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Sigma-1 receptor is involved in degradation of intranuclear inclusions in a cellular model of Huntington's disease

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ABSTRACT

The sigma-1 receptor (SIGMAR1) is one of the endoplasmic reticulum (ER) chaperones, which participate in the degradation of misfolded proteins via the ER-related degradation machinery linked to the ubiquitin–proteasome pathway. ER dysfunction in the formation of inclusion bodies in various neurodegenerative diseases has also become evident. Recently, we demonstrated that accumulation of SIGMAR1 was common to neuronal nuclear inclusions in polyglutamine diseases including Huntington's disease. Our study also indicated that SIGMAR1 might shuttle between the cytoplasm and the nucleus. In the present study, we investigated the role of SIGMAR1 in nuclear inclusion (NI) formation, using HeLa cells transfected with N-terminal mutant huntingtin. Cell harboring the mutant huntingtin produced SIGMAR1-positive NIs. SIGMAR1 siRNA and a specific inhibitor of the proteasome (epoxomicin) caused significant accumulation of aggregates in the cytoplasm and nucleus. A specific inhibitor of exportin 1 (leptomycin B) also caused NIs. Huntingtin became insolubilized in Western blot analysis after treatments with SIGMAR1 siRNA and epoxomicin. Furthermore, proteasome activity increased chronologically along with the accumulation of mutant huntingtin, but was significantly reduced in cells transfected with SIGMAR1 siRNA. By contrast, overexpression of SIGMAR1 reduced the accumulation of NIs containing mutant huntingtin. Although the LC3-I level was decreased in cells treated with both SIGMAR1 siRNA and control siRNA, the levels of LC3-II and p62 were unchanged. SIGMAR1 agonist and antagonist had no effect on cellular viability and proteasome activity. These findings suggest that the ubiquitin–proteasome pathway is implicated in NI formation, and that SIGMAR1 degrades aberrant proteins in the nucleus via the ER-related degradation machinery. SIGMAR1 might be a promising candidate for therapy of Huntington's disease.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease manifested classically as progressive chorea and psychiatric symptoms, resulting in severe dementia (Huntington, 1872). HD is now known to be one of the nine polyglutamine diseases caused by the expansion of trinucleotide repeats encoding polyglutamine in their causative genes (Orr et al., 1993; Kawaguchi et al., 1994; Koide et al., 1994; Nagafuchi et al., 1994; Pulst et al., 1996; Martindale et al., 1998). Accumulation of misfolded proteins in proteinaceous inclusions is considered to be a common feature of many neurodegenerative diseases, and clearance of abnormal proteins by the ubiquitin–proteasome system (UPS) and/or autophagy–lysosome system is paramount for cellular survival. In HD, neuronal nuclear inclusions (NNIs) containing mutant huntingtin protein is a pathological hallmark (Sapp et al., 1997). The accumulation of mutant huntingtin in neurons impairs mediators of promoter accessibility (TATA box-binding protein, TAFII130, and specificity protein I), neurotransmitter release at presynaptic nerve terminals, and mitochondrial function (Dunah et al., 2002; Schaffar et al., 2004; Zhai et al., 2005; Huang et al., 2011; Mochel and Haller, 2011). Furthermore, mutant huntingtin has adverse effects on various protein degradation pathways, such as impairment of UPS, engulfment of cytosolic cargo by autophagosomes, and gp78, an endoplasmic reticulum (ER) membrane-anchored ubiquitin ligase (E3) involved in ER-associated protein degradation (ERAD) (Bennett et al., 2007; Martinez-Vicente et al., 2010; Yang et al., 2010). However, it remains to be elucidated how aberrant proteins in the nucleus are degraded in HD.

The sigma-1 receptor (SIGMAR1), encoded by the SIGMAR1 gene, is a non-opioid ER protein with a molecular mass of 24 kDa (Martin et al., 1976; Quirion et al., 1992; Kekuda et al., 1996; Hayashi et al., 2011). In addition to the regulation of ion channels, synaptogenesis, and neuronal plasticity, SIGMAR1 functions as a molecular chaperone in the ER. ER chaperones not only facilitate the proper folding of newly synthesized proteins, but also prevent the accumulation of misfolded proteins by consigning them to ERAD, suggesting that SIGMAR1 also contributes

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to cellular survival (Hayashi et al., 2011; Schröder and Kaufman, 2005). We recently demonstrated that the accumulation of SIGMAR1 was common to NNIs in the brains of patients with five polyglutamine diseases (HD, dentatorubral-pallidoluysian atrophy, and spinocerebellar ataxia types 1–3) and intranuclear inclusion body disease (Miki et al., 2014). Double-immunocytofluorescence and Western blot analyses of cultured cells demonstrated that a specific exportin 1 inhibitor (leptomycin B) sequestered SIGMAR1 within the nucleus, acting together with p62, and that furthermore, an ER stressor (thapsigargin) caused migration of SIGMAR1 in the nucleus. These findings indicate that SIGMAR1 might shuttle between the cytoplasm and the nucleus, potentially associated with NNI clearance via ERAD (Miki et al., 2014).

We hypothesized that if expanded polyglutamine in the ER or the nucleus is cleared through ERAD, blocking a putative pathway of SIGMAR1 would provide similar pathological kinetics in NNI formation. In the present study, immunocytofluorescence, Western blot analysis, and proteasome activity assay of a cellular model of HD were performed to better understand the involvement of SIGMAR1 in the degradation of abnormal proteins in NNIs through ERAD. Our results indicated that abnormal proteins in the nucleus might be degraded by UPS through ERAD, suggesting that modulation of SIGMAR1 could have potential therapeutic applications for HD.

Material and methods

Cell culture and transfection

HeLa cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics.

siRNA-mediated knockdown

siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA). siRNA at a final concentration of 20 μM for SIGMAR1 (5′-GAAUGC GGGUGGCUGGAUG-3′, 5′-GGCUUGAGCUCACCACCUA-3′, 5′-GAGCUG GCCUUCUCUCGUC-3′, and 5′-CCAAACACAUGGAUGGUGG-3′) and non-targeting controls (D-001810-10-20) were transfected into cultured cells using siRNAMAX (Invitrogen, Carlsbad, USA).

SIGMAR1 overexpression

An expression clone of SIGMAR1 cDNA was purchased from Gene Copoeia (Rockville, Maryland, USA) and transfected into cultured cells using Fugene® 6 (Roche, Basel, Switzerland).

Antibodies and reagents

Goat polyclonal anti-SIGMAR1 (Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-p62 (BD Transduction Laboratories, San Jose, CA), mouse monoclonal anti-ubiquitin (FK1; Millipore, Bedford, NJ), rabbit polyclonal anti-microtubule-associated protein 1 light chain 3 (LC3; Sigma, St. Louis, MO), goat polyclonal anti-Bip (GRP78; Santa Cruz Biotechnology), rabbit polyclonal anti-β-actin (Sigma), and rabbit monoclonal anti-GFP antibody (Life Technologies, Carlsbad, USA) were used as primary antibodies. In addition, rabbit polyclonal anti-PML (Medical and Biological Laboratories, Nagoya, Japan), anti-Coilin (Santa Cruz Biotechnology) and anti-PSPC1 antibodies (Sigma) were also used for the detection of nuclear bodies including PML bodies, Cajal bodies, and paraspeckles, respectively (Wang et al., 2002; Spector, 2006). To investigate the role of SIGMAR1 in NNI formation, a SIGMAR1 agonist (PRE-084; Sigma), a SIGMAR1 antagonist (BD1063; Sigma), and specific inhibitors of the proteasome (epoxomicin; Peptide institute, Osaka, Japan) and exportin 1 (leptomycin B; Sigma) were utilized (Kudo et al., 1999; Meng et al., 1999; Amer et al., 2013; Hyrskyluoto et al., 2013).

Immunocytochemistry and semi-quantitative analysis

HeLa cells were treated with siRNA-SIGMAR1, epoxomicin, leptomycin B, PRE-084, and BD1063 at a final concentration of 20 μM, 20 μM, 0.1 μM, 10 nM, 0.3 μM, and 50 μM, respectively (Kudo et al., 1999; Meng et al., 1999; Amer et al., 2013; Hyrskyluoto et al., 2013). The cells were then transfected for 24 h with 1 μg of GFP-tagged plasmid DNA containing a normal (Q23; Addgene, Cambridge, MA; plasmid 40263) or pathological CAG-repeat length (Q74; Addgene; plasmid 40262) of the huntingtin exon 1 (Narain et al., 1999). The SIGMAR1 gene was also overexpressed in the cells transfected with Q74 and examined at after 12, 24, and 36 h. For double-immunostaining, the cells were fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 10 min, followed by incubation with anti-SIGMAR1 (1:200), anti-ubiquitin (1:400), anti-PML (1:400), anti-Coilin (1:100), and anti-PSPC1 (1:500) antibodies. Alexa Fluor 488- and 594-conjugated secondary antibodies (Invitrogen) were utilized as secondary antibodies. After a rinse in phosphate-buffered saline (PBS), the cells were mounted and examined as described above. In order to count the nuclear inclusions in cells with each treatment, semi-quantitative analysis was performed based on 10 randomly chosen low-power fields.

Western blot analysis

After transfection with siRNA-SIGMAR1, siRNA-control, and SIGMAR1 or treatment with epoxomicin, leptomycin B, PRE-084, and BD1063, HeLa cells were transfected with Q74 for 24 h as described above, then harvested to assess the expression levels in the total lysates. The cells were lysed with sample buffer [75 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 25% glycerol, 5% β-mercaptoethanol]. Western blot analysis was performed as reported previously (Zhang et al., 2008). Anti-SIGMAR1 (1:200), anti-GFP (1:2000), anti-β-actin (1:3000), anti-p62 (1:100), and anti-LC3 (1:4000) were used as the primary antibodies. Horseradish peroxidase-conjugated anti-goat, anti-mouse, and anti-rabbit IgG (Santa Cruz Biotechnology) were used as the secondary antibodies.

Proteasomal activity assay

Chymotryptic, tryptic, and caspase proteasome activities were measured as described previously with minor modifications (Lee et al., 2010). HeLa cells were washed with PBS and pelleted by centrifugation. The cell pellets were sonicated in homogenization buffer [25 mM Tris, pH 7.5, 100 mM NaCl, 5 mM ATP, 0.2% (v/v) NP-40 and 20% glycerol], and cell debris was removed by centrifugation at 4 °C. Protein concentration in the resulting crude cellular extracts was determined by the bicinchoninic acid method (Pierce, Rockford, IL). One hundred micrograms of protein from crude cellular extracts of each sample was diluted with buffer I [50 mM Tris–HCl, pH 7.4, 2 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP] to a final volume of 0.5 mL (assayed in quadruplicate). Fluorogenic proteasome substrates were purchased from Boston Biochem (Cambridge, MA): Suc-LLVY-7-amido-4-methylcoumarin (AMC) (chymotrypsin-like peptidase activity), Z-ARR-AMC (trypsin-like peptidase activity), and Z-LLE-AMC (caspase-like or peptidylglutamyl peptide-hydrolyzing activity). Each was dissolved in DMSO and brought to a final concentration of 80 μM. Proteolytic activities were assessed in 2 h at 37 °C by measuring the release of the fluorescent group AMC using a fluorescence plate reader (Fluoroskan Ascent; Thermo Scientific, Waltham, MA) with excitation and emission wavelengths of 380 and 460 nm, respectively.