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ORIGINAL PAPER

Current methods for investigation of the unfolded protein response

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Abstract

The endoplasmic reticulum (ER) is the cellular organelle where proteins from the secretory pathway fold. The ER is a very dinamic organelle that can respond rapidly to changes in the ER load by upregulating the ER resident proteins that are involved in the folding process. Simultaneously, less proteins from the secretory pathway are synthesized*. The whole cascade of events is called the unfolded protein response (UPR). The UPR can originate from physiological or pathological conditions and many times the ER changes in a subtle manner. Researchers working in the ER field developed various tools to assay the ER stress. Here we describe methods optimized in our laboratory and show that western blotting, quantitative real time polymerase chain reaction (RT-qPCR) and* reverse transcription-polymerase chain reaction *(RT-PCR) are reliable, cost-effective techniques that researchers can choose to evidence ER stress activation.*

Abbreviations:

BiP, immunoglobulin heavy-chain binding protein; ER, endoplasmic reticulum; TG, thapsigargin; TN, tunicamycin; UPR, unfolded protein response; IRE1, inositol-requiring kinase 1; XBP1, X-box-binding protein 1; PERK, RNA-dependent protein kinase-like ER kinase; ATF6, activating transcription factor 6; eIF2□, eukaryotic translational initiation factor 2□□□Bcl-2, B cell lymphoma 2, ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein-homologous protein; GRP, glucose-regulated proteins, CREB-RP, cyclic AMP-response-element-binding protein-related protein; S1P, site-1 protease;S2P, site-2 protease; ERSE, ER stress response elements; Grp94, Glucose-regulated protein 94; ERp44, endoplasmic reticulum resident protein 44 kDa; ERp72, endoplasmic reticulum resident protein 72 kDa; ERp57, endoplasmic reticulum resident protein 57 kDa; Ero1□, ER oxidoreductin 1□; DTT , dithiothreitol; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; ERAD, ER-associated degradation; ECL, enhanced chemiluminescence; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, quantitative real time polymerase chain reaction.

1. The endoplasmic reticulum – a short overview

The endoplasmic reticulum (ER) is essential for production of secretory proteins (BRAAKMAN [1]). Proteins from secretory pathway emerge from ER-bound ribosomes and are further modified and folded by the ER folding machinery. There are three major classes of ER resident proteins: molecular chaperones, foldases and lectins (SCHRODER [2]).

The molecular chaperones facilitate protein folding by shielding unfolded regions from surrounding proteins. BiP for instance binds to the hydrophobic patches that are exposed during folding, thus preventing aggregation. The foldases (cis–trans peptidyl–prolyl isomerases, PPI/immunophilins and PDIs) catalyze steps in protein folding such as the formation and isomerization of disulfide bonds. Lectins interact with glycoproteins due to the presence of a lectin site that can recognize an early folding oligosaccharide processing intermediate on the folding glycoprotein (WILLIAMS [3]). Correctly folded secretory proteins are exported to intracellular organelles or to the extracellular surface. Proteins

containing mutations that lead to misfolding will accumulate in the ER lumen. The misfolded proteins are further subjected to degradation by the ER-associated degradation pathway (ERAD) (BRODSKY [4]) or by autophagy (LE FOURN [5]).

ER is the main storage for calcium: the luminal concentration for calcium can reach about 5 mM, while the majority is bound to ER resident chaperones, as they require calcium for optimal function (COE [6]). Calcium is not only an important secondary messenger; it participates also to protein folding by binding directly to calcium sites within proteins structure (PENA [7]). As proteins bind calcium with certain affinity constants, calcium needs to be present in high concentration at early folding stages.

The ER also controls the synthesis of two major components of all biological membranes: sterols and phopholipids (BLOM [8]). By influencing the composition, the ER modifies the biophysical properties and subsequently the function of all biological membranes (FAGONE [9]).

In order to rapidly respond to various changes in folding demands, the ER evolved as a highly dynamic organelle. When a larger amount of proteins fold in the ER this organelle will expand and more ER resident proteins will populate it due to the increased requirement in folding enzymes and chaperones (WALTER [10]). The same adaptation occurs when misfolded proteins accumulate inside the ER lumen. The ER can adapt because of the activation of an integrated signal transduction pathway named the UPR. Briefly, UPR debuts when sensor proteins located into the ER lumen detect accumulation of unfolded or misfolded proteins (WALTER [10]). The sensors will transmit this information to other molecules in the cytosol and furthermore to nucleus where transcription factors will determine a higher expression of ER resident chaperons and a decrease of secretory proteins synthesis. The UPR is conserved from yeast to human (FOTI [11]). Below we describe the three UPR pathways that are known so far in mammals.

1.1. Ire1 pathway

In 1993 two research groups independently reported the identification of IRE1, a type 1 transmembrane protein in the ER as the sensor and transducer of ER stress (COX [12], MORI [13]). IRE1 has three distinct regions: an ER luminal domain (that binds BiP in the repressed state (BERTOLOTTI [14]), a transmembrane domain, and a cytosolic region with a serine/threonine kinase domain fused to a ribonuclease domain (SIDRAUSKI [15]). Until recently was known that BiP release activates Ire1, however *in vitro* studies showed that unfolded proteins bind directly to the luminal domain of IRE1 (GARDNER [16]), thus activating IRE1. Upon activation, IRE1 oligomerizes due to its luminal domain and clusters into distinct foci in yeast (ARAGON [17], LI [18]). This in turn activates its ribonuclease function (RNAse), cleaving the mRNA of XBP1 in two specific positions, thus excising an intron. The two fragments are further ligated giving rise to a spliced mRNA that is translated to the active form of the transcription factor XBP1 spliced (WALTER [10]). Spliced XBP1 (sXBP1) regulates many genes involved in protein entry into the ER, folding, redox metabolism, glycosylation, autophagy, ER-associated degradation (ERAD), lipid biogenesis and vesicular trafficking (GLIMCHER [19]).

1.2. PERK pathway

PERK is structurally related with IRE1, possessing an ER luminal dimerization domain and a cytosolic domain connected through a transmembrane domain. Apparently PERK also activates when BiP dissociates from its luminal domain. Active PERK phosphorylates the □ subunit of eukaryotic translational initiation factor 2□ (eIF□□), thus attenuating global protein synthesis (SHI [20], SHI [21], HARDING [22]). Another effect of eIF□□

phosphorylation is preferential translation of ATF4, a transcription factor. ATF4 induces expression of genes implicated in ER physiology (VALLEJO [23], HARDING [24]).

Under sustained ER-stress, ATF4 activates CHOP that in turn inhibits the expression of anti-apoptotic Bcl-2 (B cell lymphoma 2) protein (MCCULLOUGH [25], KILBERG [26]) and contributes to the upregulation of pro-apoptotic proteins (CAZANAVE [27]).

1.3. ATF6 pathway

Reported first time in 1998 as a basic leucine zipper protein, ATF6 is an ERSE-binding protein. ATF6 determined enhanced transcription of GRP genes when was overexpressed and this phenomenon was ERSE-dependent (YOSHIDA [28]). In mammals there are two homologous proteins, ATF6□ and ATF6□/CREB-RP/G13 (HAZE [29]). ATF6 is an ER transmembrane protein with a large ER luminal domain that has no homology with other ER proteins. Despite the differences, BiP binds ATF6, as in the case of Ire1 and PERK. When ER is under stress the unfolded proteins accumulate in the ER lumen and BiP dissociates from ATF6. Furthermore, ATF6 is packed into vesicles that are transported to the Golgi apparatus. Here two proteases – S1P and S2P – cut the full length ATF6: first by removing the luminal domain and subsequently the membrane anchored domain (HAZE [30], YE [31]). The remaining N-terminal cytosolic fragment moves into the nucleus to activate UPR target genes. ATF6 binds to the ATF/CRE element (WANG [32]) and to the ER stress response elements I and II (ERSE-I, CCAAT-N9-CCACG (ARAGON [17]), and ERSE-II, ATTGG-N-CCACG (KOKAME [33]). ER-resident proteins involved in protein folding, such as BiP, PDI, GRP94, CHOP are targets for ATF6 (SCHRODER [2]).

2. UPR in health and pathology

Cells respond to fluctuations in folding demand by activating UPR. The stimuli that affect the ER can be either physiological or pathological. It is now well known that hyperglycemia, hyperlipidemia, and inflammatory cytokines all disrupt protein folding in the ER.

UPR and diabetes

A functional UPR is necessary in cells that suddenly secrete large amounts of protein upon certain stimulation. The pancreatic β-cell for instance increase proinsulin synthesis upon glucose stimulation in a PERK-eIF2□□branch - dependent manner (HARDING [34], SCHEUNER [35]). Disruption of PERK pathway leads to hyperglycemia due to reduced insulin levels. More recent data suggest even a homeostatic role for PERK in □□cell survival (CAVENER [36]). Proinsulin misfolding, disturbed trafficking of proinsulin and apoptosis are induced when an alanine substitution at Ser51 (the phosphorylation site) of eIF2□□was□combined with the stress of a high fat diet (SCHEUNER [37]), (BACK [38]). Hypothetically improper UPR adaptive response or induction of apoptosis due to chronic ER stress can be *in vivo* the cause of β cell failure and loss of glucose homeostasis. To address these questions researchers investigated CHOP, a key protein implicated in UPR-induced cell death. The pancreatic islets produce more insulin when CHOP is deleted. CHOP deletion also precludes β cell apoptosis and at the same time enhances β cell function (SONG [39]).

The other UPR branch represented by IRE1α and its product XBP1s is required to achieve optimal insulin secretion and glucose control. This conclusion arises also from studies using cells where XBP1 was deleted. In this experimental condition were observed less insulin granules in β-cells, proinsulin processing was impaired, while the proinsulin:insulin ratio has increased in serum. Concomitantly IRE1α was hyperactivated, and this hyperactivation could be the cause of degradation of some mRNAs encoding proinsulin-processing enzymes (LEE [40]).

The third UPR branch – ATF6 pathway also seems to be involved in □□cell physiology, as shown in several reports (THAMEEM [41], MEEX [42], CHU [43], USUI [44], WANG [45]).

3. Experimental approaches for the detection of ER stress

3.1. Western Blotting for UPR target genes.

We induced ER stress with chemical agents: tunicamycin (inhibits N-acetylglucosamine, preventing N-linked glycosylation of newly synthesized proteins) (2.5 □g/ml for 3-7h) (MIZRAHI [46]) or ditiothreitol (DTT) (1 mM for 1 – 2h) (strong reducing agent, *in vivo* prevents formation of disulfide bonds). Control and stressed cells (HeLa, Hek293, or Mef cells) were lysed in sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol and bromphenol blue) containing protease inhibitors and phosphatase inhibitors (Roche) or in lysis buffer (50 mM Hepes, 200 mM NaCl, 2% CHAPS, pH 7.3), and total protein concentration of lysates was determined using Pierce BCA protein assay kit. Equal amounts of total protein were migrated in SDS-PAGE and transfered to nitrocellulose membranes. The membranes were decorated with antibodies (see Table 1 for catalog numbers) following manufacturer instructions and proteins were detected with ECL kit (Santa Cruz) or with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific).

One may think that the best way to study UPR is to directly measure activation of UPR sensors (therefore to look for phopho-PERK, phospho-IRE1, and/or cleaved ATF6). However, our experience proved us that this is not always easy to accomplish. We tested several antibodies and more lysis conditions until we got reproducible blots for phospho-PERK, and with only one antibody in mouse cells (Figure 1c). In human cells it appears that an antibody that recognize endogenous levels of total PERK protein is also capable to recognize two different forms of PERK (presumably un-phosphorylated and phosphorylated PERK), as a doublet appears after tunicamycin treatment. We propose the use of the appearance of this doublet as prove for PERK activation in human cells (see Figure 1b).

The antibody we purchased for phospho-Ire1 gives a lot of background bands (Figure 1b). As a consequence we would rather test it more extensively before recommending it to other researchers.

For ATF6 we tested three different commercial antibodies and with all of them we could not obtain a reproducible detection for endogenous ATF6. However, we overexpressed HA-tagged ATF6 (Addgene) (ZHU [47]) in Mef and Hek293 cells and obtained a robust detection (Figure 1d) for the full length protein with several commercial antibodies (see table 1 for catalog numbers). We failed to detect the 50 kDa activation product after treatment with either tunicamycin or thapsigargin for various time intervals, most probably because it is unstable and rapidly degraded protein. Another possibility is that transfection of ATF6 results in high levels per cell and only very low levels of the cleaved product are generated in each cell (WANG [32]). However, after 7h treatment with tunicamycin (2.5 □g/ml) or thapsigargin (1 □M) we obtained faster migrating forms of full-length ATF6, most probably underglycosilated forms. It has been proposed already that glycosylation status of 90kDa ATF6 can serve as a sensor for ER homeostasis (HONG [48], BADIOLA [49]). Therefore we also recommend the use of this technique in evidencing more reproducibly the activation of ATF6 pathway.

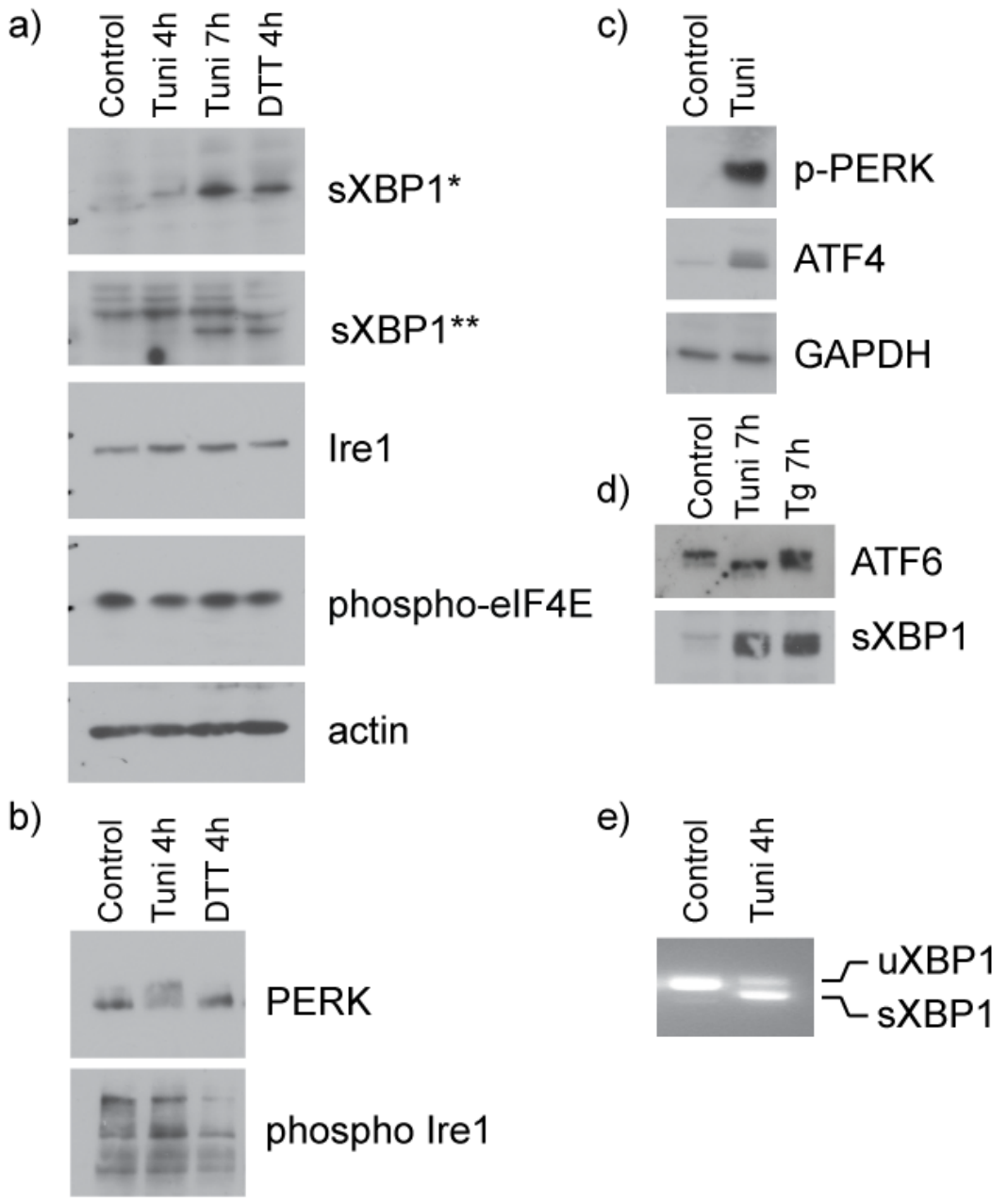

Figure 1. Detection of UPR activation. a) HeLa cells were treated with tunicamycin for 4 and 7h (2.5 μg/ml) or with dithiothreitol (DTT) (1 mM) for 4h. Spliced XBP1 appears after all treatments and its amount increases with time. sXBP1* was detected with Biolegend antibody (recognizes only human sXBP1) and sXBP1** with Santa Cruz antibody (recognizes both murine and human sXBP1 but the antibody gives a higher background). Ire1 is slightly upregulated by tunicamycin stress. b) PERK and phosphorylated Ire1 were detected in HeLa cell lysates treated with tunicamycin or DTT. c) Mef cells were treated with tunicamycin (2.5 μg/ml) for 4h. Phosphorylated PERK (phospho-PERK) and ATF4 were detected in total lysates. d) Mef cells, control and tunicamycin (Tuni) (2.5 μg/ml) or thapsigargin (Tg) (1 μM) treated, were transiently transfected with HA-tagged ATF6α. ATF6 was detected with CosmoBio antibodies and sXBP1 is shown as stress control. e) Unspliced (uXBP1) and spliced XBP1 (sXBP1) is shown in an agarose gel in control and tunicamycin (Tuni) (2.5 μg/ml) – A375 treated cells.

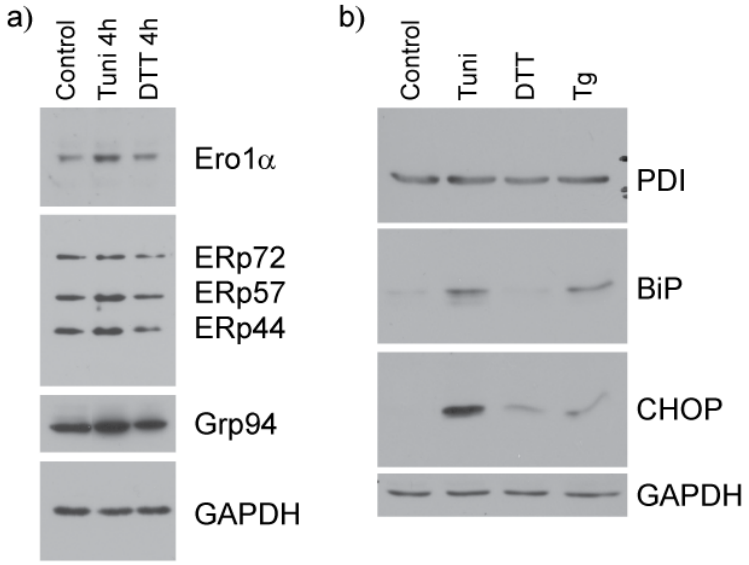

Figure 2. Downstream activation of UPR. a) HeLa cells were incubated with tunicamycin (TUNI) (2.5 μg/ml) and dithiotreithol (DTT) (1 mM) for 4h. ER resident proteins were detected by western bloting. b) Mef cells were incubated with tunicamycin (TUNI) (2.5 μg/ml), dithiotreithol (DTT) (1 mM) or thapsigargin (1 μM) for 3h, and PDI, BiP and CHOP were detected by western bloting. GAPDH was used as loading control.

Because of all these difficulties we propose the use of downstream protein markers for ER stress as a better way of action. We obtained accurate proves of ER induction under chemical stress (tunicamycin and thapsigargin exposure) by looking at: CHOP, ATF4, spliced XBP1, BiP, PDI, ERp44, ERp57, ERp72, Ero1, and Grp94 in western blot. Figure 1a shows undetectable levels of sXBP1 in control cells. sXBP1 could be detected after 4h treatment with tunicamycin and the amount increases at longer exposure to this stressor. We detected sXBP1 with two different antibodies as one only detects the human sXBP1 (Biolegend) with very low background while the other can recognize both murine and human protein (Santa Cruz), though with a higher background. ATF4 is strongly induced by tunicamycin treatment (Figure 1c). Ero1α, ERp72, ERp44, ERp57, Grp94 are upregulated after 4h of tunicamycin treatment, but at a much lesser extent after incubation in 1mM DTT (Figure 2a). Interestingly, BiP is induced by tunicamycin and thapsigargin stress, and not by DTT. CHOP is also reaching high levels after tunicamycin treatment but there is a lower induction after DTT and thapsigargin stress (Figure 2b).

Table 1. Antibodies that reproducibly detect markers of UPR.

Protein	**Antibody Catalog Number**
Ero1	Cell Signaling #3264
ERp72	Cell Signaling #2798
ERp57	Cell Signaling #2881
ERp44	Cell Signaling #2886
Grp94	Cell Signaling #2104
PDI	Cell Signaling #3501
BiP	Cell Signaling #3177
sXBP1	Santa Cruz SC-7160 Biolegend 647502
ATF4	Santa Cruz SC-200

CHOP	Cell Signaling #2895
PERK total	Cell Signaling #5683
phospho-PERK	Cell Signaling #3179
Ire1	Cell Signaling #3294
phospho-Ire1	Abcam ab124945
ATF6	Imgenex IMG-273 CosmoBio BAM-73-505-EX
GAPDH	Fitzgerald 10R-G109A
actin	Abcam ab8226

3.2. mRNA levels of UPR target genes.

ER stress leads to upregulation of ER chaperones and enzymes and of ERAD components as a result of the activation of specific sequences in the promotor of ER stress genes. As explained above, experimentally it may be difficult sometimes to determine activation of ER stress sensors by western blot. As a complementary method we used real-time PCR successfully to compare expression of ER genes in various stress conditions. We induced ER stress with tunicamycin. Control or stressed cells were harvested directly in TRIzol (Life Technologies) and stored at -80 ^{o}C or used immediately for RNA purification using manufacturer recommendations. SensiMix SYBR No-Rox one-step Kit (Bioline) was used for RT-qPCR reactions that contain 100 ng total RNA template and 250 nM primers (see table 2 for primer sequences). Some primers were used previously (FENG [50]). As internal reference we used beta actin expression and found that ER resident proteins are upregulated upon ER stress also at mRNA level. We show in Figure 3 induction of several ER resident proteins after treatment with tunicamycin in Mef cells. BiP is overexpressed in higher folds than ERp72 and ERp57, as well as Herpud1 that was shown as being involved in both UPR and ERAD.

Table 2. Sequence of primers used for several murine ER resident proteins (purchased from Eurofins MWG Operon GmbH).

Gene	**Primers**
BiP	Forward: 5'GCTTCGTGTCTCCTCCTGAC3' Reverse: 5'TAGGAGTCCAGCAACAGGCT3'
ERp57	Forward: 5'CAAGAGGCTTGCCCCTGAG3' Reverse: 5'GGTGTTTGTGTTGGCAGTGC3'
ERp72	Forward: 5'TTCCACGTGATGGATGTTCAG3' Reverse: 5'AGTCTTACGATGGCCCACCA3'
Herpud1	Forward: 5'CCCACCTGAGCCGAGTCTAC3' Reverse: 5'CTTGGAGACACTGGTGATCCAA3'
□-Actin	Forward: 5'GGCACCACACCTTCTACAATG3' Reverse: 5'GGGGTGTTGAAGGTCTCAAAC3'

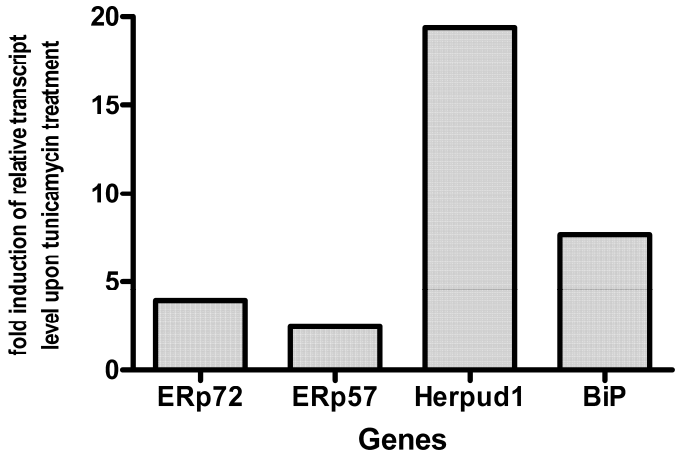

Figure 3. Induction of UPR genes upon ER stress. Mef cells were cultured in the presence of tunicamycin (TUNI) (2.5μg/ml) for 4 hours. Each mRNA was quantified by RT-qPCR using β-actin as reference gene in control and tunicamycin-treated cells. The graph shows how many times a gene is up-regulated after tunicamycin treatment.

3.3. **XBP-1 reverse transcription PCR (RT-PCR) splicing assay.**

Upon ER stress, transcribed XBP-1 mRNA is converted to its active form by unconventional cytoplasmic splicing mediated by IRE-1 (YOSHIDA [51]). XBP1 protein derived from spliced XBP1 mRNA functions as a potent transcription factor. We evidenced the protein product by western blot and described the protocol used above. However, we successfully employed another cheap, semi-quantitative method to measure expression of spliced XBP1: agarose analysis after RT-PCR, based on the length difference between unspliced and spliced XBP1. The protocol we used belongs to David Ron lab (HARDING [52]).

Cells (control and stressed cells with tunicamycin) were dissolved in TRIzol (Life Technologies) and total RNA was purified following the manufacturer recommendations. We used DEPC treated water to dissolve RNA. RNA purification was followed by the reverse transcription using Promega ImProm-II™ Reverse Transcription System kit for RT and spliced and non-spliced XBP1 were amplified with a PCR using specific primers. Table 3 contains the detailed sequence of the primers we used. PCR product was visualized on a 2.5% agarose gel (see Figure 1e).

Table 3. Primers used to detect XBP1 mRNA (HARDING [52]).

Primer	Sequence
hXBP1 forward	**5'** AAACAGAGTAGCAGCTCAGACTGC **3'**
mXBP1 forward	**5'** AAACAGAGTAGCAGCGCAGACTGC **3'**
mhXBP1 reverse (works for both human and mouse)	**5'** TCCTTCTGGGTAGACCTCTGGGAG **3'**

PCR program : 4 min at 94°C, (10 sec at 94°C, 30 sec at 63-68°C, and 30 sec at 72°C) × 35, and 10 min at 72°C.

3.4. Other methods

We listed above three methods we currently use in our laboratory to analyze UPR activation. Beside those, several other laboratories developed alternative experimental approaches for investigating UPR. Based on nuclear translocation of soluble ATF6 and of sXBP1, many data emerged from immunofluorescence experiments. We found these experiments partly difficult when looking at endogenous ATF6 activation, as the technique rely on both good antibodies for IEF and translocation of a substantial amount of ATF6 into the nucleus. Overcoming these disadvantages and the well-known effects of overexpressing ATF6, we propose to look at ATF6 translocation into the nucleus using an assay developed by Thermo Scientific (Thermo Scientific ATF6 Redistribution Assay). The system is based on recombinant U2OS cells stably expressing human ATF6 fused to the C-terminus of enhanced green fluorescent protein (EGFP). However, we found the price of this assay prohibitive.

There are also several reporter assays which can be used to detect ATF6 and XBP1 activation. These techniques make use of luciferase gene that was engineered under the control of transcription factors consensus binding sites. In p4xXBPGL3 reporter for instance, the luciferase gene is under the control of four tandem copies of the XBP1 consensus binding 5'-CGCG(TGGATGACGTGTACA)$_4$-3' (LEE [53]). Although there are some problems regarding these reporter systems (several other ERSE reporters, the need to be used in combination with the corresponding function-ablating mutant promoter, and so on), the reporter assays remain sensitive techniques to detect UPR activation.

Concluding remarks:

UPR functions as the major response to ER stress, but also plays indispensable roles in normal cellular function, involving both transcriptional and nontranscriptional mechanisms. In order to study UPR activation in health and disease is crucial to establish simple methods that can insure reproducible data about different levels of UPR activation. We showed that western blotting, Q-PCR and RT-PCR are reliable, cost-effective techniques that can contribute to our current knowledge regarding cellular physiology.

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