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Research Article

Heat stress promotes the down-regulation of IRE1α in cells: An atypical modulation of the UPR pathway

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ABSTRACT

Heat stress induces intracellular protein denaturation and endoplasmic reticulum (ER) stress, which elicits unfolded protein response (UPR) in cells. UPR involves three ER-localized sensor proteins: the inositol-requiring protein 1α (IRE1α), the dsRNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor-6 (ATF6). However, the precise mechanism by which cells deal with heat stress remains to be elucidated. We report herein that heat stress effectively activates all branches of the UPR. While splicing of the XBP-1 mRNA is usually triggered by activated IRE1α, the level of this protein was found to be decreased immediately after the occurrence of heat stress and the down-regulation gradually recovered thereafter. The protein levels of other UPR sensors or ER luminal proteins were unaffected. The down-regulation of IRE1α was independent of cellular viability. It thus appears that the heat-stress induced down-regulation of the IRE1α protein may lead to the termination of the IRE1α signaling pathway in an attempt to avoid excessive responses. We investigated the machinery causing IRE1α down-regulation and found that heat stress effectively promoted the activation of autophagy. Importantly, the inhibition of either proteasomes or autophagic flux failed to rescue the loss of IRE1α following heat stress. These collective results suggest that heat stress simultaneously activates both the UPR and autophagy, followed by the activation of a negative feedback system in UPR by modulating the responses related to the IRE1α–XBP-1 axis.

1. Introduction

In eukaryotic cells, secretory proteins and membrane proteins are synthesized on the endoplasmic reticulum (ER) membrane and undergo oxidative folding in the ER. Various signaling pathways, which are referred to as an unfolded protein response (UPR), are activated to deal with the excessive loading of proteins to the ER or an impairment in ER homeostasis [1]. Upon ER stress, the UPR is initiated by ER-resident sensor proteins: the inositol-requiring protein 1α (IRE1α), dsRNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor-6 (ATF6).

The ER chaperone BiP [2], also known as the glucose-regulated protein 78, binds these sensors, rendering them inactive under non-stressed conditions. However, when unfolded and misfolded proteins accumulate in the ER, BiP dissociates from these sensors to perform its chaperone function. As a consequence, three major UPR branches are activated as follows. (I) The activation of PERK induces the phosphorylation of eukaryotic translation initiation factor 2 (eIF2α), resulting in the global suppression of protein translation [3]. Upon prolonged ER stress, eIF2α initiates the expression of activating transcription factor 4 (ATF4) [4], which induces the expression of the proapoptotic transcription factor C/EBP-homologous protein (CHOP) [5]. (II) ATF6 [6], another transcription factor, is activated and translocated to the Golgi membrane where it is cleaved by site 1/2 proteases [7], and then becomes relocated to the nucleus, where it induces the transcription of ER chaperone genes. (III) IRE1α is the most conserved branch of the UPR [8,9]. IRE1α dimerizes and transphosphorylates itself in the UPR. This mediates the activation of endoribonuclease activity in IRE1α, leading to the splicing of the X-box binding protein-1 (XBP-1) mRNA in the cytosol [10,11]. sXBP-1, the protein that is translated from the spliced XBP-1 mRNA, becomes localized in the nucleus and induces the expression of proteins that are involved in ER-associated degradation (ERAD), chaperones, and lipid synthesis [12]. Activated IRE1 also mediates the rapid degradation of a specific subset of mRNAs called Regulated IRE1-Dependent Decay (RIDD) [13,14], which have been implicated in cell death [15].

Abbreviations: ATF6, activating transcription factor-6; CHX, cycloheximide; eIF2α, phospho-eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; IRE1α, inositol-requiring protein-1α; MEF, mouse embryonic fibroblast; PDI, protein disulfide isomerase; PERK, dsRNA-activated protein kinase-like ER kinase; Prx4, peroxiredoxin 4; UPR, unfolded protein response; XBP-1, X-box binding protein-1

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Heat stress is one of the types of environmental stress that activates the UPR pathway [16–19]. Heat stress triggers the formation of protein aggregates in the cytosol as well as ER. Macroautophagy is a vacuolar self-digestion mechanism and functions to remove protein aggregates and damaged organelles by autophagosomes [20]. Results reported by several studies indicate that cancer cells are more vulnerable to heat stress than normal cells [21,22], although the precise mechanism responsible for the enhanced heat sensitivity in tumors remains to be determined.

Some classes of normal cells are also highly sensitive to heat stress. It is well known that a lower scrotal temperature is required for normal spermatogenesis in mammals [23] because germ cells are more susceptible to heat stress and cell death occurs in response to higher temperatures [24–26]. It has been demonstrated that testicular hyperthermia induces the UPR and repetitive cycles of hyperthermia have been reported to lead to cell death in mouse testis [18]. Heat stress in the testes triggers autophagy and cell death preferentially in germ cells [27]. Because a deficiency of superoxide dismutase 1 (SOD1) enhances the heat sensitivity in the testes, elevated levels of reactive oxygen species appear to be involved in the heat-induced death of germ cells [28].

In this study, we report that heat stress activates all three of the major UPR branches: IRE1α–XBP-1, PERK–eIF2α, and ATF6 to protect cells against heat stress-triggered ER stress, while heat stress decreased the expression of IRE1α in both somatic and germ cell models. This study reports on a novel mechanism by which cells react to heat stress-induced protein aggregation by means of UPR.

2. Materials and methods

2.1. Antibodies

Preparation of the Prx4 antibody was described elsewhere [29]. Other antibodies were purchased as follows: anti-IRE1α (#3294), anti-phospho-eIF2α (#3398), anti-cleaved caspase-3 (#9664), and anti-LC3B (#3868) from Cell Signaling Technology; anti-Hsp70 (ab181606) from abcam; anti-ATF6 (NBP1-40256) from Novus Biologicals; anti-PERK (sc-13073), anti-GRP78/Bip (sc-13968), anti-PDI (sc-20132), and anti-β-actin (sc-69879) from Santa Cruz Biotechnology; anti-eIF2α (GTX101241) from GeneTex; anti-Human/Mouse Caspase-3 (AF-605-NA) from R & D Systems; anti-ATF6 (A303-719A) from Bethyl Laboratories; Horseradish peroxidase (HRP)-conjugated anti-mouse (sc-2005) and anti-rabbit (sc-2004) IgG antibodies from Santa Cruz Biotechnology.

2.2. Cell culture and heat stress

HeLa cells, HepG2 cells, DU145 cells, A549 cells, and mouse spermatocyte-derived GC-2spd(ts) cells (GC-2 cells) were obtained from the American Type Culture Collection (ATCC). Mouse embryonic fibroblasts (MEFs) were generated, as previously described [30]. All cells were maintained in Dulbecco's Modified Eagle's Medium (Wako, 044–29765) supplemented with 10% fetal bovine serum (Biowest), 100 U/ml penicillin, and 100 μg/ml streptomycin (168–23191, Wako) at 37 °C with 5% CO_2. To induce heat stress, confluent cells were transferred to an incubator at a temperature of 40 °C or 43 °C under an atmosphere of 5% CO_2. Where indicated, the cells were treated with DMSO (Wako), MG132 (Calbiochem), NH_4Cl (Wako), 3-MA (InvivoGen), 4 μ8 C (Calbiochem), cycloheximide (Sigma), actinomycin D (Sigma), thapsigargin (Sigma), tunicamycin (Sigma), dithiothreitol (Sigma), Calpeptin (Calbiochem), or zVAD-fmk (Promega).

2.3. Immunoblotting

Cells were lysed in cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA) containing a protease inhibitor cocktail (Sigma, P8340) for 30 min at 4 °C. After centrifugation at 15,000g for 10 min, the supernatant was collected, and the concentration of total protein was determined using a Pierce® BCA™ Protein Assay Kit (Pierce). The proteins were separated on 10–15% SDS-polyacrylamide gels and transferred to Immobilon-P Membranes (Millipore). The blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), and were then incubated overnight with the primary antibodies diluted in TBST containing 1% skim milk. The blots were reacted with the HRP-conjugated second antibody (dilution 1:10,000) and analyzed in the form of an Immobilon western chemiluminescent HRP substrate (Millipore) on an image analyzer (ImageQuant LAS500, GE Healthcare).

2.4. Phos-tag electrophoresis

To visualize the phosphorylation of IRE1α, cell lysates were separated by 5% SDS-polyacrylamide gel containing 25 μM Phos-tag (AAL-107, Wako) and 50 μM $MnCl_2$. After electrophoresis, the gel was washed in transfer buffer containing 1 mM EDTA followed by immunoblotting.

2.5. Cell survival assay

Cell viability was determined using a CellTiter-Blue™ Cell Viability Assay (Promega) according to the manufacture's instructions.

2.6. Caspase activity assay

Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5, with 5 mM CHAPS, and 5 mM DTT). After centrifugation, twenty μg of total protein was incubated with 100 μM of Ac-DEVD-MCA (fluorogenic substrate for caspase-3) or Ac-LEHD-MCA (a fluorogenic substrate for caspase-9) in 100 μl assay buffer (20 mM HEPES, pH 7.5, with 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) for 60 min at 37 °C. All substrates were purchased from the Peptide Institute, Inc. (Osaka, Japan). After incubation, the fluorescence intensity was measured using a microplate reader (Varioskan Flash, Thermo Fisher Scientific) with an excitation filter of 365 nm and an emission filter of 460 nm.

2.7. Mice and testicular hyperthermia

Wild-type (C57BL/6, WT; eight-weeks old) male mice were purchased from Japan SLC (Hamamatsu, Japan). The local heating of mouse testis (43 °C for 15 min; n=3 for each group) was performed as described previously [26]. After the heat treatment, mice were dried and returned to their cages. The mice were sacrificed after 12 h, and the testes were then removed for further analysis. Animal experiments were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee at Yamagata University.

2.8. Immunostaining

Cells were washed with PBS and fixed in ice-cold 100% methanol for 15 min at −20 °C. After washing twice with PBS, the cells were blocked for 30 min by treatment with TBST containing 5% skim milk at room temperature, and incubated overnight at 4 °C with anti-LC3B (dilution 1:200) or ATG16L1 (GeneTex, GTX129098, dilution 1:200) antibodies in IMMUNO SHOT immunostaining (Cosmo Bio). After three washes in PBS, the cells were further incubated with an Alexa Fluor®-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, dilution 1:200) for 90 min at room temperature. All images were obtained using a BZ-X700 microscope (KEYENCE, Osaka, Japan).