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RAPID REPORT

ENDOPLASMIC RETICULUM STRESS RESPONSE IN DENDRITES OF CULTURED PRIMARY NEURONS

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Abstract—The endoplasmic reticulum (ER) is an organelle in which secretory and transmembrane proteins are folded or processed, and is susceptible to various stresses that provoke the accumulation of unfolded proteins in the ER lumen. Recently, ER stress has been reported to be linked to neuronal death in various neurodegenerative diseases. Neurons contain the ER not only in the soma, but also in the dendrites, thus presenting a different case to non-neuronal cells. The ER in the dendrites has potential functions in local protein synthesis and sorting of synthesized proteins to postsynaptic membranes. It raises the possibility that ER stress could occur locally in the dendrites. Here we showed that ER stress sensors, inositol-requiring 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) exist in the ER of both soma and dendrites in primary mouse neurons, and that under ER stress conditions, GRP78/BiP and phosphorylated eIF2α are induced. Furthermore, XBP1 mRNA was localized in the proximal dendrites where IRE1 was rapidly phosphorylated in response to ER stress. These results indicate that the ER in dendrites could respond to ER stress and retain the capacity of protein quality control.
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Key words: ER stress, dendrite, unfolded protein response, protein quality control.

The endoplasmic reticulum (ER) is a critical cellular compartment responsible for proper localization and folding of transmembrane and secreted proteins. Unfolded proteins accumulate in the ER lumen under physiological or pharmacological ER stresses such as a block in protein glycosylation, expression of mutated proteins, or disturbance of calcium homeostasis. If the stress is excessive or long-term, apoptotic cell death occurs involving activation of the caspase-12 and caspase-3, and ASK1–JNK pathways (Nakagawa et al., 2000; Urano et al., 2000; Nishitoh et al., 2002). However, cells first attempt to adapt for survival by transmitting signals from the ER to the nucleus and cytoplasm. This regulatory system is termed the unfolded protein response (UPR) (Mori, 2000; Kaufman, 2002; Schroder and Kaufman, 2005).

ER stress transducers play important roles in signal transduction of the UPR. In mammalian cells, the three major transducers of the UPR are inositol-requiring 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), which sense the presence of unfolded proteins in the ER lumen. The transcriptional induction of UPR-target genes encoding ER-resident chaperones facilitates correct folding of unfolded proteins in order to protect cells from ER stress.

ER stress has been reported to be linked to neuronal death in various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Katayama et al., 1999; Imaizumi et al., 2001; Imai et al., 2001). However, it remains unclear whether ER stress indeed occurs in neurons of the brains of patients. It will be necessary to image ER stress in the individual neurons in order to demonstrate the relationship between ER stress and neuronal death in neurodegenerative diseases.

Neurons contain the ER not only in the soma, but also in the dendrites. The ER in the dendrites has the capacity for local protein synthesis (Torre and Steward, 1996; Ju et al., 2004), but it is unclear whether ER stress and UPR occur in the dendrites. ER stress in dendrites may link to neuronal cell death or synaptic dysfunction such as found in the pathology of Alzheimer's disease (Terry et al., 1991). In this study, to investigate ER stress and the UPR in dendrites, we examined localization of ER stress sensors in primary neurons. Moreover, the activation of the IRE1-XBP1 (X-box binding protein 1) pathway, one of the UPR signaling pathways, was evaluated in dendrites under ER stress conditions.

EXPERIMENTAL PROCEDURES

Primary cultures of hippocampal neurons were prepared from the hippocampus of E18 ddY mouse embryos. The ddY mice were purchased from Nihon SLC, Shizuoka, Japan. The dissected tissues were treated with papain (Sigma, St. Louis, MO, USA) to dissociate the cells, and the cells were plated on polyethyleneimine (Sigma) -coated glass coverslips. The medium was changed every other day with Neurobasal medium supplemented with B27 and L-glutamine (all Invitrogen, Carlsbad, CA, USA). 5-Fluoro-2′-

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Abbreviations: ATF6, activating transcription factor 6; eIF2α, eukaryotic initiation factor-2α; eIF2α-P, phosphorylated form of eukaryotic initiation factor-2α; ER, endoplasmic reticulum; IRE1, inositol-requiring 1; IRE1-P, phosphorylated inositol-requiring 1; NMDA, *N*-methyl-D-aspartate; PERK, PKR-like endoplasmic reticulum kinase; Tm, tunicamycin; UPR, unfolded protein response; UTR, untranslated region; XBP1, X-box binding protein 1.

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deoxyuridine and uridine (10 μM) were added to the medium for 4–5 days to prevent proliferation of glial cells. Seven days after plating, neurons were transfected with each expression plasmid using TransFectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

GFP-tagged plasmids were generated by a PCR-based approach using pc-FLAG-human ATF6 (kindly provided by Dr. Ron Prywes, Columbia University, New York, NY, USA), pc-human IRE1-α (kindly provided by Dr. Fumihiko Urano, University of Massachusetts Medical School, Worcester, MA, USA) and pUB6-mouse PERK-Myc (kindly provided by Dr. David Ron, New York University School of Medicine, New York, NY, USA) as the templates. To avoid interfering with the localization of the GFP fusion proteins, we constructed each protein with the GFP tag at the terminus of the cytoplasmic domain. Mouse XBP1 cDNA containing the untranslated region (UTR) was generated by PCR via reverse transcription of RNA from primary cultures of hippocampal neurons. All products were inserted into pcDNA3.1 (+) (Invitrogen).

Western blotting was performed as described previously (Murakami et al., 2006). Antibodies used were anti-KDEL (StressGen Biotechnologies, Collegeville, PA, USA), anti-phosphorylated forms of eIF2α (anti-eIF2α-P) (StressGen), and anti-β-actin (Sigma). Quantifications of blots were performed with Scion Image software (Scion, Frederick, MD, USA).

Cells were fixed with 4% paraformaldehyde and permeabilized in PBS containing 0.1% Triton X-100 for 20 min at 4 °C. For immunostaining of primary neurons, we stained the cells with mouse anti-MAP2 monoclonal antibody (Sigma), anti-phosphorylated forms of eIF2α, anti-GRP78/BiP (MBL, Nagoya, Japan), anti-KDEL, and anti-phosphorylated forms of IRE1 (kindly provided by Dr. Urano). Primary antibodies were visualized with alexa-conjugated goat anti-rabbit IgG, alexa-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA), and fluorescein-conjugated goat anti-mouse IgG antibodies (ICN Pharmaceuticals, Costa Mesa, CA, USA). Stained cells were viewed using a fluorescence microscope or a confocal microscope (Leica DMIRE2, Wetzlar, Germany). For quantitative analysis, images were obtained with a fluorescence microscope (Eclipse TE2000-U) (Nikon, Kanagawa, Japan). Images obtained were analyzed with Lumina Vision software (Mitani Corporation, Fukui, Japan). To quantify the fluorescence intensity of dendrites, we used Line Profile of Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

In situ hybridization was performed as described previously (Mori et al., 2000). The XBP1-specific probe (1266 bp), which extends from the region following the sites spliced out by IRE1 to the 3′-UTR containing the polyA signal, and which excludes the coding region of bZIP, was generated by a PCR-based approach using pc-XBP1 as the template. The XBP1 probe was subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Digoxigenin-labeled antisense and sense probes were synthesized from the linearized plasmids with DIG RNA labeling mix (Roche, Basel, Switzerland).

RESULTS

As a cell survival response in mammalian cells under ER stress conditions, ER-resident chaperones such as GRP78/BiP are up-regulated and eukaryotic initiation factor-2α (eIF2α) is phosphorylated (Ron, 2002). So the induction and phosphorylation of these molecules are fundamental indicators of stress in the ER lumen, and that the UPR signaling pathways are activated. To evaluate whether ER stress and the UPR occur in the dendrites, we first analyzed the immunostaining of primary cultures of hippocampal neurons after ER stress, with antibodies against GRP78/BiP and phosphorylated forms of eukaryotic initiation factor-2α (eIF2α-P). Tunicamycin (Sigma), an inhibitor of *N*-glycosylation, was used as the ER stressor. The immunoreactivity of GRP78/BiP was elevated in primary neurons during ER stress, which overlapped with MAP2 immunoreactivity (Fig. 1A). The elevated signals were also found in the dendrites. Quantitative analysis of fluorescence intensity for the dendrites confirmed that GRP78/BiP fluorescence was elevated in the dendrites, at least within 90 μm from the soma (Fig. 1A, B). By Western blotting of the lysates from primary cultures, the total amount of GRP78/BiP protein was also found to be increased during ER stress (Fig. 1C). The immunoreactivity of phosphorylated forms of eIF2α after ER stress was also elevated not only in the soma but also in the dendrites (Fig. 1D, E), similar to the case of GRP78/BiP, and the total amount of eIF2α-P protein also increased (Fig. 1F). These results indicate that ER stress and the UPR could occur both in the soma and dendrites of neurons.

Since downstream signaling from the ER stress response was activated in dendrites, it was possible that the ER stress sensors could localize there. Therefore, we investigated the cellular localizations of three major ER stress sensors, IRE1, PERK, and ATF6 in primary neurons. The neurons were transiently transfected with GFP-tagged ER stress sensors and we then determined the localization of these ER stress sensors using confocal microscopy. Strong autofluorescence of each GFP-tagged ER stress sensor was observed in the soma (Fig. 2). In addition, the autofluorescence was detected in MAP2-positive neurites, indicating that these neurites containing GFP autofluorescence were dendrites; that is, the ER stress sensors have the potential to localize in the dendrites. To confirm that each ER stress sensor was distributed in the ER of dendrites, we performed immunostaining of the ER with anti-KDEL antibody in primary neurons transfected with GFP-tagged ER stress sensors. The GFP signal of the sensors overlapped with the immunoreactivity of the ER marker, in soma and dendrites (Fig. 3A, B). These results suggest that ER stress sensors could localize in the ER of dendrites of primary neurons.

To investigate whether the stress sensors in the dendrites are activated, we focused on IRE1 because IRE1 signaling is the most conserved over evolution, and phosphorylated forms of IRE1, i.e. activated forms, were easily detected by using an antibody against phosphorylated inositol-requiring 1 (IRE1-P). We performed immunostaining of primary neurons transfected with the expression vector for IRE1-GFP (Fig. 4A). Under normal conditions, immunoreactivity in transfected neurons using anti-IRE1-P antibody was observed in a relatively low percentage of neurons. In contrast, under ER stress conditions, immunoreactivity was observed in the soma and dendrites in approximately 50% of neurons (Fig. 4B), indicating that IRE1 is phosphorylated and activated in the dendrites as well as the soma. However, from the available data, we could not completely rule out the possibility that IRE1 is phosphorylated in the soma and then transported into dendrites. The detailed reasons why phosphorylation of

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