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YOD1 attenuates neurogenic proteotoxicity through its deubiquitinating activity

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ABSTRACT

Ubiquitination, a fundamental post-translational modification of intracellular proteins, is enzymatically reversed by deubiquitinase enzymes (deubiquitinases). > 90 deubiquitinases have been identified. One of these enzymes, YOD1, possesses deubiquitinase activity and is similar to ovarian tumor domain-containing protein 1, which is associated with regulation of the endoplasmic reticulum (ER)-associated degradation pathway. Indeed, YOD1 is reported to be involved in the ER stress response induced by mislocalization of unfolded proteins in mammalian cells. However, it has remained unclear whether YOD1 is associated with pathophysiological conditions such as mitochondrial damage, impaired proteostasis, and neurodegeneration. We demonstrated that YOD1 possesses deubiquitinating activity and exhibits preference for K48- and K63-linked ubiquitin. Furthermore, YOD1 expression levels increased as a result of various stress conditions. We demonstrated that the neurogenic proteins that cause Huntington disease and Parkinson's disease induced upregulation of YOD1 level. We observed that YOD1 reduced disease cytotoxicity through efficient degradation of mutant proteins, whereas this activity was abolished by catalytically inactive YOD1. Additionally, YOD1 localized to Lewy bodies in Parkinson's disease patients. Collectively, these data suggest that the deubiquitinase YOD1 contributes to pathogenesis of neurodegenerative disease by decreasing ubiquitination of abnormal proteins and their subsequent degradation.

1. Introduction

Ubiquitination is a widespread post-translational modification that affects many biological processes, including cell division, antigen presentation, and longevity (Hershko and Ciechanover, 1998; Kevei and Hoppe, 2014; Shi et al., 2012). This process is tightly regulated through the actions of specialized enzymes called ubiquitin ligases and deubiquitinases. Deubiquitinases cleave the isopeptide bond between lysine residues (K) on target proteins and the C-terminal glycine on ubiquitin. Analyses of the human genome revealed that over 90 deubiquitinases exist (Komander et al., 2009) and can be divided into six groups according to amino acid homology: 1) ubiquitin-specific proteases, 2) ubiquitin carboxy-terminal hydrolases, 3) monocyte chemotactic protein-induced proteins, 4) Machado-Joseph disease protein domain proteases, 5) JAMM/MPN domain-associated metallopeptidases, and 6) ovarian tumor (OTU) proteases (Nijman et al., 2005; Sowa et al., 2009).

YOD1 (also known as OTUB2), is a highly conserved deubiquitinase similar to yeast OTU1 that is associated with regulation of the endoplasmic reticulum (ER)-associated degradation pathway (Rumpf and Jentsch, 2006). Indeed, YOD1 is reported to be involved in the ER stress response induced by mislocalization of unfolded proteins in mammalian cells (Bernardi et al., 2013; Claessen et al., 2010; Ernst et al., 2009; Sasset et al., 2015). Recently, YOD1 was identified as a regulator of the inflammatory cytokine, interleukin-1 by binding with TRAF6 (Schimmack et al., 2017). However, it is unclear whether YOD1 is involved in pathophysiological conditions such as mitochondrial damage, impaired proteostasis, and neurodegeneration.

In this study, we found that YOD1 protein levels were significantly increased by inhibition of proteostasis and mitochondrial damage. Furthermore, abnormal huntingtin and α-synuclein protein expression caused upregulation of YOD1, which can reduce cytotoxicity through its efficient degradation of proteins incorporated with K48- and K63-linked ubiquitin. We also observed elevated YOD1 protein levels in lesions associated with Parkinson's disease (PD). Because YOD1 localizes to Lewy bodies, YOD1 may contribute to the pathogenesis of PD through its deubiquitinase activity.

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2. Materials and methods

2.1. Cell culture and transfection

Human uterine cervical carcinoma (HeLa) cells, hepatic carcinoma cells (JHH5) (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), and human neuroblastoma SH-SY5Y cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Cultured cells were transfected with the following cDNAs using X-tremeGENE 9 DNA Transfection Reagent (Roche, Basel, Switzerland): catalytically active YOD1 (pcDNA3-Flag-YOD1) and catalytically inactive YOD1 (pcDNA3-Flag-YOD1 C160S) (49784 and 49785, respectively, Addgene, Cambridge, MA, USA) (Ernst et al., 2009); wild-type (pHM6-Q23, 40263, Addgene) and pathological CAG-repeat length (pHM6-Q74, 40262, Addgene) of the Huntingtin (Htt) exon 1 (Narain et al., 1999); wild-type pRK5-HA-Ubiquitin (Ub-wt, 17608, Addgene), mutant ubiquitin mutated at all lysine (K) residues into alanine Ub-K0 (17603, Addgene), except for K48 (Ub-K48, 17605, Addgene), except for K63 (Ub-K63, 17606, Addgene) (Lim et al., 2005). Ub-K33 was generated using standard mutagenesis and confirmed by sequencing.

For the formation of Lewy body-like inclusions, the previously described plasmid (Tanji et al., 2006), designed for simultaneous expression of Flag-α-synuclein and synphilin-1 tagged with enhanced green fluorescent protein (EGFP) (Engelender et al., 1999), was used. After 24 and 48 h incubation periods, cells were observed with either a 20× objective lens microscope (Eclipse Ti, Nikon, Tokyo, Japan) equipped with a spinning disc system (TI-S-EJOY, Nikon) and a CCD camera (CoolSNAP HQ2, Photometrics, Tucson, AZ, USA), or a FlexStation3 (Molecular Devices, Sunnyvale, CA, USA) for measuring EGFP intensity. Cells expressing EGFP or synphilin-1-EGFP were counted to determine the number of transfected cells. The transfected cells containing cytoplasmic inclusions were also counted. The value of '% cells with inclusions' was calculated as the ratio of 'the number of transfected cells containing inclusions' to 'the total number of transfected cells.' Cells were treated with epoxomicin (Calbiochem, San Diego, CA, USA), bafilomycin A (Wako, Osaka, Japan), leptomycin B (Sigma, St. Louis, MO, USA), thapsigargin (Wako), and 2-[2-(3-Chlorophenyl) hydrazinylyidene] propanedinitrile (CCCP, Abcam, Cambridge, UK). For immunoblotting analysis, cells were harvested at the indicated times, and lysed as indicated below Section 2.9.

2.2. Human subjects

Tissue samples from 18 autopsies were obtained from the Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, and the Department of Pathology, Brain Research Institute, University of Niigata, Niigata, Japan. Written informed consent for autopsy, sample collection, and subsequent analysis was obtained from the patients and next of kin of the deceased. This study was approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine, Hirosaki, Japan. For immunohistochemistry, formalin-fixed and paraffin-embedded sections from patients with Alzheimer's disease (AD) (n = 5), PD (n = 5) (Braak et al., 2003), and Huntington disease (HD; n = 3), and normal controls (n = 5), were used. For biochemical analysis, brain tissues were dissected during autopsy and rapidly frozen at −70 °C. The substantia nigra and putamen of patients with PD (n = 3) and normal controls (n = 3) were used.

2.3. Animals

All animal experiments were performed in accordance with the Guidelines for Animal Experimentation and were approved by the Animal Research Committee of Hirosaki University. Animals (C57BL/6J) were housed under standard conditions (12 h light, 12 h dark; food and water available ad libitum).

2.4. Antibodies

The following antibodies were used in this study: rabbit antibodies against YOD1-N, YOD1-C (Abcam), β-actin (Sigma), and GFP (Thermo Fisher Scientific Inc., Waltham, MA, USA), p62 (MBL, Nagoya, Japan), valosin-containing protein (VCP, Thermo Fisher Scientific Inc.), LC3 (Sigma), specifically K48- or K63-linked ubiquitin antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA). Mouse antibodies against phosphorylated α-synuclein (pSyn#64, Wako), HA (BioLegend-Covance, San Diego, CA, USA), Ubiquitin (FK2, MBL; 1B3, MBL), NeuN (Millipore Billerica, MA, USA) and Flag (M2, Sigma) were also used. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (Santa Cruz Biotechnology) were used as secondary antibodies for Western blot analyses. Donkey anti-rabbit antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) and anti-mouse antibody conjugated with Alexa Fluor 594 (Invitrogen) were also used in this study.

2.5. Immunohistochemistry

Mice were transcardially perfused with phosphate-buffered saline (PBS). The brain was removed, and the right hemisphere was fixed with 4% paraformaldehyde for 48 h. After dehydration through a graded ethanol series, the brain sample was embedded in paraffin and cut into 4-μm-thick sections.

The left hemisphere was frozen at −80 °C for subsequent biochemical analyses. Tissue sections were heated by an autoclave for 15 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. These sections were then subjected to immunohistochemical staining using the avidin-biotin-peroxidase complex method with the chromagen, diaminobenzidine. Sections were counterstained with hematoxylin.

2.6. 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 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 bicinchonic acid method (Pierce, Rockford, IL, USA). One hundred micrograms of protein from each sample of crude cellular extracts 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). The following fluorogenic proteasome substrates were purchased from Boston Biochem (Cambridge, MA, USA): 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 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 FlexStation3 with excitation and emission wave lengths of 380 and 460 nm, respectively.

2.7. In vitro deubiquitination assay

YOD1 was immunoprecipitated with a Flag tag in HeLa cells after transfection with pcDNA3 Flag-YOD1. Deubiquitinating reactions (Fig. 1E) were performed in a 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM DTT, 10 mM $MgCl_2$, and di-ubiquitin (40 nM, UBP Bio, Aurora, CO, USA) mixture at 30 °C for 30 min with the indicated amounts of YOD1. Reactions were terminated by the addition of sodium dodecyl sulfate (SDS) sample buffer. Products were analyzed by Western