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Polyglutamine expansion disturbs the endoplasmic reticulum formation, leading to caspase-7 activation through Bax

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ABSTRACT

The endoplasmic reticulum (ER) plays a pivotal role in cellular functions such as the ER stress response. However, the effect of the ER membrane on caspase activation remains unclear. This study reveals that polyglutamine oligomers augmented at ER induce insertion of Bax into the ER membrane, thereby activating caspase-7. In line with the role of ER in cell death induced by polyglutamine expansion, the ER membrane was found to be disrupted and dilated in the brain of a murine model of Huntington's disease. We can conclude that polyglutamine expansion may drive caspase-7 activation by disrupting the ER membrane.

1. Introduction

Apoptosis is necessary for development and tissue homeostasis, but its dysfunction can initiate disorders such as cancer and neurodegenerative diseases [1]. Apoptosis is mediated by caspases, which are activated by signals from the plasma membrane and mitochondria. Caspase-9 activity is regulated by the apoptosome that contains Apaf-1 and cytochrome c, whose release from mitochondria is controlled by members of the Bcl-2 family [2]. Research reveals that the endoplasmic reticulum (ER) is a site that initiates caspase activation [3,4], as demonstrated in the experiment where translocation of cell death abnormal (CED)-4 protein to the nuclear envelope was shown to be crucial for CED-3 activation in the nematode worm *Caenorhabditis elegans* [5]. Caspase-7 (gene symbol *CASP7*) is an effector caspase, whose activated form is observed in the microsomal fraction after apoptotic stimuli (incidentally, an inactivating mutation of *CASP7* is found in human cancers) [6,7].

Polyglutamine (polyQ) expansion is a mutation that increases the length of a polyglutamine sequence in a normal protein. The unusual length of polyQ causes conformational changes in the protein, and consequently pathological changes within the cell. PolyQ expansion is observed in some diseases, such as Huntington's disease (HD). PolyQ expansion may be caused by several aberrations in axonal transport, the ubiquitin–proteasome system, or transcription [8]. These anomalies cause neuronal cell death through caspase activation initiated via aggregation of the polyQ-containing protein [9] and via p53-mediated mitochondrial mechanism of apoptosis (cytochrome c release) [10]. An HD-related protein, huntingtin (gene symbol *HTT*), contains a short sequence targeting it to ER [11]. A study on laboratory animals revealed that concanavalin A immunoreactivity of ER membranes is increased *in vitro* in striatal neurons that express mutant Htt^{Q111} [12]. At present, the role of the ER membrane in caspase activation by polyglutamine remains obscure.

In this study, we tested whether polyglutamine expansion affects conformation of the ER membrane and its ability to regulate caspase-7 activity.

2. Materials and methods

2.1. Materials

We purchased the following antibodies and chemicals: anti-FLAG (M2) and anti-α-tubulin antibodies were from Sigma Aldrich (St. Louis, MO); thapsigargin was from Sigma Aldrich and Research Biochemicals International (Natick, MA); anti-BiP (grp78) antibody was from Stressgen (Victoria, BC, Canada); anti-calreticulin

Abbreviations: Apaf-1, Apoptotic protease activating factor 1; cb5, cytochrome b5; polyQ79, polyQ82, polyglutamine repeats containing proteins with 79 or 82 glutamine residues; xbp-1, x-box binding protein 1; FRET, fluorescence resonance energy transfer.

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antibody was from Abcam (Cambridge, MA); anti-cleaved caspase-7 and anti-lamin A/C antibodies were from Cell Signaling Technology (Beverly, MA); anti-huntingtin (mEM48) antibody was from Chemicon (Temecula, CA); anti-Bax antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-SRP54 antibody was from Proteintech Group (Chicago, IL); anti-HA (HA.11) antibody was from COVANCE (Richmond, CA); HRP-conjugated anti-rat, anti-mouse, and anti-rabbit IgG (H + L) antibodies were from Southern Biotech (Birmingham, AL); and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (H + L) and Cy3-conjugated anti-chicken IgY antibodies were from Jackson ImmunoResearch (West Grove, PA). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Sigma Aldrich. The R6/2 HD exon 1 transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

2.2. DNA constructs

The transmembrane domain of cb5 (amino acids 100–134, AF007108), which contains a static ER retention signal [13], was fused to murine Bcl-xL lacking its C-terminal region (amino acids 1–211, L35049) to generate ER-targeted Bcl-xL (ER-Bcl-xL). ER-Bcl-xL tagged with FLAG was subcloned into the pBabe-Puro retroviral vector. ER-targeted Bax (ER-Bax) was generated by fusing human Bax lacking its C-terminal region (amino acids 1–171, L22473) to cb5, which had been subcloned into the pcDNA6/myc-His (Invitrogen, Carlsbad, CA) or pEGFP-N3 (Clontech, Mountain View, CA) vector. ER-polyQ82 was generated by fusing polyQ82 to cb5, which had been fused to mRFP. polyQ82 was subcloned into the pEYFP-N1 and pECFP-C1 vectors (Clontech) for the FRET assay. The vector pmRFP-Mito was generated by substituting mRFP for EYFP in pEYFP-Mito (Clontech). The polypeptide pQ79-APER is a chimeric type I transmembrane protein that contains the N-terminal 79 repeat polyglutamine (pQ79), the transmembrane region of the amyloid precursor protein (APP), YFP, a nuclear localization signal (NLS), and the ER retrieval motif of p28Bap31 at the cytosolic side (APER). APER, which lacks the pQ79 part of pQ79-APER, was used as a control. Seventy-nine-CAG repeat was inserted within the 3′ untranslated region of the pEGFP vector to generate a CAG repeat RNA expression plasmid, named GFPstopCAG80.

2.3. Reverse transcription PCR (RT-PCR)

RT-PCR was performed according to a previously described procedure [14]. PCR of xbp-1 and β-actin was performed for 25 cycles. Splicing of xbp1 mRNA was detected by electrophoresis using a 3% agarose 21 gel (Wako). The following PCR primer pairs were used: rat xbp-1, 5′-GCTTGTGATTGAGAACCAGG-3′ and 5′-GAGGCTTGGTGTATACATGG-3′ and β-actin, 5′-GTTTGAGACCTTCAACACC-3′ and 5′-GTGGTGGTGAAGCTGTAG-3′.

To detect mRNA of transfected plasmids, total RNA was purified from HEK293T cells after transfection with GFPstopCAG80 or GFP-GST and ER-mRFP, and then treated with RNase-free DNase (1 U; Promega, Madison, WI) for 30 min at 37 °C. We synthesized cDNA by reverse transcription using an oligo$(dT)_{20}$ primer. The following primer pair was used in the PCR to amplify GST and CAG-repeat cDNAs (GST cDNA from the GFP-GST plasmid; 80 CAG-repeat cDNA from the GFPstopCAG80 plasmid): 5′-TGCCCGACAACCACTACCTGAG-3′ and 5′-TAAAGCAAGTAAAACCTCTAC-3′.

2.4. Cell culture and induction of cell death

Induction of cell death was performed as described previously [3]. To generate PC12 cells stably expressing ER-Bcl-xL, the cells were infected with the viral supernatant generated by transfection of the pBabe-Puro vector (control) and ER-Bcl-xL/pBabe-Puro vector (ER-Bcl-xL) in the presence of 8 μg/ml polybrene (Sigma). Infected cells were selected via growth in the presence of 1.5 μg/ml puromycin (Sigma). Time lapse analysis was performed 6 h after transfection with polyQ82-GFP and ER-mRFP using an inverted fluorescence microscope (Axio Observer.Z1 with AxioCaMRm and PM-S1 incubator system; Carl Zeiss, Oberkochen, Germany). HEK293 Tet-Off Advanced Cell Line (Clontech) was transfected with 18 μg of pTRE-Tight-HA-polyQ82-GFP and 0.9 μg of pTK-Hyg by the calcium phosphate method. Selection of stable cell clones was initiated 50 h after the transfection using hygromycin B (250 μg/ml; Wako). For induction of HA-polyQ82-GFP, the cells were washed twice with HBSS (−), and then incubated in doxycycline free medium.

2.5. Immunostaining

Immunocytochemical and immunohistochemical analyses were performed as described previously [15]. PolyQ82-GFP and ER-mRFP were cotransfected into HEK293T cells and rat primary hippocampal neurons using a Lipofectamine 2000 and by the calcium phosphate DNA precipitation method, respectively. Mouse brains were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) followed by incubation in 15% sucrose. Sections (14 μm) were incubated with mEM48 and anticalreticulin antibodies, followed by FITC-conjugated anti-mouse or Cy3-conjugated anti-chicken immunoglobulin antibodies. Samples were examined under an inverted fluorescence microscope (Axiovert 200; Carl Zeiss) or a confocal microscope (LSM510; Carl Zeiss).

2.6. Western blotting

Western blotting was performed as described previously [14]. Quantification of signals was performed using a LAS-4000 (Fujifilm).

2.7. Transmission electron microscopy

Mouse brains (control and R6/2 mice) were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After postfixation with 1% osmium, samples were embedded in Epon-812. Ultrathin sections were obtained with glass knives on a Porter Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT) and collected onto copper mesh grids. The sections were analyzed using transmission electron microscopy (Hitachi H-800) after staining with 0.1% uranyl acetate in sodium acetate buffer and lead salts.

To combine fluorescence and electron microscopy, polyQ82-GFP-transfected HEK293T cells, which were cultured on coverslips with grids, were fixed in 1.5% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min. After examination using confocal microscopy, the cells were fixed with 1% osmium and 1.5% potassium ferrocyanide for 50 min on ice, dehydrated, and the embedded in Epon-812.

2.8. The FRET assay

HEK293T cells were sonicated in cold PBS (−) supplemented with protease inhibitors 24 h after transfection with CFP-polyQ82, CFP-YFP fusion, or CFP-polyQ82 plus polyQ82-YFP plasmids by the calcium phosphate DNA precipitation method. Supernatants were separated by centrifugation. Congo red (100 μM) was added to the supernatant 6 h after the transfection. The reported FRET value is the ratio of emission at 538 nm to emission at 485 nm after excitation at 440 nm on a fluorometer (Fluoroskan Ascent).

2.9. Alkaline treatment

The microsomal fraction was harvested and treated with 100 mM sodium carbonate pH 11.5 at 0 °C for 30 min, and