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Research paper

ER and Golgi stresses increase ER–Golgi SNARE Syntaxin5: Implications for organelle stress and βAPP processing

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HIGHLIGHTS

- Effect of ER and Golgi stress on ER–Golgi SNARE expression was studied in neurons.
- ER and Golgi stress upregulated the synthesis of Syx5 proteins.
- Syx5 knockdown enhanced cell vulnerability to ER stress and apoptosis.
- Syx5 induced by such stress modulates neuronal βAPP processing and viability.

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ABSTRACT

Unresolved endoplasmic reticulum (ER) stress causes neuronal death and has been implicated in neurodegenerative conditions such as Alzheimer's disease (AD). However, the mechanisms by which stress signals propagate from the ER through the Golgi apparatus and their effects on the transport and processing of AD-related proteins, such as β-amyloid precursor protein (βAPP), are unknown. We recently found that in the NG108-15 cell line, ER stress upregulates ER–Golgi-soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors (ER–Golgi SNAREs) Syx5 and Bet1. In the present study, we examined the effects of apoptosis and ER stress inducers on the expression of ER–Golgi SNARE proteins and cell viability in a primary culture of rat hippocampal neurons. An apoptosis inducer significantly downregulated the expression of ER–Golgi SNARE Syx5. ER-stress inducers upregulated the expression of Syx5 isoforms and Bet1 proteins via *de novo* synthesis of their mRNA transcripts. Knockdown of Syx5 during apoptosis or ER stress induction enhanced vulnerability of neurons. Additionally, we examined the effects of Golgi stress on Syx5 expression and βAPP processing. Golgi stress also induced upregulation of ER–Golgi SNARE Syx5, and concomitantly, suppressed amyloid-β peptide secretion. These findings suggest that Syx5 is a potential stress responsive factor that participates in βAPP processing and the survival pathways of neuronal cells.

1. Introduction

Endoplasmic reticulum (ER) stress occurs under a variety of conditions, such as accumulation of misfolded proteins, perturbation of the secretory pathway, or disruption of Ca^{2+} homeostasis [13]. Much attention is given to ER stress because it is especially involved in several chronic neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease (for review, see [8]). ER stress has also been documented in neurons under acute pathological conditions, including cerebral ischemia [17]. However, despite severe ER stress being a cause of neuronal death, the mechanism by which such stress signals propagate from the ER through the Golgi apparatus within individual cells is unknown. Recently, although the sensor molecules have not been defined yet, the cellular stress that originates from the Golgi apparatus, the existence of a so-called "Golgi stress" has been proposed [15]. Neither the regulatory mechanism of intracellular transport and AD-related protein (e.g., βAPP) processing nor the involvement of soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs) during Golgi stress has been studied. Intracellular transport of vesicles along the

Abbreviations: Aβ peptide, amyloid-β peptide; AD, Alzheimer's disease; BFA, brefeldin A; βAPP, β-amyloid precursor protein; DIV, day in vitro; ELISA, enzyme linked immunosorbent assay; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; siRNA, small interfering RNA; SNARE, soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor; STS, staurosporine; Syx5, syntaxin 5; Tg, thapsigargin; Tm, tunicamycin.

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secretory pathway is governed by various proteins, including Rab and ADP ribosylation factor GTPases, SNAREs, and Sec1/Munc proteins [1,4,9]. SNAREs are highly conserved proteins that are major players in the final docking and in subsequent fusion stages in a diverse array of vesicle-mediated transport processes [9]. Syntaxin 5 (Syx5) is a member of ER–Golgi SNAREs that reside in the ER, ER–Golgi-intermediate-compartment/vesicular tubular clusters (ERGIC/VTC), and the Golgi compartments. There are two mammalian Syx5 isoforms that are generated from alternative translation initiation sites on the same mRNA [10]. Relative to the 35-kDa isoform (designated Syx5), the 42-kDa isoform (designated Syx5L) has an extended N-terminal region [7,10,22]. We previously showed that downregulation of Syx5 by small interfering RNA (siRNA) results in Golgi fragmentation [18], as is observed in the neurons of AD-affected brains [5]. Furthermore, we found that Syx5 isoforms specifically interact with the presenilin holoprotein, which is a key player in the pathogenesis of AD [21,23]. In addition, we showed that Syx5 overexpression causes accumulation of βAPP in the ER and suppresses its further processing into the C-terminal fragment of βAPP and Aβ peptides [22]. Recently, we identified Syx5 as a novel ER stress responsive factor and demonstrated the significance of Syx5 expression on βAPP processing during ER stress of the immortalized neuroblastoma–glioma hybrid cell line NG108-15 [19]. In the present study, to investigate the involvement of Syx5 in the processing and transport of AD-related proteins in the nervous system, we examined whether the phenomena observed in the cell line occur in primary culture neurons. In addition, since it is likely that the Golgi compartment is susceptible to ER homeostasis, we examined the effect of Golgi stress on the expression of ER–Golgi SNAREs, cell survival, and the processing of βAPP in rat hippocampal neuron cultures.

2. Materials and methods

2.1. Materials

Tunicamycin (Tm), Hoechst 33342, and brefeldin A (BFA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO); monensin and nigericin were obtained from Merck (Darmstadt, Germany). Monensin, nigericin, and BFA were dissolved in methanol. Staurosporine (STS) and thapsigargin (Tg) were also purchased from Merck and dissolved in dimethyl sulfoxide. A protease inhibitor cocktail was purchased from Wako Chemicals (Osaka, Japan). All other reagents were of the highest grade available, unless otherwise noted.

2.2. Antibodies

Mouse anti-Syx5 monoclonal antibody (clone 1C5) was prepared as described previously [18]. The preparation of other antibodies was described previously [19].

2.3. Hippocampal primary culture

Pregnant Wistar Kyoto rats were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). All of the experimental procedures using animals were approved by the Experimental Animal Ethics Committee of the Kyorin University School of Medicine, and performed in accordance with the guidelines for handling laboratory animals. Primary rat hippocampal cultures were prepared as described previously [11]. In brief, hippocampi from postnatal day-0 pup brains were harvested in L15 medium (Sigma–Aldrich), dissociated in 2.5 mg/mL trypsin (DIFCO, Detroit, MI) and DNase (Sigma–Aldrich) for 15 min at 37 °C, and triturated with a siliconized pipette. Cells were plated at a density of 4–8 × 10^4 cm^{-2} in either a glass-bottomed 35-mm dish, in polyethyleneimine-coated 96-well plates (Greiner Bio-One, Frickenhausen, Germany), or in 12-well plates (Corning, NY) and cultured in DMEM (Sigma–Aldrich) containing 10% fetal bovine serum at 37 °C with 95% air, 5% CO_2 in a humidified incubator. After 24 h of *in vitro* culture, the medium was replaced with serum-free, DMEM-containing GlutaMax and 2% B-27 supplement (Life Technologies, Rockland, MD) either with or without 2 μM Ara-C (Sigma–Aldrich) and used after 13–16 days of in vitro culture.

2.4. Duplex siRNA

The sequences and preparation of Syx5 siRNA#1 (R424442) and control siRNA were described previously [19].

2.5. Extract preparation, SDS-PAGE, western blot analysis, and transfection

Biochemical analyses were carried out as described previously [19,22–24]. Transfection of annealed duplex siRNA (final concentration of 30 nM) was performed using Lipofectamine 2000 transfection reagent from Life Technologies.

2.6. RNA preparation and reverse transcription polymerase chain reaction

Total RNA was prepared with an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To quantitate specific mRNAs by reverse transcription polymerase chain reaction (RT-PCR), 0.2–0.8 μg of total RNA was used to first generate 40 μL of cDNA using an oligo(dT)-20 primer (100 pmol, Life Technologies), PrimeScriptR® Reverse Transcriptase (Takara Bio, Otsu, Japan), RNase Plus Inhibitor (Promega, Madison, WI), and dNTP mixture (Stratagene, San Diego, CA). Reverse transcription was performed at 42 °C for 1 h and terminated by incubating at 70 °C for 15 min. The resultant 2 μL of cDNA template was used in the 25-μL PCR using each primer pair for Syx5 and Bet1. PCR was carried out using PrimeScript® HS DNA polymerase (Takara Bio) and a GeneAmp9700 thermal cycler (Life Technologies). The optimized PCR conditions and the cycling parameters for the detection of Syx5 and Bet1 were as follows: 5 min at 94 °C, 30 cycles of 94 °C for 30 s for denaturation, 30 s at 55.5 °C for primer annealing, 45 s at 72 °C for polymerization, and 7 min at 72 °C for final extension. The same amount of template cDNA was amplified for 30 cycles using β-actin-specific primers. All of the primer sequences and analysis of PCR products were described previously [19].

2.7. Immunocytochemistry

Immunocytochemical analyses were carried out as described previously [19,22].

2.8. Cell viability and cytotoxicity assays

Cell viability and cytotoxicity of cells were measured with a CellTiter-Glo Luminescent Cell Viability Assay and CytoTox-Glo Cytotoxicity Assay systems (Promega) according to the manufacturer's instructions. Luminescence was measured using a luminometer (GloMax; Promega).

2.9. Quantification of rat Aβ peptides by sandwich enzyme-linked immunosorbent assay

Hippocampal neurons were cultured in 12-well plates (in 2 mL of medium) for 13 days before treatment. After the medium was replaced with fresh medium, the cells were treated with monensin or nigericin for 16 h. The culture medium was collected, and cell