Biochemical and Biophysical Research Communications 377 (2008) 550–555

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ER stress is the initial response to polyglutamine toxicity in PC12 cells

Hitoshi Nakayama [a,*], Masashi Hamada [b], Nobuhiro Fujikake [c], Yoshitaka Nagai [c], Jing Zhao [a], Osamu Hatano [d], Koji Shimoke [b], Minoru Isosaki [a], Masanori Yoshizumi [a], Toshihiko Ikeuchi [b]

[a] Department of Pharmacology, Nara Medical University School of Medicine, Kashihara, Nara 634-8521, Japan
[b] Laboratory of Neurobiology, Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering and High Technology Research Center (HRC), Kansai University, Suita, Osaka 564-8680, Japan
[c] Division of Clinical Genetics, Department of Medical Genetics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
[d] Department of Anatomy, Nara Medical University School of Medicine, Kashihara, Nara 634-8521, Japan

ARTICLE INFO

Article history:
Received 16 September 2008
Available online 12 October 2008

Keywords:
ER stress
PC12 cells
Polyglutamine
Proteasome
Ubiquitin
UPS

ABSTRACT

Persistent endoplasmic reticulum (ER) stress and impairment of the ubiquitin–proteasome system (UPS) cause neuronal cell death. However, the relationship between these two phenomena remains controversial. In our current study, we have utilized an expanded polyglutamine fusion protein (polyQ81) expression system in PC12 cells to further examine the involvement of ER stress and UPS impairment in cell death. The expression of polyQ81-induced ER stress and cell death. PolyQ81 also induced the activation of c-Jun N-terminal kinase (JNK) and caspase-3 and an increase in polyubiquitin immunoreactivity, suggesting UPS impairment. ER stress was induced prior to the accumulation of polyubiquitinated proteins. Low doses of lactacystin had almost similar effects on cell viability and on the activation of JNK and caspase-3 between normal cells and polyQ81-expressing cells. These results suggest that ER stress mediates polyglutamine toxicity prior to UPS impairment during the initial stages of these toxic effects.

Endoplasmic reticulum stress (ER) is a common phenomenon in a number of neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and polyglutamine disease [1,2]. The accumulation of misfolded and unfolded proteins in the ER leads to stress conditions in this organelle and activates the unfolded protein response (UPR). UPR is activated by binding of the ER chaperon protein, binding protein (Bip)/glucose-regulated protein 78 (GRP78) to malfolded proteins and is then mediated by the three ER resident sensor proteins inositol requiring 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). In normal cells, GRP78 binds and inactivates these three sensor proteins in the ER. Once GRP78 has dissociated from the ER sensor proteins, PERK phosphorylates and inactivates the α-subunit of eukaryotic translational initiation factor 2 (eIF2α). This consequently attenuates protein synthesis. IRE1 activates the induction of a number of proteins including chaperone proteins and components of ER-associated degradation of unfolded proteins (ERAD). ATF6 also activates the transcription of ER chaperone genes. As an adaptation to ER stress through ERAD, accumulated malfolded proteins in the ER are retrotranslocated to the cytosol and degraded by the ubiquitin–proteasome system (UPS). The impairment of UPS is likely to inhibit the degradation of many cellular proteins, including misfolded proteins produced in the ER, which in turn enhances ER stress.

Expansion of the glutamine-encoding trinucleotide CAG repeats is the underlying cause of nine inherited degenerative neurological diseases [3]. The onset and severity of the neurodegenerative disease in question is dependant on the length of polyglutamine tract. The expression of proteins containing long polyglutamine tracts leads to their aggregation, inclusion formation and neuronal dysfunction. A number of hypotheses have been proposed regarding the mechanisms of polyglutamine-induced toxicity [4–6]. Based on the findings of a reduction of proteasome activity and the failure of the proteasomes to degrade polyglutamine sequences, the impairment of the UPS has been suggested to be associated with polyglutamine toxicity. In addition, polyglutamine-containing proteins in an inclusion body are resistant to degradation by UPS [7]. UPS impairment with long polyglutamine repeats has been proposed to lead to ER stress-induced cell death [8]. In contrast, recent reports have indicated no involvement of UPS in polyglutamine disease [9,10] and no impairment of proteasome activity was reported in a mouse model of Huntington's disease [11,12]. Furthermore, soluble and aggregated polyQ-containing proteins were found not to inhibit *in vitro* proteasome activity [6]. More recently, it has reported that N-terminal mutant huntingtin proteins induce ER stress and inhibition of the ER stress increases cell survival [13].

* Corresponding author. Fax: +81 744 29 0510.
E-mail address: hitoshin@naramed-u.ac.jp (H. Nakayama).

Abbreviations: Dox, doxycycline; ER, endoplasmic reticulum; ERAD, ER-associated degradation of unfolded proteins; eIF2a, eukaryotic translational initiation factor 2; EYFP, yellow fluorescent protein; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-phenyl]-2,5-diphenyl-tetrazolium bromide; UPR, unfolded protein response; UPS, ubiquitin–proteasome system.

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.10.006

There are few studies to date that have examined the possibility that the expression of expanded polyglutamine tracts causes ER stress without UPS impairment. In our present study, using the expression of fusion proteins with expanding polyglutamine tracts, we have shown the polyglutamine-induced signaling and cell death in PC12 cells. We have further evaluated the effects of proteasome inhibitors upon cell death signaling and cell survival. Our data strongly suggest that ER stress is the initial response to polyglutamine toxicity and that this precedes UPS impairment in polyglutamine-expressing PC12 cells.

Materials and methods

Materials. The following antibodies were used for Western blot analyses: Actin (Santa Crutz Technology); cleaved caspase-3 (Cell Signaling Technology); phosphorylated-eIF2α (Cell Signaling Tech., Beverly, MA); GFP (GF200) (Nacalai Tesque, Kyoto, Japan); GRP78/Bip (Santa Cruz Biotech., CA); polyubiquitin (P4D1) (Santa Cruz Biotech); phosphorylated JNK (Cell Signaling Tech.). ECL and ECLplus systems were purchased from GE Healthcare UK Limited.

Constructs. The CAG repeats of the polyQn-enhanced yellow fluorescent protein (EYFP) vector ($n = 19$ or 81) were inserted into the XhoI and EcoRI sites of the pEYFP-N1 vector (Clontech, Palo Alto, CA) as described previously [14,15].

Cell culture. The stable PC12 cell line expressing polyQn-EYFP-Myc fusion proteins (polyQn) was constructed according to the protocol supplied with the Tet-Off gene expression system (Clontech, Palo Alto, CA). The colonies were selected by incubation with 50 μg/ml hygromycin and 500 ng/ml blasticidin, as described previously [15]. The cells were maintained in 75-cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with 5% precolostrum newborn calf serum, 5% heat-inactivated horse serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml hygromycin, and 500 ng/ml blasticidin (5/5/DMEM), in the presence of 10 ng/ml doxycycline (Dox) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The expression of polyQ81 was induced in the absence of Dox. To investigate the effects of lactacystin, this compound was always added 24 h prior to the termination of the cell culture.

Western blot analysis. For Western blot analysis, PC12 cells were plated at a density of $1–1.2 \times 10^5$ cell/cm^2 onto collagen-coated 35-mm dishes in culture medium. Western blotting analysis of lysate proteins was performed as described previously [16]. Blots were developed using the ECL or ECLplus system. The density of the bands was quantified with a scanner and NIH Image software. Differential loading was corrected for by comparing the values obtained for phosphorylated JNK or cleaved caspase-3 with those obtained for actin. The relative expression ratio of the protein

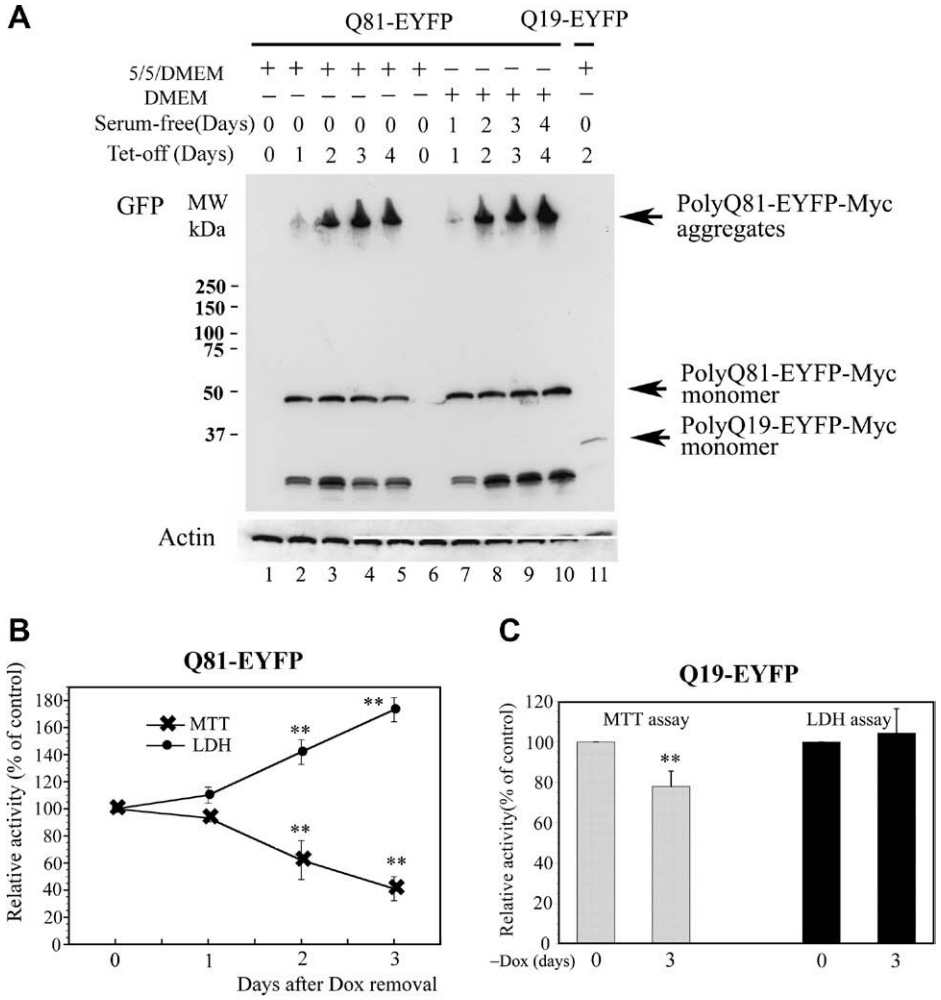

Fig. 1. The expression and toxicity of polyQ19-EYFP-Myc and polyQ81-EYFP-Myc fusion proteins (polyQ19, polyQ81) in PC12 cells. Fusion protein expression was induced by the removal of doxycycline (Dox) from 5/5/DMEM or serum-free DMEM PC12 cultures for the indicated time periods. (A) Time course of polyQ81 and polyQ19 expression in PC12 cells by Western blotting with an antibody against GFP. (B) Time course analysis of the changes in MTT reduction and LDH-release following the initiation of polyQ81 expression. (C) Effects of polyQ19 on MTT reduction and LDH-release. The activity levels are expressed as a percentage of the control measurements (MTT reduction or LDH-release in the presence of Dox). Values are means ± SD of three or four independent cultures, each performed in quadruplicate. $^{**}P < 0.01$ compared with the control.