European Journal of Cell Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

Neuropeptide Y mitigates ER stress–induced neuronal cell death by activating the PI3K–XBP1 pathway

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ARTICLE INFO

Keywords:
Neuropeptide Y
ER stress
XBP1
PI3K
Grp78/BiP

ABSTRACT

The unfolded protein response (UPR) is an evolutionarily conserved adaptive reaction that increases cell survival under endoplasmic reticulum (ER) stress conditions. ER stress–associated neuronal cell death pathways play roles in the pathogenesis of neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease. Neuropeptide Y (NPY) has an important role in neuroprotection against neurodegenerative diseases. In this study, we investigated whether NPY has a protective role in ER stress–induced neuronal cell death in SK-N-SH human neuroblastoma cells. An ER stress–inducing chemical, tunicamycin, increased the activities of caspase-3 and -4, whereas pretreatment with NPY decreased caspase-3 and -4 activities during the ER stress response. In addition, NPY suppressed the activation of three major ER stress sensors during the tunicamycin-induced ER stress response. NPY-mediated activation of PI3K increased nuclear translocation of XBP1s, which in turn induced expression of Grp78/BiP. Taken together, our data indicated that NPY plays a protective role in ER stress–induced neuronal cell death through activation of the PI3K–XBP1 pathway, and that NPY signaling can serve as therapeutic target for ER stress–mediated neurodegenerative diseases.

1. Introduction

The endoplasmic reticulum (ER) is involved in maintenance of cellular homeostasis, including protein folding and quality control, intracellular calcium balance, and cholesterol synthesis (Ron and Walter, 2007). Many genetic and environmental insults, such as disruption of calcium homeostasis, inhibition of protein glycosylation or disulfide bond formation, hypoxia, and the accumulation of unfolded or misfolded proteins in ER lumen, can disturb the function of ER and induce ER stress (Boyce and Yuan, 2006). Neurons are especially vulnerable to factors that induce ER stress, including accumulation of unfolded proteins and disturbances in redox and calcium homeostasis (Matus et al., 2011). A number of studies demonstrate that ER stress increases in common neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (Matus et al., 2011).

To overcome ER stress, mammalian cells activate an adaptive intracellular signaling cascade known as the unfolded protein response (UPR) that is involved in attenuation of protein translation, induction of molecular chaperones, and promoting the clearance of misfolded proteins in the ER (Ron and Walter, 2007). Three distinct UPR signaling pathways exist in mammalian cells, mediated by ER transmembrane inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Wu and Kaufman, 2006). During the UPR, these three ER stress sensors are activated, leading to three independent pathways involved in attenuation of protein translation, induction of molecular chaperones, and clearance of misfolded proteins in the ER (Ron and Walter, 2007). In particular, phosphorylation and dimerization of the transmembrane sensor IRE1 activates its cytoplasmic RNase domain, which cleaves the X-box binding protein 1 (XBP1) pre-mRNA in the cytoplasm (Calfon et al., 2002). Under ER

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1, ER transmembrane inositol-requiring enzyme 1; PERK, PKR-like ER kinase; ATF6, activating transcription factor 6; XBP1s, spliced XBP1; eIF2α, eukaryotic translation initiator factor 2α; ERAD, ER-associated degradation; NPY, neuropeptide Y; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; TM, tunicamycin

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https://doi.org/10.1016/j.ejcb.2018.04.003
Received 10 March 2018; Received in revised form 4 April 2018; Accepted 5 April 2018
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stress, IRE1 induces the unconventional splicing of XBP1 pre-mRNA, which eliminates a 26-nucleotide intron that changes the reading frame of the 3′ exon. The evolutionarily conserved cytoplasmic splicing of XBP1 produces transcriptionally active spliced XBP1 (XBP1s) (Yoshida et al., 2006). XBP1s directly activates ER stress target genes to facilitate the refolding and degradation of misfolded proteins, including ER chaperones such as Grp78/BiP, Grp58, Grp94; ER-associated degradation (ERAD) components such as EDEM and HRD1; and lipid synthesis and ER biogenesis pathways (Lee et al., 2003).

Activated PERK inhibits general protein translation through the phosphorylation of eukaryotic translation initiator factor 2α (eIF2α) (Harding et al., 2000a,b). Phosphorylated eIF2α augments the translation of activation of transcription-4 (ATF4), a transcription factor that controls the expression of a subset of UPR target genes involved in redox homeostasis, amino acid metabolism, apoptosis, and autophagy (Schroder and Kaufman, 2005). Upon UPR induction, ATF6 is released by Grp78/BiP and exported to the Golgi, where it is cleaved by S1P proteases (Haze et al., 1999; Yoshida et al., 2001). Cleaved ATF6 acts as a transcriptional activator that increases the expression of ER chaperones and ERAD-related genes (Haze et al., 1999; Yoshida et al., 2001).

Neuropeptide Y (NPY) is widely expressed in the central and peripheral nervous system and plays important roles in a variety of physiological processes, including the neuroendocrine mechanisms, cognitive functions, feeding behavior, cardiovascular activity, and regulation of neuronal excitability (Berglund et al., 2003; Hokfelt et al., 1998). These diverse actions of NPY are mediated by G protein–coupled receptor (GPCR) subtypes named NPY Y1, Y2, Y4, and Y5. Five subtypes of the NPY receptor have been identified in mammals, four of which are functional in humans (Mannon and Mele, 2000). The activation of NPY1R is generally associated with reduction of cAMP accumulation, an increase in intracellular free calcium concentration, and modulation of the MAPK pathway (Pedrazzini et al., 2003). Through NPY Y1 or Y5 receptor, NPY also activates ERK, protein kinase A (PKA)-CREB, and PI3K in various cell types (Lee et al., 2008b; Son et al., 2011; Zhou et al., 2008).

Several lines of evidence suggest that NPY plays a neuroprotective role against excitotoxic and ischemic neurodegeneration, as well as chronic neurodegenerative diseases. Neutralization of endogenous NPY by intracerebroventricular administration of anti-NPY antibody accelerates the onset of kainate-induced seizure and increases seizure-induced neuronal damage in the dentate gyrus (Veliskova and Velisek, 2007). Administration of NPY ameliorates AMPA and kainate-induced excitotoxicity in rat hippocampal slice cultures (Silva et al., 2003; Xapelli et al., 2007). Moreover, changes in NPY levels have been observed in brain ischemia and neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's diseases (Cannizzaro et al., 2003; Diez et al., 2003; Duszczyk et al., 2009; Oddo et al., 2004). In addition, NPY was shown to protect SH-SY5Y neuroblastoma cells from 6-hydroxydopamine–induced toxicity (Decressac et al., 2011). These studies suggest that NPY and NPY receptors in the nervous system may be therapeutic targets in various pathological conditions. In this study, we found that NPY plays a protective role against ER stress–induced neuronal cell death through activation of the PI3K–XBP1 pathway.

2. Materials and methods

2.1. Cell culture and NPY treatment

SK-N-SH and SK-N-MC human neuroblastoma cell lines were obtained from American Type Culture Collection and cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS in a humidified 95% air/5% CO_2 incubator. Cell culture reagents were purchased from Gibco BRL. Human NPY (Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2; Sigma) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 1 mM, stored as aliquots in the deep freezer (−70 °C), and thawed once. Prior to use, they were diluted with culture medium to yield the desired final concentration (100 nM). Tunicamycin (TM), LY294002, and wortmannin were purchased from Assay Designs, Calbiochem, and Sigma Chemical, respectively. The NPY1R inhibitor BIBO3304 (10 nM) and NPY2R inhibitor BIIE0246 (50 nM) were purchased from Tocris.

siRNA oligoribonucleotides corresponding to the human p85 and XBP1 were purchased from Dharmacon. Non-targeting scrambled siRNA (siCtrl) was used as the negative control. Total cells were transfected with 100 pmol siRNA in six-well plates by using RNAi Max (Invitrogen) as recommended by the supplier. At 24 h after transfection, cells were treated with the indicated agents. The efficiency of siRNA was checked by western blotting analysis at 48 h after transfection.

2.2. Cell viability and caspase substrate cleavage assays

For assessing apoptosis, alamarBlue assay and Hoechst staining were performed as described previously (Lee et al., 2008a). Caspase-3 and -4 activities were measured using colorimetric assay kits (BioVision Lab) as described previously (Lee do et al., 2010). Ac-DEVD-CHO (casp-3) or z-LEVD-FMK (casp-4) was used as a caspase-specific inhibitor (Sigma-Aldrich).

2.3. Primary cortical neuron culture

Primary cortical neuron cultures were prepared from embryos derived from pregnant C57BL/6 mice on embryonic day 18. Brains were isolated from embryos and placed into a dish containing calcium-, magnesium-, and bicarbonate-free Hank's balanced salt solution (CMF-HBSS, Welgene). Under a dissecting microscope, the cerebral hemisphere was separated in ice-cold CMF-HBSS. The meninges surrounding the cortex were taken away. Cortices were digested in 0.25% Trypsin (Welgene) and DNase (Takara) in a 37 °C water bath for 4 min. The cells were then triturated with a 10 ml pipet and centrifuged for 5 min at 700 rpm. The supernatant was discarded, and the cell pellet was re-suspended in Neurobasal medium (NBM, Thermo Fisher Scientific) containing B27 serum-free supplement (Thermo Fisher Scientific). Cortical neurons were plated onto Poly-D-Lysine coated dishes in NBM with B27 serum-free supplement. At 14 days *in vitro* (DIV), cortical neurons were cultured with TM and NPY. Subsequently, images of cortical neuron morphology were obtained on a Leica DMi1 microscope (Leica). C57BL/6 mice were maintained in a pathogen-free facility at Korea Research Institute of Bioscience and Biotechnology (KRIBB). Animal care was conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the KRIBB Institutional Animal Care and Use Committee.

2.4. CCK cell viability

After exposure of cortical neurons to TM and NPY for 12 h, 100 μl D-Plus™ CCK (Dongin Biotech) per 1 ml media was added to cortical neurons, and the cells were incubated at 37 °C for 1 h. The media were transferred to 96-well dishes, followed by gentle shaking for 1 min. Absorbance was measured at 450 nm on a Magellan Infinite F50 plate reader (Tecan).

2.5. RT-PCR and real-time RT-PCR analysis

Total RNA was isolated from cells using the Trizol Reagent (Invitrogen). cDNA was synthesized using the Superscript II Reverse Transcription system (Invitrogen). PCR reactions were performed using AccuPower PCR premix (Bioneer). For quantitative reverse transcription–PCR (RT-PCR) analysis, an ABI Prism 7900 Sequence Detection System (Applied Biosystems) and SYBR Green PCR Core reagents (Applied Biosystems) were used. mRNA levels were expressed as

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