FEBS Letters 588 (2014) 3062–3067

journal homepage: www.FEBSLetters.org

Polynomial algebra reveals diverging roles of the unfolded protein response in endothelial cells during ischemia–reperfusion injury

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ARTICLE INFO

Article history:
Received 15 February 2014
Revised 23 May 2014
Accepted 30 May 2014
Available online 16 June 2014

Edited by Takashi Gojobori

Keywords:
Ischemia–reperfusion injury (IRI)
Unfolded protein response (UPR)
Endothelial cells (EC)
Murine embryonic cells (MEC)
Probabilistic polynomial dynamical systems (PDS)
Gene regulatory networks (GRN)

ABSTRACT

The unfolded protein response (UPR) – the endoplasmic reticulum stress response – is found in various pathologies including ischemia–reperfusion injury (IRI). However, its role during IRI is still unclear. Here, by combining two different bioinformatical methods – a method based on ordinary differential equations (Time Series Network Inference) and an algebraic method (probabilistic polynomial dynamical systems) – we identified the IRE1α–XBP1 and the ATF6 pathways as the main UPR effectors involved in cell's adaptation to IRI. We validated these findings experimentally by assessing the impact of their knock-out and knock-down on cell survival during IRI.
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1. Introduction

This study proposes a new application of polynomial algebra in biology by determining which gene from a specific signaling pathway could have the greatest impact on cell's response to a stimulus.

1.1. The unfolded protein response

The endoplasmic reticulum (ER) is involved in the synthesis and maturation of secreted and membrane proteins which represent 30% of cell's proteins. It also presents numerous functions such as gluconeogenesis, calcium storage, and lipid synthesis [1]. Cells are frequently stressed by their environment, e.g., when pancreatic β-cells respond to hyperglycemia by synthesizing and secreting large amounts of insulin to reduce glycemia to its normal level. This stress is caused by an increase in the load of newly synthesized proteins that are unfolded. As the folding machinery is insufficient to cope with this increase, there is an accumulation of unfolded proteins. This triggers a series of complementary adaptive mechanisms to resolve ER's stress which are together known as the unfolded protein response (UPR). It is now known that UPR dysfunctions could lead to various pathological states [2,3].

In mammalian cells, the UPR is composed of three distinct pathways namely, IRE1α–XBP1 (also named ERN1–XBP1), PERK-eIF2α-ATF4 (also named EIF2AK3-EIF2S1-ATF4), and ATF6 which act to reestablish proteostasis (see Supplementary Fig. 1). If this stress is irreversible, the ER – through the UPR – eliminates these cells by triggering cell death [4]. Herein, we explored simultaneously these three pathways by studying the mRNA expression of IRE1α, XBP1 isoforms (total, XBP1t; spliced, XBP1s; unspliced, XBP1u), and one of the downstream targets of XBP1, Erdj4, for the IRE1α–XBP1 pathway; PERK, ATF4, and its direct targets GADD34 (also named PPP1R15A) and CHOP (also named DDIT3) for the PERK-eIF2α-ATF4 pathway; ATF6 and two of its downstream targets, GRP78 (also named BiP or HSPA5) and HerpUD, for the ATF6 pathway [5].

1.2. Ischemia–reperfusion injury (IRI)

Ischemia–reperfusion injury (IRI) is a syndrome combining the cessation of the blood flow followed by its reestablishment [6]. It is

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http://dx.doi.org/10.1016/j.febslet.2014.05.065
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encountered in different contexts such as surgery, cardiovascular diseases or organ transplantation. In addition, longer durations of ischemia have been correlated with delayed graft function, acute rejection, chronic dysfunction, and graft loss [7,8]. It has been reported that IRI could trigger the UPR [9–11]. Nevertheless, the roles of each UPR pathway in this pathology remain to be determined. We focused our study on endothelial cells (EC) which are known to be the first target of IRI [12,13].

1.3. Polynomial dynamical system (PDS) and times series identification network (TSNI)

The identification of dynamic models for gene regulatory networks (GRN) from proteome and transcriptome is a hot research topic. In this regard, various methods are available [14]. We focused here on two methods recently developed to infer GRN from short time series: a method based on ordinary differential equations (TSNI) which has been successfully used to determine genes under the control of a specific transcription factor [15] and an algebraic method developed to uncover possible interactions between genes without prior hypotheses namely the polynomial dynamical systems (PDS) also named algebraic models [16].

PDS are a multistate generalization of Boolean networks allowing a more subtle description of the expression level of the genes than two-states Boolean systems (expressed vs not expressed). It has been demonstrated that K-bounded Petri nets, Boolean networks, and logical models can be translated into PDS [17]. As for probabilistic Boolean networks [18], PDS evolved to a probabilistic form called probabilistic polynomial dynamical systems (pPDS) [19] to circumvent the issue of model selection by introducing the Gröbner fan and its cones' relative volumes [20,21].

Finally, the dependency between different genes is determined by defining an appropriate threshold on the Deegan–Packel indices of power (DPIp) calculated as in [21,22].

1.4. Approach

To identify which gene presents the greatest probability to influence the cell's response to a particular stimulus, we hypothesized that the higher the number of genes depending on a single gene, the higher the impact of this single gene on the response of a cell to a stimulus (a case study is presented in the Supplementary Material and Method). Herein, the combination of both methods allowed the determination of the most perturbed genes (TSNI) and the most disruptive genes (pPDS).

2. Methods

2.1. Cell culture

All cell culture media, sera, and supplements were purchased from Invitrogen. STF083010 (10 μM) and 4μ8c (10 μM) were purchased from Axon MedChem. Tunicamycin (2 μg/L) and 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF, 300 μM) were purchased from Sigma. All chemicals were dissolved in DMSO (Sigma). IRE1$\alpha^{-/-}$ and IRE1$\alpha^{-/-}$-rescued murine embryonic cells (MEC) were produced as previously described [23]. The wild-type murine embryonic fibroblasts (MEF) and MEF knocked-out for ATF6 and PERK were kindly provided by Pr. Randal J. Kaufman (MTA 13-141). Primary human aortic endothelial cells (HAEC) were obtained from Gibco (Lot No: #765093 and #999999) and cultured on 1% gelatin (Sigma) coated flasks of 75 cm^2 in M200 medium supplemented with LSGS, 8% fetal bovine serum (FBS), and 100 μg/mL penicillin and streptomycin in a humidified atmosphere at 21% O_2, 5% CO_2 and 37 °C. The HAEC were split at a ratio of 1:4 every 5 days and every 3 days for the MEF and MEC. Cells up to passage 5 were used in this study. MEF and MEC were cultured in DMEM high-glucose media supplemented with 10% FBS, non-essential amino acids, penicillin and streptomycin, L-glutamine, and puromycin (3 μg/mL). β-Mercaptoethanol (50 μM) was added to culture PERK$^{-/-}$ cells.

2.2. Hypoxia-reoxygenation experiments

After PBS washing, HAEC were incubated in cold UW preservation solution in a chamber with hypothermic (4 °C) hypoxic atmosphere obtained by flushing the chamber atmosphere with Bactal 2 (0% O_2, 95% N2 and 5% CO_2) until the reach of 0% O_2 in the chamber atmosphere. The oxygen level was controlled with the oximeter Oxy-4 micro from PreSens Precision Sensing GmbH with channels present in the outside's atmosphere, the chamber's atmosphere and the cell's supernatant. To perform the normothermic reoxygenation, cells were washed with PBS and then incubated with M200 supplemented with 2% FBS in a humidified atmosphere at 21% O_2, 5% CO_2 and 37 °C. The same protocol was applied to MEF and MEC except that the hypothermic hypoxia and the warm reperfusion were performed with their standard media to avoid perturbations due to their specific culture conditions (see http://www.ron.medschl.cam.ac.uk/protocols/GrowingISRdefCELLS.html).

2.3. Quantitative PCR (qPCR)

RNA was extracted from cells using the NucleoSpin RNA kit (Macherey–Nagel) containing a DNase treatment to remove potentially contaminating genomic DNA. RNA quality was verified by resolution on a 1.5% (wt/vol) agarose gel and measurement of A260 nm/A280 nm and A260 nm/A230 nm ratios, using a NanoDropTM 2000 (Thermo Scientific). A quantity of 1 μg of total RNA was reverse-transcribed with High Capacity cDNA Reverse Transcription kit (ABsystems). qPCR was performed in triplicate with negative template controls, negative enzyme controls, and the use of a calibrator to limit inter-run variations as recommended by the MIQE guidelines [24]. Two reference genes, RPS5 and RPS15, were validated under hypoxia and hypoxia-reoxygenation conditions by using the geNorm algorithm [25]. The amplification efficiencies were tested by a five log-dilution and were comprised between 1.85 and 2.15. The runs were performed with Rotor-Gene 3000 (Qiagen) and results were analyzed using the EasyqpcR package [26]. The lists of the primers used in this study are available in the Supplementary Material and Method.

2.4. Immunoblotting

The HAEC were washed in cold PBS and resuspended in cOmplete lysis buffer (Roche Diagnostics, #04719956001) before being sonicated at output power of 2 for 3 s (Branson Sonifier 450). The protein concentration of cell lysate supernatant was measured by BCA protein assay (Bio-Rad, #23225). 10–20 μg of cell lysate was applied to SDS/PAGE and transferred to Hybond PVDF membrane (Amersham Biosciences), followed by standard Western blot procedure. The bound primary antibodies were detected with ChemiDoc™ MP imager (Bio-Rad) by the use of HRP-conjugated secondary antibody and the ECL detection system (Amersham Biosciences). The band density was semiquantified with Image Lab Software (Bio-Rad). We used primary antibodies from Cell Signaling for PERK (#3192, 1:1000), IRE1α (#3294, 1:1000), from Abcam for XBP1 (ab37152, 1:500), ATF4 (ab1371, 1:1000), and ATF6 (ab37149, 1:1000), and from Millipore for GAPDH (CB1001, 1:30000). The secondary antibodies were from Invitrogen (G-21040 and G-21234, 1:4000) and Santa-Cruz (sc-2922, 1:4000).

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