Cleavage activation of caspase-3, leading to activation of PARP, are final steps in the execution process of apoptosis, and their inhibition can prevent apoptotic cell death (Qiu et al., 2000). In accordance with earlier work showing that proteasome inhibition induces apoptosis (Feuillet et al 2005, Papa et al., 2007), we also found that Epoxomicin causes apoptosis in SH-SY5Y cells, in association with mitochondrial dysfunction and oxidative stress.

Although IGF-1 reversed the apoptotic effect of Epoxomicin at low concentrations, it did not affect the accumulation of poly-ubiquitinated proteins in the treated cells (Fig 6). The results presented here suggest that IGF-1 blocks signals generated downstream of the accumulated proteins targeted for turnover. Taken together, the data suggests that IGF-1 is likely to protect neurons from apoptosis induced by proteasome inhibition. It will be of further interest to determine whether the accumulation of poly-ubiquitinated proteins in the absence of cell death results in the formation of Lewy Body-like aggregates in the SH-SY5Y cells. It is likely that proteasome inhibition-induced toxicity may not only be mediated by protein ubiquitination alone, but may also block certain survival factors or signaling pathways. Based on our results, the activated PI3/AKT pathway by IGF-1 may have a critical and compensatory role in overriding the damage caused by proteasome inhibition. Our data suggests that PI3/AKT signaling pathway participates in IGF-1-mediated neuroprotection against proteasome inhibition-induced cytotoxicity. Inhibiting this pathway significantly reversed the rescue effects mediated by IGF-1. Moreover, the data presented here also show that IGF-1 affords only partial protection to cells exposed to higher concentrations of Epoxomicin, thereby also indicative of a cell death mechanism independent of Akt phosphorylation and caspase activation.

In summary, proteasome inhibition by Epoxomicin induced SH-SY5Y cells to undergo apoptosis, in association with cytochrome c release, activation of caspase-3 leading to PARP cleavage, mitochondrial dysfunction and apoptosis. To some extent, the potent anti-apoptotic small molecule IGF-1 enhances cell survival in this UPS-deprived human neuronal model. The mechanism by which this protection occurs involves the PI3/AKT pathway, prevention of mitochondrial dysfunction and oxidative stress, and overall protection from apoptosis. Notably, IGF-1 did not affect abnormal accumulation of ubiquitinated proteins in Epoxomicin treated cells, even though the cells were protected; thus, IGF-1 appears to activate PI3/AKT pathway elements that protect Epoxomicin-treated cells from the consequences of ubiquitinated protein accumulation. IGF-1 could provide a novel and promising therapeutic strategy for the treatment of diseases or toxic injuries induced by failure of the UPS, including AD and PD.

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Abbreviations used

AD	Alzheimer's Disease
CT-L	Chymotrypsin-like
DMSO	Dimethyl sulfoxide
ECL	Chemiluminescences
IGF-1	Insulin-like growth factor-1
MEM	Minimum essential medium
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl etrazolium bromide]
PARP	poly adenosine diphosphate-ribose polymerase
PD	Parkinson's Disease
PGPH	Peptidyl-glutamyl peptide hydrolyzing
SNc	Substantia Nigra pars compacta
TH	Tyrosine Hydroxylase
T-L	Trypsin-like
UPS	Ubiquitin-proteasome system

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Fig. 1. Prolonged exposure of SH-SY5Y cells to Epoxomicin induces cell loss and accumulation of ubiquitinated proteins

(A) SH-SY5Y cells were treated with 0, 10, 20 25, 50, 100, 150 and 200nM concentrations of Epoxomicin for 24hr. Cell viability was measured by MTT assay as described in the “Methods” section. Results are expressed as percentage mean $\pm$ SEM of controls (treated with DMSO alone) and are for four independent experiments. The level of significance is denoted as * $P < 0.05$, ** $P < 0.01$, respectively. (B) Cells were treated as described in (A) with varying concentrations of Epoxomicin for 24 hours, and chymotrypsin-like activity (proteasome activity) was measured using a fluorogenic assay. A dose-dependent loss in proteasomal activity was observed, with less than 5% activity remaining in cells treated with 100nM and 200nM of Epoxomicin. (C) Cells were treated as described above for 4 and 24 hours, after which lysates were prepared and analyzed by Western blotting analysis to detect and measure amounts of ubiquitinated proteins. Actin served as the loading control. (D) Accumulation of ubiquitinated proteins is also a function of time, as determined by Western blot analysis, wherein SH-SY5Y cells were treated with 100nM Epoxomicin from 0 min – 4 hours, as well as for longer durations of 8 and 24 hours. Actin served as the internal loading control.

Fig. 2. Proteasome inhibition by Epoxomicin causes mitochondrial damage, generates oxidative stress and causes apoptosis of SH-SY5Y cells

(A) SH-SY5Y cells were treated with 100nM Epoxomicin for 0, 15min, 30min, 1h, 2h, 4h and 8h, and cytosolic proteins were collected in a specific sucrose-PIPES extraction buffer (containing protease inhibitor cocktail) as detailed in the “Methods” section. Cytochrome c release into the cytosol from mitochondria was detected and quantitated by Western blotting, and the densitometry graph plotted as relative densities of protein bands normalized to actin levels. Actin served as the loading control. (B) To detect late stage apoptosis, cells were treated with 100nM Epoxomicin for 0, 1, 2, 4, 8, 12, 18 and 24 hours, and total protein lysates prepared from each culture for Western blot analysis of cleavage of the PARP protein from its 116kDa native length to its 85kDa fragment. The right panel is a densitometry graph of the 85kDa cleaved PARP as normalized to actin expression. Actin served as the internal control. (C) SH-SY5Y cells were treated with 100 Epoxomicin for 24 hours, and caspase-3 and PARP cleavage (both endpoint apoptotic markers) were detected and measured by Western blot analysis. The right panel is a densitometry graph of cleaved PARP (85kDa) as normalized to actin expression. (D) Cells were treated in the absence and presence of 100nM Epoxomicin for 24 hours, loaded with a fluorescent probe DCF-DA for 30 minutes and then imaged using a widefield microscope to assess the augmentation of oxidative stress. The left panel shows SH-SY5Y cells treated with the DMSO solvent, showing low background levels of ROS. The right panel shows cells treated with Epoxomicin, which emit increased amounts of fluorescence, reflecting elevated intracellular ROS levels. Both panels represent images from three independent experiments. Densitometry graphs show mean values $\pm$ SEM of three independent experiments. The level of significance is denoted as * $P < 0.05$.

Fig. 3. IGF-1 protects SH-SY5Y cells from toxicity caused by Epoxomicin proteasome inhibition, and induces AKT phosphorylation

(A) Morphological changes in SH-SY5Y cell cultures were compared after treatment with: DMSO (diluent control), 100nM Epoxomicin for 24hours (EPX), 20nM IGF-1 treatment for 24 hours, pretreatment with 20nM IGF-1 for 30min followed by 100nM Epoxomicin for 24 hours (I + EPX), 20uM LY294002, 24 hours (LY), and cells treated with 20uM LY294002 for 30 min prior to pretreatment with 20nM IGF-1 30 min, followed by 100nM Epoxomicin for 24hours (LY + IGF + EPX). (B) Cells were treated with 50nM or 100nM Epoxomicin for 24hours, and their viability was determined by MTT assay as described in the “Methods” section. Results are expressed as percentage means $\pm$ SEM of controls (treated with DMSO alone) and are averaged data for four independent experiments. Level of significance is denoted as $**P<0.01$.

Fig. 4. IGF-1 pretreatment inhibits apoptosis in SH-SY5Y cells treated with Epoxomicin (A) Western blot analysis for cytochrome c expression (reflective of mitochondrial damage) was performed using cytosol lysates from three groups; control SH-SY5Y cells, cells treated with 50nM (top) or 100nM (bottom) Epoxomicin, and cells pretreated with 20nM IGF-1 followed by exposure to either 50nM or 100nM Epoxomicin for 24hours. Right panels depict densitometry graphs plotted as relative values of cytochrome c expression normalized to actin expression levels. Actin served as the loading control. (B) To assess mitochondrial function, cells were treated with; vehicle- DMSO diluent control, 100nM Epoxomicin, alone for 24 hours, 20nM IGF-1 alone, and 20nM IGF-1 treatment followed by exposure to 100nM Epoxomicin for 24hours. Mitochondrial membrane potential ($\Delta\psi_m$) fluorescence intensity plots were generated after loading cells with 20nM TMRM and imaged using a confocal microscope. (C) Lysates were collected from cells treated as follows: DMSO (control), IGF-alone, 100nM Epoxomicin for 24 hours, and pretreatment with 20nM IGF-1, followed by 100nM Epoxomicin for 24 hours. Western blotting analysis was performed to identify and compare the extent of PARP and Caspase -3 cleavage in the differently treated cultures. Actin served as the loading control. Blots are representative of three independent experiments. The level of significance is denoted as * $P < 0.05$, ** $P < 0.01$, respectively.

Fig. 5. IGF-1 primarily activates the PI3/AKT pathway within minutes

(A) SHSY5Y cells were treated as follows: 20 μ M LY294003 alone for 2 hours, treated with 20nM IGF-1 for 2 hours, or pretreated with 20 μ M LY294003 for 30 min, followed by 20nM IGF-1 for 2 hours. Cell lysates were used for western blot analysis to determine that IGF-1 regulates phosphorylation of AKT. (B) Western blotting analysis of phosphorylated vs. unphosphorylated AKT in SH-SY5Y cells treated with 20nM IGF-1 for 0, 15min, 30min, 1h, 2h, 4h, 8h and 24h. The densitometry plots show that p-AKT/AKT ratios rise to peak levels at 30 minutes to 2 hours, and remain high as a function of time, even after 24 hours of IGF-1 treatment. The level of significance is denoted as * $P < 0.05$.

Fig. 6. IGF-1 has no effect on accumulation of ubiquitinated proteins in Epoxomicin-treated SH-SY5Y cells

(A) SH-SY5Y cells were treated as follows: DMSO (control), 20nM IGF-1 alone for 18h, 100nM Epoxomicin-24hours, and pretreatment with 20nM IGF-1 for 18 hours prior to 100nM Epoxomicin exposure for 24 hours. Lysates were collected and Western blot analysis performed, both to detect and compare amounts of accumulated ubiquitinated proteins, and to measure p-AKT/AKT ratios. Actin served as the loading control. (B) Densitometry analysis of the P-AKT blots shown in (A). The P-AKT intensities (arbitrary units) were normalized to the corresponding actin band. Blots are representative of three independent experiments. * $P < 0.05$ denotes level of significance.