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Chapter 3

A quantitative method for detection of spliced X-box binding protein (XBP1) mRNA as a measure of endoplasmic reticulum stress

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Abstract

Endoplasmic reticulum (ER) stress is increasingly recognised as an important mechanism in a wide range of diseases including cystic fibrosis, α₁-antitrypsin deficiency, Parkinson’s and Alzheimer’s disease. Therefore, there is an increased need for reliable and quantitative markers for detection of ER stress in human tissues and cells. Accumulation of unfolded or misfolded proteins in the endoplasmic reticulum can cause ER stress, which leads to the activation of the unfolded protein response (UPR). UPR signaling involves splicing of X-box binding protein-1 (XBP1) mRNA, which is frequently used as a marker for ER stress. In most studies, the splicing of the XBP1 mRNA is visualised by gel electrophoresis which is laborious and difficult to quantify. In the present study we have developed and validated a quantitative RT-PCR method to detect the spliced form of XBP1 mRNA.
Introduction

Endoplasmic reticulum (ER) stress induced by protein misfolding is an important mechanism in cellular stress in a variety of diseases. When protein folding in the ER is compromised, the unfolded proteins accumulate in the ER which leads to ER stress. ER stress triggers the unfolded protein response (UPR), a transcriptional induction pathway which is aimed at restoring normal ER functioning (1).

The UPR is mediated by three ER stress receptors: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE1) and activating transcription factor-6 (ATF6). In the absence of ER stress, all three ER stress receptors are maintained in an inactive state through their association with the ER chaperone protein GRP78 (BiP). ER stress results in the dissociation of BiP from the three receptors, which subsequently leads to their activation (2). Dissociation of BiP from PERK leads to autophosphorylation and thereby activation of PERK and subsequent phosphorylation of translation initiation factor eIF2α, resulting in an inhibition of mRNA translation, and eventually in the translation of the transcription factor ATF4. Dissociation of BiP from ATF6 leads to translocation of ATF6 to the Golgi complex where it is cleaved by proteases into an active transcription factor. Active ATF6 moves to the nucleus and induces expression of genes with an ER stress response element (ERSE) in their promoter such as the ER chaperone BiP and the transcription factors C/EBP homologous protein (CHOP) and X-box binding protein-1 (XBP1). Dissociation of BiP from IRE1 leads to the activation of IRE1 which cleaves a 26-nucleotide intron from the XBP1 mRNA. The spliced XBP1 mRNA encodes a stable, active transcription factor that binds to the UPRE or ERSE sequence of many UPR target genes, leading to transcription of ER-chaperone proteins (2, 3).

The UPR can be induced experimentally by chemicals like thapsigargin and tunicamycin. Thapsigargin blocks the ER calcium ATPase pump, leading to depletion of ER calcium stores and tunicamycin blocks N-linked glycosylation of proteins. Both chemicals lead to high levels of stressors which are expected to rapidly activate all three components of the UPR (4).
An increasing number of studies have reported the involvement of ER stress in a variety of diseases, including cystic fibrosis, α,-antitrypsin deficiency, Parkinsons’ and Alzheimers’ disease. Therefore there is a growing demand for quantifiable markers to measure ER stress. The splicing of XBP1 mRNA is considered to be an important marker for ER stress, however the quantification is difficult because the splicing is mainly visualised by gel electrophoresis after conventional RT-PCR. We have now developed a simple and quantitative method to measure spliced human XBP1 by using quantitative real-time RT-PCR, and we show that the results obtained with this method correlate with data for BiP and CHOP mRNA.
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Results and discussion

Several publications described methods for monitoring ER stress and the UPR, and spliced XBP1 mRNA is generally considered to be a relevant marker for ER stress. However, the semi-quantitative conventional RT-PCR method is currently used to assess the splicing of the XBP1 mRNA. Only Hirota et al. (5) developed a quantitative real-time RT-PCR method for measuring spliced XBP1, but this method does involve an additional step with a restriction enzyme during the PCR reaction, which we consider to be more laborious and more complex. Both Samali et al. (6) and Zhao et al. (7) recently reviewed methods for monitoring ER stress, and recommended the analysis of spliced XBP1 by conventional RT-PCR for detection of ER stress.

In our study we have designed primers that span the 26bp intron of the XBP1 mRNA, in order to amplify only the spliced XBP1 mRNA. Because of the similarities between the sequence of the XBP1 mRNA just before this intron and the last part of the intron itself, only a very few options were possible to design a specific forward primer for the spliced XBP1 mRNA (Figure 1). We tested the specificity of the XBP1spl primers by sequencing. The PCR product of the thapsigargin treated, as well as the DMSO treated cells, both matched the spliced XBP1 mRNA and no unspliced XBP1 mRNA was detected with the new primers. These results also indicate that in DMSO treated cells there is a low level of spliced XBP1 mRNA present.

Next, we compared the semi-quantitative conventional RT-PCR method with the newly developed quantitative real-time RT-PCR method by conducting a time course experiment with thapsigargin and DMSO on primary bronchial epithelial cells. In both methods the spliced XBP1 mRNA was mostly expressed after 6 hours, and its levels were found to be comparable between the two methods (Figure 2). Next, we used the XBP1spl primers to perform a quantitative RT-PCR in a dose-response experiment on primary bronchial epithelial cells, using various concentrations of thapsigargin as well as two concentrations of tunicamycin. In addition, CHOP and BiP mRNA were analysed and correlated with levels of spliced XBP1 mRNA (Figure 3A and 3B). We found a significant and high correlation between CHOP and spliced XBP1 mRNA (r=0.962, p<0.001) as well
as between BiP and spliced XBP1 mRNA \((r=0.884, p<0.001)\). Since both CHOP and BiP genes contain an ERSE region which is recognised by the XBP1 protein, a good correlation between the spliced XBP1 and the more downstream ER stress markers CHOP and BiP was anticipated.

To evaluate whether the new XBP1spl primers could also be used in other cell lines, experiments were performed in two different epithelial cell lines, HK-2 (a proximal tubule epithelial cell line from normal adult human kidney) and A549 (a human lung carcinoma cell line with type II alveolar epithelial cell characteristics). We repeated the dose-response experiments on these cell lines and compared the results with those
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Figure 3. Effect of thapsigargin and tunicamycin on markers of ER stress in primary bronchial epithelial cells.

Primary bronchial epithelial cells were exposed to various concentrations of thapsigargin or tunicamycin for 6 hours, and next total RNA was isolated for quantitative RT-PCR-based detection of spliced XBP1, CHOP and BiP mRNA. The results showed a dose-dependent increase in spliced XBP1 mRNA after exposure to both stimuli (A), that showed a significant correlation with levels of CHOP and BiP mRNA (B). Data are mean ± SEM using cells from 3 different donors (A), and data points represent the result of a single experiment (B).

previously obtained with the primary bronchial epithelial cells (Figure 4). We found a dose-dependent increase of spliced XBP1 mRNA in both cell lines, similar to the results found in primary bronchial epithelial cells.
In conclusion, the novel quantitative real-time RT-PCR method described in this report is a reliable quantitative method to measure spliced human XBP1 mRNA as a marker for ER stress.

**Figure 4. Effect of thapsigargin and tunicamycin on spliced XBP1 mRNA in different cell lines.**

Primary bronchial epithelial cells, HK-2 and A549 cells were exposed to various concentrations of thapsigargin or tunicamycin, and next total RNA was isolated for quantitative real-time RT-PCR-based detection of spliced XBP1 mRNA. The results showed a dose-dependent increase in spliced XBP1 mRNA after exposure to both stimuli for all cell lines. Data are mean ± SEM using cells from 3 different donors (primary bronchial epithelial cells) or 3 separate experiments (HK-2, A549). Spliced XBP1 mRNA data for primary bronchial epithelial cells are the same as those shown in Figure 3A.
Materials and methods

Cell culture and stimulation

Primary bronchial epithelial cells were isolated from resected lung tissue obtained from patients undergoing surgery for lung cancer as described previously (8). Briefly, the cells were cultured in a 1:1 mixture of DMEM (Invitrogen, Carlsbad, CA, USA) and BEGM (Clonetics, San Diego, CA, USA), supplemented with 0.4% w/v BPE, 0.5 ng/ml EGF, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 10 μg/ml transferrin, 1 μM hydrocortisone, 6.5 ng/ml T3, 0.5 μg/ml epinephrine (all from Clonetics), 1.5 μg/ml BSA (Sigma-Aldrich, St Louis, MO, USA), 1 mM Hepes (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Cambrex, East Rutherford, NJ, USA).

Immortalised human renal PTEC (HK-2, kindly provided by M. Ryan, University College Dublin, Dublin, Ireland) were grown in serum-free DMEM/HAM-F12 (Bio-Whittaker, Walkersville, MD) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Breda, The Netherlands), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), triiodothyronine (40 ng/ml), epidermal growth factor (10 ng/ml), and hydrocortisone (36 ng/ml, all purchased from Sigma).

Cells from the A549 human lung carcinoma cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Cambrex, East Rutherford, NJ, USA) and 10% (v/v) heat-inactivated FCS (Gibco) at 37°C in a 5% CO2-humidified atmosphere.

ER stress was induced in cells by exposure to thapsigargin or tunicamycin (both Sigma). After reaching near-confluence, primary bronchial epithelial cells were exposed to thapsigargin (50 nM) for various time periods. For the dose-response experiment, primary bronchial epithelial cells from 3 different donors were stimulated with various concentrations of thapsigargin or tunicamycin for 6 hours. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) served as a solvent control for both thapsigargin and tunicamycin. The dose-response experiments were repeated on HK-2 cells and A549 cells.
with 2 hours stimulation instead of 6 hours. This shorter duration of exposure in HK-2 and A549 cells was based on pilot experiments using these cell lines.

**Total RNA isolation and reverse-transcription**

After stimulation, the cells were washed twice with PBS and total mRNA was isolated using the RNeasy mini kit (Qiagen, Valencia CA, USA). DNase I amplification grade (Invitrogen) was used to remove genomic DNA. Total RNA concentration and purity were measured on a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington USA). Next, cDNA synthesis was performed with M-MLV Reverse Transcriptase (Promega, Madison WI, USA).

**Semi-quantitative RT-PCR**

To amplify the spliced and unspliced XBP1 mRNA, XBP1 primers were used as described previously (3). PCR products were electrophoresed on 2.5% agarose gel. GAPDH (forward 5’ GGATGATGTTCTGGAGAGCC 3’, reverse 5’ CATCACCATCTTCCAGGAGC 3’) was used as a loading control. The size difference between the spliced and the unspliced XBP1 is 26 nucleotides.

**Quantitative real-time RT-PCR**

Primers were designed to span the 26 base pair intron that is removed by IRE1 to obtain the spliced XBP1 mRNA (XBP1spl; forward 5’ TGCTGAGTCCGCAGGTG 3’ and reverse 5’ GCTGGCAGGCTCTGGGAAG 3’). Also specific primers for CHOP mRNA (forward 5’ GCACCTCCAGAGCCCTCACTCC 3’ and reverse 5’ GTCTACTCCAAGCCTTCCCCCTGCG 3’) and BiP mRNA (5) were used. Quantitative real time RT-PCR was carried out at 95°C for an initial 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C for 15 seconds and extension at 72°C for 30 seconds using IQ SYBRGreen supermix (Bio-Rad, Hercules, CA, USA). Each assay was run on a Bio-Rad CFX Real-time PCR system in triplicates and arbitrary mRNA concentrations were calculated by the Bio-Rad software, using the relative standard curve method. Stable housekeeping genes were selected
using the Genorm software (9). Relative mRNA concentrations of ATP5B and RPL13A (GeNorm, Primerdesign, Southampton, UK) were used as reference genes to calculate the normalised expression of the spliced XBP1 mRNA. The identity of the PCR products obtained with the XBP1spl primers was verified by DNA sequencing.

**Statistical analysis**

The results of the dose-response experiment were expressed as mean ± SEM. The correlation coefficient was determined by the use of Pearson's correlation statistics. The correlation coefficient was considered significant at p-values < 0.05.

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References


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