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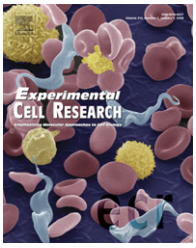

Research Article

Inhibition of endoplasmic reticulum stress counteracts neuronal cell death and protein aggregation caused by N-terminal mutant huntingtin proteins

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ABSTRACT

Accumulation of abnormal proteins occurs in many neurodegenerative diseases including Huntington's disease (HD). However, the precise role of protein aggregation in neuronal cell death remains unclear. We show here that the expression of N-terminal huntingtin proteins with expanded polyglutamine (polyQ) repeats causes cell death in neuronal PC6.3 cell that involves endoplasmic reticulum (ER) stress. These mutant huntingtin fragment proteins elevated Bip, an ER chaperone, and increased Chop and the phosphorylation of c-Jun-N-terminal kinase (JNK) that are involved in cell death regulation. Caspase-12, residing in the ER, was cleaved in mutant huntingtin expressing cells, as was caspase-3 mediating cell death. In contrast, cytochrome-c or apoptosis inducing factor (AIF) was not released from mitochondria after the expression of these proteins. Treatment with salubrinal that inhibits ER stress counteracted cell death and reduced protein aggregations in the PC6.3 cells caused by the mutant huntingtin fragment proteins. Salubrinal upregulated Bip, reduced cleavage of caspase-12 and increased the phosphorylation of eukaryotic translation initiation factor-2 subunit-α (eIF2α) that are neuroprotective. These results show that N-terminal mutant huntingtin proteins activate cellular pathways linked to ER stress, and that inhibition of ER stress by salubrinal increases cell survival. The data suggests that compounds targeting ER stress may be considered in designing novel approaches for treatment of HD and possibly other polyQ diseases.

Introduction

HD is a neurodegenerative disorder that belongs to the family of polyQ diseases [1]. HD is caused by the expansion in the first exon of the *huntingtin* gene that leads to the formation of intracellular and intranuclear aggregates of misfolded huntingtin protein [1,2]. HD is characterized by the selective loss of neurons, particularly in the striatum, but the underlying mechanisms causing cell death are not fully understood [1,3]. Previous studies in cell cultures and in brain tissue in mouse models of HD suggest that caspase-dependent and caspase-independent pathways are both activated during cell death [3].

Mitochondria play a crucial role in the control of cell death and apoptosis in most cells including neurons [4,5]. Apart from

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mitochondria, other cell organelles take part in cell death and its regulation [6]. Recently, alterations in the function of the endoplasmic reticulum (ER) membrane have been shown to be involved in cell stress and in different types of human disorders [7–9]. The ER exerts an important function in lipid and protein metabolism and regulates intracellular calcium levels and cell stress responses [7,10]. Dysfunction in the ER can be caused by variety of genetic and environmental factors and toxins that leads to abnormal protein folding, transport and breakdown with changes in calcium homeostasis that impairs cell viability [7–10]. Prolonged ER stress is linked to induction of specific signaling pathways that are normally under the control of ER sensor proteins, such as the chaperone, Bip/Grp78 (Bip) [11,12]. These cellular pathways include the PRK-like ER protein kinase/pancreatic eIF2α kinase (PERK/PEK), the activating transcription factor-6 (ATF6), and the inositol-requiring enzyme-1 (IRE1) that in complex cascades influence gene transcription and may cause cell degeneration [13,14].

Among proteins involved in cell death regulation, the leucine zipper-containing transcription factor Chop/Gadd153 is activated during ER stress [15,16]. Chop is translocated to the cell nucleus after ER stress and activates transcription of different genes, including DR5 that is a membrane bound death receptor belonging to the tumor necrosis factor-α superfamily of receptors [15,16]. ER stress is also associated with the cleavage of caspase-12 that resides in the ER, and other cellular caspases, including caspase-9 and caspase-3 involved in cell death execution [17–21]. Previous studies have shown that huntingtin and huntingtin-associated protein 1 can influence calcium signaling mediated by the inositol-(1,4,5) triphosphate receptor type 1 in the ER, and that apoptosis signal-regulating kinase 1 (ASK1) is essential for ER stress-mediated neuronal death induced by expanded polyQ repeats [22,23].

In this work, we have studied the role of ER stress in the pathogenesis of HD by the expression of N-terminal huntingtin fragment proteins with expanded polyQ tract. Expression of these proteins in neuronal PC6.3 cells was found to induce ER stress with elevation of Bip, cleavage of caspase-12 and the activation of different ER signaling pathways including the Chop and the c-Jun-N-terminal kinase (JNK) pathways. Inhibition of ER stress by the compound salubrinal [24,25] reduced ER stress and counteracted cell death caused by the mutant huntingtin proteins. Salubrinal also reduced aggregates of these proteins in PC6.3 cells, suggesting that inhibition of ER stress may directly or indirectly regulate the accumulation of protein aggregates in the cell and in the membrane.

Experimental procedures

Cell culture and cell viability

PC6.3 cells were cultured on Nunc dishes in RPMI-1600 (Biochrom) medium supplemented with 10% fetal calf serum and 5% horse serum. Cells were transfected with expression vectors encoding various CAG-repeat lengths of huntingtin exon-1 fused to EGFP [26]. Transfectin reagent (BioRad) was used following the instructions provided by the vendor using 0.5 μg DNA per 24 well plates, and 4 μg per 6 well plates. Controls were transfected with EGFP expression plasmid (Clontech) and cells were incubated for 24–30 h, fixed for 20 min using 4% paraformaldehyde (PFA), and nuclei were stained for 1 min with Hoechst 33342 (4 μg/ml, blue color; Sigma). Cells were examined under a Zeiss fluorescence microscope and the number of transfected cells with distorted or condensed nuclei was counted as an estimate of cell death [21,25,27]. In each experiment, about 100 cells in four different fields per well using triplicates cultures were counted, and experiments were repeated more than three times. Results are expressed as percentage of mock-transfected controls. Statistical analyses were done using Students *t*-test or ANOVA together with the Bonferoni post hoc test. To study the role of ER stress, 5 μM salubrinal was added 2 h after transfection and cells were analyzed as above. In some experiments, 0.5 μg/ml Brefeldin-A inducing ER stress and 200 ng/ml cycloheximide inhibiting protein synthesis were also used.

Immunocytochemistry

PC6.3 cells plated on collagen coated coverslips were transfected and fixed as above followed by incubation for 1 h using 0.1% Triton X-100 in phosphate based saline (PBS), and 5% bovine serum albumin (Sigma) [27]. Following washing, primary antibodies were added overnight. These included anti-cytochrome-*c* (diluted 1:200, BD Biosciences), anti-AIF (1:200, Santa Cruz Biotechnology), anti-Chop (1:200; Santa Cruz), anti-ATF6 (1:100, Pierce), anti-p-eIF2α (1:100, Cell Signaling) and anti-p-PERK (1:100, Santa Cruz), anti-active-caspase-3 (1:150, Cell Signaling) and anti-caspase-12 (1:200, Chemicon). Cells were then washed with PBS, and incubated for 2 h using Cy2- and Cy3-conjugated secondary antibodies (1:300, Jackson Laboratories, CA, USA). Cells were counterstained for 1 min using Hoechst 33342 blue (4 μg/ml, Sigma), and mounted in gel mounting medium (Sigma). Controls without primary antibodies showed no positive staining. For double staining, mouse anti-cytochrome-*c* antibodies were combined with rabbit anti-Hsp-60 antibody (1:300, Stressgen), a mitochondrial marker, followed by incubation with Alexa 596 and Cy5-conjugated secondary antibodies (1:500, Molecular probes). Cells were visualized using a Zeiss fluorescence microscope or a Zeiss LSM 510 Meta confocal microscope.

Western blots

Cells were lysed in either RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl and 0.1% SDS, pH 8.0), 1× SDS loading buffer (62 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM DTT, 0.01% bromophenol blue) or in the PARP lysis buffer (62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% 2-mercaptoethanol) [21,25,27]. Protein concentrations were determined (BioRad) and an equal amount of protein was subjected to SDS-PAGE, and blotted onto a nitrocellulose filter (Amersham).

Filters were blocked for 1 h in 5% milk-TBS followed by an overnight incubation at + 4 °C using primary antibodies. These included anti-EGFP (diluted 1:5000, Roche), anti-BiP/Grp78 (1:1000, BD Biosciences), anti-p-eIF2α (1:3000, Cell Signaling), anti-Chop (1:500, Santa Cruz), anti-p-JNK (1:250, Cell Signaling),