



Oxidative stress induced by glutathione depletion reproduces pathological modifications of TDP-43 linked to TDP-43 proteinopathies

Yohei Iguchi^a, Masahisa Katsuno^a, Shinnosuke Takagi^a, Shinsuke Ishigaki^{a,d}, Jun-ichi Niwa^b, Masato Hasegawa^c, Fumiaki Tanaka^a, Gen Sobue^{a,d,*}

^a Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan

^b Stroke Center, Aichi Medical University, Aichi 480–1195, Japan

^c Departments of Molecular Neurobiology, Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156–8585, Japan

^d CREST, Japan Science and Technology Agency, 4-1-8, Honcho, Kawaguchi, Saitama 332–0012, Japan

ARTICLE INFO

Article history:

Received 30 March 2011

Revised 29 August 2011

Accepted 4 December 2011

Available online 13 December 2011

Keywords:

TAR DNA-binding protein 43 kDa (TDP-43)

TDP-43 proteinopathy

Oxidative stress

Glutathione depletion

Post-translational modification

Protein phosphorylation

ABSTRACT

TAR DNA-binding protein 43 (TDP-43) is a major component of ubiquitin-positive inclusion of TDP-43 proteinopathies including amyotrophic lateral sclerosis and frontotemporal lobar degeneration with ubiquitinated inclusions, which is now referred to as FTLD-TDP. TDP-43 in the aberrant inclusion is known to be hyperphosphorylated at C-terminal sites, to be truncated at the N-terminal region, and to re-distribute from nucleus to cytoplasm or neurite. The pathogenic role of these modifications, however, has not been clarified. Furthermore, there is no evidence about the initial cause of these modifications. Herein we show that ethacrynic acid (EA), which is able to increase cellular oxidative stress through glutathione depletion, induces TDP-43 C-terminal phosphorylation at serine 403/404 and 409/410, insolubilization, C-terminal fragmentation, and cytoplasmic distribution in NSC34 cells and primary cortical neurons. In the investigation using a nonphosphorylatable mutant of TDP-43, there was no evidence that C-terminal phosphorylation of TDP-43 contributes to its solubility or distribution under EA induction. Our findings suggest that oxidative stress induced by glutathione depletion is associated with the process of the pathological TDP-43 modifications and provide new insight for TDP-43 proteinopathies.

© 2011 Elsevier Inc. All rights reserved.

Introduction

TAR DNA-binding protein 43 (TDP-43) is a major component of ubiquitin-positive inclusion, a pathological hallmark of TDP-43 proteinopathies including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with ubiquitinated inclusions, which is now referred to as FTLD-TDP (Arai et al., 2006; Neumann et al., 2006). Both diseases occur in sporadic or familial forms, and are characterized by late-onset progressive deterioration of motor and/or cognitive function. TDP-43 is a heterogeneous nuclear ribonucleoprotein (hnRNP), which is known to regulate gene transcription and exon splicing through interactions with RNA, hnRNPs, and nuclear bodies (Ayala et al., 2005; Buratti et al., 2005; Wang et al., 2002,

2004). In addition, this protein has also been reported to stabilize human low molecular weight neurofilament (hNFL) mRNA through direct interaction with its 3'UTR (Strong et al., 2007), regulate retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 (Cdk6) expression (Ayala et al., 2008), regulate activity of Rho family GTPases (Iguchi et al., 2009), and alter the expression of selected microRNAs, such as let-7b and miR-663 (Buratti et al., 2010). Furthermore, very recent works using cross-linking immunoprecipitation sequencing show that multiple RNAs interact with TDP-43 (Polymenidou et al., 2011; Sephton et al., 2011; Tollervey et al., 2011).

Although it mostly localizes in the nucleus under normal conditions, TDP-43 is distributed from nucleus to cytoplasm or neurite, and forms aggregates consisting mainly of C-terminal fragments in affected neurons of patients with TDP-43 proteinopathies. In addition, TDP-43 in the aberrant aggregation is hyperphosphorylated at multiple C-terminal sites (Hasegawa et al., 2008). However, neither the pathogenic role nor the initial cause of these abnormal modifications of TDP-43 has been elucidated. The fact that the majority of patients with TDP-43 proteinopathies are sporadic suggests that exogenous factors induce post-translational modifications of TDP-43 that are seen in the disease. Furthermore, TDP-43 inclusions have also been observed in Alzheimer disease (AD), Parkinson disease (PD),

Abbreviations: TDP-43, TAR DNA-binding protein of 43 kDa; ALS, amyotrophic lateral sclerosis; hnRNP, heterogeneous nuclear ribonucleoprotein; hNFL, human low molecular weight neurofilament; Cdk6, cyclin-dependent kinase 6; ROS, reactive oxygen species; EA, ethacrynic acid; NAC, N-acetylcysteine; CK1, casein kinase 1; CK2, casein kinase 2; WT-TDP-43, wild type TDP-43; SA-TDP-43, nonphosphorylatable TDP-43.

* Corresponding author at: Department of Neurology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan. Fax: +81 52 744 2785.

E-mail address: sobueg@med.nagoya-u.ac.jp (G. Sobue).

Available online on ScienceDirect (www.sciencedirect.com).

dementia with Lewy bodies (DLB), and Huntington disease (HD), argyrophilic grain disease, suggesting that the aggregation of this protein may be a secondary feature of neurodegeneration (Amador-Ortiz et al., 2007; Arai et al., 2009, 2010; Geser et al., 2008; Hasegawa et al., 2007). These findings complicate understanding of the pathogenic role of TDP-43. On the other hand, there is considerable evidence that reactive oxygen species (ROS) and oxidative stress are associated with many neurodegenerative conditions including ALS (Abe et al., 1995, 1997; Beal et al., 1997; Butterfield et al., 2007; Ferrante et al., 1997; Lovell and Markesbery, 2007; Nunomura et al., 2002; Shaw et al., 1995). Herein we show that oxidative stress induced by glutathione depletion reproduces the pathological modifications of TDP-43, that are seen in TDP-43 proteinopathies, in motor neuron-like cells and primary cortical neurons.

Materials and methods

Cell culture and treatment

Mouse NSC34 motor neuron-like cells (a kind gift of N.R. Cashman, University of British Columbia, Vancouver, Canada) were cultured in a humidified atmosphere of 95% air–5% CO₂ in a 37 °C incubator in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To differentiate the cells, the medium was changed to DMEM containing 1% FBS and 1% NEAA, and was cultured for 24 h. For the interventions, the cells were then incubated with ethacrynic acid (EA) (Sigma-Aldrich, St. Louis, MO), with or without N-acetylcysteine (NAC) (Sigma-Aldrich), casein kinase 1 (CK1) inhibitor (D4476), or casein kinase 2 (CK2) inhibitor (TBCA) (Sigma-Aldrich). Primary cultures of mouse embryonic cortical neurons that were dissociated from embryonic cortex of embryonic day 15 (E15) C57BL/6J pregnant mice were plated onto poly-L-lysine-coated plates or glass bottom dishes, and maintained in neuron culture medium (Sumilon, Osaka, Japan). Five days after the incubation, the indicated interventions were performed. In both NSC34 cells and primary cortical neurons, the transfections of the intended plasmids were performed using Lipofectamine 2000 (Invitrogen, Eugene, OR), according to the manufacturer's instructions.

DNA constructs

Human wild type TDP-43 (WT-TDP-43) (accession number NM007375) cDNA was amplified by PCR from cDNA of human spinal cord using the following primers: 5'-CACCATGTCTGAATATATTCGGG-TAAC-3' and 5'-CTACATTCCCAGCCAGAAGACTTAGAAT-3'. The PCR product was cloned into the pENTR/D-TOPO vector (Invitrogen). For nonphosphorylatable TDP-43 (SA-TDP-43) vector, primers containing the mutant substitution of TDP-43 serine 403/404 and 409/410 to alanine were used to mutagenize WT-TDP-43 (KOD-Plus-Mutagenesis kit; Toyobo, Osaka, Japan). The entry vector of WT- or SA-TDP-43 was transferred into pcDNA6.2/N-EmGFP-DEST Vector or pcDNA3.1/nV5-DEST using Gateway LR Clonase II enzyme mix (Invitrogen). The sequences of all constructs were verified using CEQ 8000 genetic analysis system (Beckman Coulter, Brea, CA).

Immunoblot analysis

For whole lysate analysis, NSC34 cells and primary cortical neurons were lysed in 2% SDS sample buffer. For analysis of protein solubility, cells cultured in 6-well plates were lysed in 100 µl of Tris (TS) buffer (50 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 5 mM EGTA, protein phosphatase inhibitors, and protease inhibitor cocktail). Lysates were sonicated and centrifuged at 100,000 ×g for 15 min. To prevent carryover, the pellets were washed with TS buffer, followed by sonication and centrifugation. TS-insoluble pellets were lysed in 50 µl of Triton-X100 (TX) buffer (TS buffer containing 1% Triton X-

100), sonicated, and centrifuged at 100,000 g for 15 min. The pellets were washed with TX buffer, followed by sonication and centrifuge. TX-insoluble pellets were lysed in 50 µl of Sarkosyl (Sar) buffer (TS buffer containing 1% Sarkosyl), sonicated and centrifuged at 100,000 ×g for 15 min. Sar-insoluble pellets were lysed in 25 µl of SDS sample buffer. After denaturation, 3 µl of each cell lysate was separated by SDS-PAGE (5%–20% gradient gel) and analyzed by western blotting with ECL Plus detection reagents (GE Healthcare, Buckinghamshire, UK). Primary antibodies used were as follows: anti-TDP-43 rabbit polyclonal antibody (1:1000, ProteinTech, Chicago, IL), anti-TDP-43 (405–414) rabbit polyclonal antibody (1:1000, Cosmo Bio Co. Ltd., Tokyo, Japan), anti-TDP-43 (phospho Ser403/404, Cosmo Bio) rabbit polyclonal antibody (1:1000, Cosmo Bio), anti-TDP-43 (phospho Ser409/410, Cosmo Bio) rabbit polyclonal antibody (1:1000, Cosmo Bio), anti-GAPDH mouse monoclonal antibody (1:2000, Temecula, CA), anti-GFP mouse monoclonal antibody (1:2000, MBL, Nagoya, Japan), and anti-V5 mouse monoclonal antibody (1:2000, Invitrogen).

Assay of ROS production

NSC34 cells to be treated with intended agents were incubated in 96-well plates with 5-(and-6)-chloromethyl-2',7'-dichlorodihydro fluoresceindiacetate acetyl ester (CM-H2DCFDA) (Molecular Probes, Eugene, OR, USA) for 1 h. Oxidation in the cells was then measured in a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical, Japan) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. The assays were carried out in 6 wells for each condition.

Immunocytochemistry

NSC34 cells and primary cortical neurons were fixed with 4% paraformaldehyde, incubated with PBS containing 0.2% Triton X-100 for 5 min, blocked, and incubated overnight with anti-TDP-43 rabbit polyclonal antibody (1:1000, ProteinTech), anti-TDP-43 (phospho Ser409/410) mouse monoclonal antibody (1:2000, Cosmo Bio) and anti-TIAR mouse monoclonal antibody (1:1000, BD Transduction Laboratories, Milan, Italy). After washing, samples were incubated with Alexa-488-conjugated goat anti-rabbit IgG (1:1000, Invitrogen) and Alexa-564-conjugated goat anti-mouse IgG (1:1000, Invitrogen) for 30 min, mounted with (Vector Laboratories, Inc. Burlingame, CA), then imaged with a laser confocal microscope (Nikon A1, Nikon, Tokyo, Japan).

Time lapse analysis

NSC34 cells or mouse primary cortical neurons were grown on glass base dishes, transfected with GFP-WT-TDP-43, and treated with EA. GFP and phase contrast imaging was done every 10 min using a 40X objective lens on a laser scanning confocal microscope.

Cell viability analysis

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based cell proliferation assay (MTS assay) was carried out using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's instructions. Absorbance at 490 nm was measured in a multiple-plate reader (PowerscanHT, Dainippon Pharmaceutical, Japan). The assays were carried out in 6 wells for each condition.

Statistical analysis

Statistical differences were analyzed by ANOVA and Bonferroni post hoc analyses for three group comparisons (SPSS version 15.0, SPSS Inc., Chicago, IL). Two-tailed $p < 0.05$ was regarded as statistically significant.

Download English Version:

<https://daneshyari.com/en/article/6022658>

Download Persian Version:

<https://daneshyari.com/article/6022658>

[Daneshyari.com](https://daneshyari.com)