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Proteome-wide study of endoplasmic reticulum stress induced by thapsigargin in N2a neuroblastoma cells

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ABSTRACT

Disturbances in intraluminal endoplasmic reticulum (ER) Ca^{2+} concentration leads to the accumulation of unfolded proteins and perturbation of intracellular Ca^{2+} homeostasis, which has a huge impact on mitochondrial functioning under normal and stress conditions and can trigger cell death. Thapsigargin (TG) is widely used to model cellular ER stress as it is a selective and powerful inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases. Here we provide a representative proteome-wide picture of ER stress induced by TG in N2a neuroblastoma cells. Our proteomics study revealed numerous significant protein expression changes in TG-treated N2a cell lysates analysed by two-dimensional electrophoresis followed by mass spectrometric protein identification. The proteomic signature supports the evidence of increased bioenergetic activity of mitochondria as several mitochondrial enzymes with roles in ATP-production, tricarboxylic acid cycle and other mitochondrial metabolic processes were upregulated. In addition, the upregulation of the main ER resident proteins confirmed the onset of ER stress during TG treatment. It has become widely accepted that metabolic activity of mitochondria is induced in the early phases in ER stress, which can trigger mitochondrial collapse and subsequent cell death. Further investigations of this cellular stress response in different neuronal model systems like N2a cells could help to elucidate several neurodegenerative disorders in which ER stress is implicated.

1. Introduction

The endoplasmic reticulum (ER) is a membrane-bound organelle presented in all eukaryotic cells, where it has a role in storing, modifying and transporting newly synthesized proteins and in the regulation of intracellular Ca^{2+} homeostasis (Stutzmann and Mattson, 2011). Owing to its role as an intracellular Ca^{2+} reservoir, the ER functions as an excitable system that is capable of spreading Ca^{2+}-related signals throughout the cell (Berridge, 2002). Changes in intracellular Ca^{2+} concentration regulate the activity of numerous proteins with roles in different cellular processes (Berridge et al., 2003). Disturbances in intraluminal ER Ca^{2+} concentration lead to protein unfolding because of the Ca^{2+}-dependent nature of several ER chaperones, such as glucose-regulated protein 78 (Grp78), Grp94 and calreticulin (Ma and Hendershot, 2004). These proteins are involved in protein folding, post-translational modification, Ca^{2+} storage and release, and lipid synthesis and metabolism (Michalak et al., 2002). A recent study suggests that ER may contain transient receptor potential cation channel subfamily V member 1 inducing Ca^{2+} leak (Gallego-Sandin et al., 2009). In addition, calcium homeostasis modulator 1 is able to decrease the Ca^{2+} content of the ER, which produces ER stress leading to cell death (Gallego-Sandin et al., 2011). Accumulation of unfolded proteins triggers the unfolded protein response (UPR), which is an evolutionarily conserved cell stress response that initially aims to compensate for damage, but can trigger cell death if the ER dysfunction is severe or prolonged (Xu et al., 2005).

Abbreviations: 2-DE, two-dimensional electrophoresis; AD, Alzheimer's disease; BSA, bovine serum albumin; Calr, calreticulin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CV, coefficient of variation; DAVID, Database for Annotation, Visualisation and Integrated Discovery; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; ER, endoplasmic reticulum; ERp, ER resident protein; GO, gene ontology; Grp, glucose-regulated protein; IAA, iodoacetamide; IDH, isocitrate dehydrogenase; IP_3R, inositol trisphosphate receptor; MAM, mitochondrial associated membrane; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PAGE, polyacrylamide gel-electrophoresis; PDH, pyruvate dehydrogenase; PDI, protein disulphide isomerase; RuBPs, ruthenium (II) bathophenanthroline disulfonate; SDS, sodium dodecyl sulphate; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SGoF, sequential goodness of fit; TG, thapsigargin; UPR, unfolded protein response; VDAC, voltage-dependent anion channel.

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Thapsigargin (TG) is a sesquiterpene alkaloid that is a highly selective and powerful inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCAs) pumps. By the inhibition of SERCAs, TG prevents Ca^{2+} transport into the ER lumen and subsequently increases the Ca^{2+} level in the cytosol (Lytton et al., 1991; Sagara and Inesi, 1991). TG induced a rapid rise followed by a sustained increase in free cytosolic Ca^{2+} in the murine hypothalamic cell line, GT1-7 (Wei et al., 1998). Furthermore TG triggered ER stress-induced apoptotic cell death in primary rat cortical neurons (Chung et al., 2011).

Besides many other human diseases, ER stress and UPR are also implicated in different neurological disorders (Matus et al., 2011). Investigations of clinical samples confirm that ER stress is one of the main pathological events in Parkinson's disease, Alzheimer's disease and multiple sclerosis (Hoozemans et al., 2007; Hoozemans et al., 2009; Cunnea et al., 2011). In addition, very recent studies provide direct evidence of upregulation of UPR in motor neuron disease models suggesting that ER stress plays a major role in amyotrophic lateral sclerosis (Prell et al., 2012). Despite the accumulated knowledge, the exact mechanism of the neuronal ER stress remains obscure. However, the UPR-related signalling pathways and related metabolic processes could be a potential therapeutic target for the treatment of neurological disorders.

In this study, we used two-dimensional electrophoresis (2-DE) along with mass spectrometry (MS) to identify protein expression changes in TG-treated N2a neuroblastoma cells. We showed that several ER chaperone proteins were upregulated on TG treatment, which could confirm the activation of TG-induced ER stress. Here Grp78 expression was validated meticulously by Western blot analysis. Besides ER chaperones, majority of the upregulated proteins belonged to the mitochondrial proteome suggesting a strong crosstalk between ER stress and mitochondrial bioenergetic changes. Morphological changes of cells were also observed after TG treatment resulting in a marked reduction in the number of neurites. This correlated well with the fact that a significant portion of the downregulated proteins control cytoskeletal organisation.

2. Materials and methods

2.1. Materials

High performance liquid chromatography grade acetonitrile, formic acid, dimethyl sulfoxide (DMSO), Tris, urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithioerythritol (DTE), iodoacetamide (IAA), glycerol and TG were obtained from Sigma–Aldrich (Budapest, Hungary). Sodium dodecyl sulphate (SDS) and piperazine diacrylamide were purchased from Bio-Rad (Hercules, CA, USA). Acrylamide was procured from GE Healthcare (Little Chalfont, UK).

2.2. Cell culture, treatment and cell viability assay

A slightly modified method of Datki et al. (2003) was used for cell culturing. Mouse N2a cells (Sigma–Aldrich, Hungary) were grown for 2 days on 96-well plates reaching a density of 3×10^4 cells per well to confluency with 5% CO_2 in a humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM). L-Glutamine (4 mM; Gibco, Europe), penicillin (200 units/mL; Gibco), streptomycin (200 μg/mL; Gibco), MEM non-essential amino acid solution (100 × liquid mg/L; Gibco) and 10% fetal bovine serum (FBS; Gibco) were added to the medium. The cells were treated with 1 μM TG dissolved in DMSO (stock solution 1 mM) or 0.1% DMSO as control. EZ4U assay was performed to measure the toxic effect of TG on cell viability. N2a cells grown on 96-well plates were treated with 1 μM TG or 0.1% DMSO for 24 or 48 h, and the media was changed to FBS-free media during treatments. After 24 or 48 h incubation, 10 μL of EZ4U stock solution (one kit dissolved in 2.5 mL activator solution and 2.5 mL distilled water; Biomedica, Hungary) was added to each well, containing a 90 μL FBS-free medium, and the mixture was incubated for 2 h. The optical density was measured with a 96-well plate ELISA reader at 490 nm, with the reference filter set to 620 nm. Each cell viability assay was triplicated.

2.3. Sample preparation, 2D gel-electrophoresis and image analysis

For 2-DE and Western blot analysis N2a cells were grown in 60 mm Petri-dishes and were treated with TG as described above. After 24 h of treatment cells were washed three times with phosphate-buffered saline (PBS), then collected in 1.5 mL ice cold PBS. The suspensions were centrifuged at 1000*g* for 10 min at 4 °C. The supernatants were removed and the cell pellets were stored at −80 °C until lysis. Each pellet was lysed in 100 μL 2-DE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTE) supplemented with a 1% (v/v) protease inhibitor cocktail (Sigma–Aldrich). The samples were incubated on ice for 15 min then the cells were physically disrupted by sonication for 6 cycles (10 s sonication/10 s break) on ice. The lysates were centrifuged at 14,000*g* for 30 min at 4 °C. The supernatants were then pipetted into clean Eppendorf tubes. Four samples per group were pooled to obtain a sufficient amount of protein for 2-DE (12 Petri-dishes/group). On the whole three independent gels were made and analysed per group. The protein concentrations were determined by a Non-Interfering Protein Assay Kit (Calbiochem, Gibbstown, NJ, USA). A volume of samples containing 400 μg of total protein was supplemented with a 2-DE lysis buffer to a total volume of 450 μL plus 2.5 μL Bio-Lyte 3–10 buffer (Bio-Rad, Hercules, CA, USA) and left on 24 cm, pH 3–10, NL IPG strips (Bio-Rad) for overnight rehydration. Isoelectric focusing and subsequent SDS–polyacrylamide gel-electrophoresis (SDS–PAGE) were performed as described previously (Foldi et al., 2011). After SDS–PAGE, the gels were stained with ruthenium (II) bathophenanthroline disulphonate (RuBPs) according to the protocol of Rabilloud et al. (2001). Following staining, the gels were scanned on a FLA-5100 laser scanner (Fujifilm, Tokyo, Japan) using a 473 nm laser and the 575 nm lp filter at 100 μm resolution, and the photomultiplier (PMT) was adjusted to 400 V. Digitised 2-D gel images were analysed by Progenesis Samespots software version 3.3.3420.25059 (NonLinear Dynamics, Newcastle Upon Tyne, UK). Software-based analysis focused on those spots that were 100% matched across all the gels.

2.4. Mass spectrometric analysis and protein identification

RuBPs stained gels were overstained with colloidal Coomassie blue G-250 according to the "Silver Blue" staining protocol (Candiano et al., 2004). Individual spots of interest were excised from the gel, destained, and then subjected to in-gel digestion with trypsin for 24 h at 37 °C using modified protocol of Shevchenko et al. (1996). Tryptic peptides extracted from gel pieces using 50% acetonitrile and 5% formic acid were dried under a vacuum. MS analysis was performed on a Waters NanoAcquity UPLC system coupled with a Micromass Q-TOF premier mass spectrometer (Waters, Millford, MA, USA) as described previously (Foldi et al., 2011). All the obtained data were processed and peaklists were generated by the Waters Proteinlynx Global Server, ver. 2.4 software using default settings. Each MS/MS sample was analysed using a Mascot 2.2.07 (Matrix Science, London, UK). The Mascot device was set up to search in a Swissprot 2011_05 database (528048 entries) where the selected digestion enzyme was trypsin. A database search was performed with a fragment ion mass tolerance of 0.15 Da and a