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4-Hydroxynonenal induces persistent insolubilization of TDP-43 and alters its intracellular localization

Chihana Kabuta [a, 1], Kanako Kono [a, b, 1], Keiji Wada [a], Tomohiro Kabuta [a, *]

[a] Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan
[b] Department of Electrical Engineering and Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by progressive degeneration of motor neurons. TDP-43 has been found to be a major component of ubiquitin-positive inclusions in ALS. Aberrant TDP-43, which is found in inclusions, is phosphorylated and is re-distributed from the nucleus to the cytoplasm. Alterations of TDP-43 protein, particularly insolubilization/aggregation and cytosolic distribution are thought to be involved in the pathogenesis of ALS. Levels of 4-hydroxynonenal (HNE), a marker of oxidative stress, have been reported to be elevated in sporadic ALS patients. However, the effects of HNE on TDP-43 are unclear. In this study, we found that HNE treatment of cells causes insolubilization, phosphorylation, and partial cytosolic localization of TDP-43. HNE-induced cytosolic TDP-43 was diffusely localized and only a small proportion of TDP-43 localized to stress granules, which are transient structures. HNE-induced TDP-43 insolubilization and phosphorylation were even observed 24 h after washout of HNE. We also showed that the cysteine residues of TDP-43 are responsible for HNE-induced insolubilization of TDP-43. Our results indicate that HNE can cause biochemical changes of TDP-43, which resemble the aberrant alterations of this protein in ALS, and suggest that upregulation of HNE could be a risk factor for ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by degeneration of both upper and lower motor neurons. Approximately 90% of ALS cases are sporadic and the remaining cases are familial. Although the exact causes of motor neuron degeneration in sporadic ALS remain unclear, oxidative stress has been thought to be relevant to the pathogenesis of the disease. This is because markers of oxidative stresses, including 4-hydroxynonenal (HNE), have been reported to be elevated in spinal cord, motor cortex, cerebrospinal fluid, or serum of sporadic ALS patients [1–3] compared with that of other neurodegenerative diseases, non-neurodegenerative controls, or healthy groups. HNE is produced by lipid peroxidation of polyunsaturated fatty acids and is one of the most toxic products of lipid peroxidation [4,5]. It is therefore a candidate for mediation of oxidative stress in sporadic ALS.

The presence of aggregated proteins or inclusion bodies in neurons are a common feature of affected regions in many neurodegenerative diseases including ALS. In ALS and frontotemporal lobar degeneration, TDP-43 is a major component of ubiquitin-positive inclusions [6]. TDP-43 is an RNA-binding protein normally localized to the nucleus. In aberrant inclusions, TDP-43 is phosphorylated and re-distributes from the nucleus to the cytosol [7]. Additionally, missense mutations in the TDP-43 gene have been reported to be causative in familial ALS [8,9]. Thus, alterations of TDP-43, particularly insolubilization/aggregation and cytosolic distribution are thought to be involved in the pathogenesis of sporadic ALS.

However, the relationship between HNE and TDP-43 is unclear and it is worth evaluating the effects of HNE on TDP-43. In this study, we investigated the effects of HNE on intracellular localization, insolubilization, and phosphorylation of TDP-43.

Abbreviations: ALS, amyotrophic lateral sclerosis; HNE, 4-hydroxynonenal.

* Corresponding author. Fax: +81 42 346 1745.
E-mail address: kabuta@ncnp.go.jp (T. Kabuta).

[1] These authors equally contributed to this work.

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2. Materials and methods

2.1. Cell culture and treatment

COS-7 cells were cultured as described previously [10]. Transient transfections of COS-7 cells were performed using Lipofectamine LTX (Life Technologies) as described previously [11]. HNE treatment of cells were performed as described previously [12,13]. Briefly, cells were incubated at 37 °C for 60 min with 100 μM HNE in PBS containing 5 mM glucose, 0.3 mM $CaCl_2$, and 0.62 mM $MgCl_2$.

2.2. Plasmids

The pCI-neo-hTDP-43 plasmids containing human wild-type TDP-43 and TDP-43 variants, with a C-terminal FLAG tag, were prepared as described previously [14], or generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) or PrimeSTAR Max DNA Polymerase (Takara). All resulting constructs were confirmed by sequencing.

2.3. Cell fractionation

The preparation of detergent (1% Triton X-100)-soluble and insoluble fractions was performed as described previously [15]. For detection of phospho-TDP-43, cell lysates were prepared using phosphatase inhibitors (PhosSTOP, Roche).

2.4. SDS-PAGE and immunoblotting

SDS-PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures as described previously [16,17]. Anti-reduced HNE-Michael Adducts (rabbit) was purchased from Calbiochem. Anti-TARDBP/TDP-43 (rabbit) was purchased from Protein Tech Group, Inc. Anti-phospho-TDP-43 (Ser 409/410) (mouse) was purchased from Cosmobio Co., Ltd. Anti-TIAR (mouse) was purchased from BD Transduction Laboratories. Anti-β-actin (mouse) was purchased from Sigma Aldrich. Anti-DYKDDDDK (FLAG, mouse) was purchased from Wako. For immunoblotting with an anti-reduced HNE-Michael Adducts antibody, proteins were transferred to a PVDF membrane and reduced with 10 mM $NaBH_4$ in Tris-buffered saline for 30 min at room temperature prior to incubation with the antibody.

2.5. Immunoprecipitation

Immunoprecipitation was performed as previously described [12] with a slight modification. In brief, cells were harvested in cold RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate) containing protease inhibitors (Complete, EDTA-free, Roche). FLAG-tagged TDP-43 proteins were immunoprecipitated using anti-FLAG M2 affinity gel (Sigma).

2.6. Immunofluorescence analysis

Immunofluorescence was performed as described previously [18,19] with some modifications. Cells were fixed with 3.7% formaldehyde for 30 min, washed with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 10 min. Cells were washed with PBS again and then blocked with 3% bovine serum albumin in PBS before incubating overnight at 4 °C with primary antibodies. After another PBS wash, cells were incubated with Alexa Fluor 488- or 594-labeled secondary antibodies (Life Technologies) for 1 h at room temperature. Finally, cells were washed with PBS and mounted in ProLong Gold Antifade Reagent with DAPI (Life Technologies). Confocal microscopy was performed using a FLUOVIEW system (Olympus, Tokyo, Japan).

3. Results

To examine the effects of HNE on insolubilization of TDP-43, we used COS-7 cells in which alternations of TDP-43 can be observed in response to oxidative stress [20]. COS-7 cells were treated with 100 μM HNE for 60 min, and the levels of TDP-43 in the insoluble fraction of cells was analyzed. We found that HNE treatment increased the levels of insoluble TDP-43 while decreasing the levels of soluble TDP-43 (Fig. 1A and B). Using the antibody specific to phosphorylated TDP-43, we observed that in HNE-treated cells, insoluble TDP-43 was phosphorylated and soluble TDP-43 was not phosphorylated (Fig. 1A). In HNE-untreated cells, phosphorylation of TDP-43 was not detected in either insoluble or soluble fractions (Fig. 1A).

We then tested the effect of HNE on intracellular localization of TDP-43. In HNE-untreated cells, endogenous TDP-43 was predominantly localized to the nuclei (Fig. 1C). We found that in HNE-treated cells, a considerable proportion of TDP-43 localized to the cytoplasm with a diffuse pattern (Fig. 1C). Collectively, HNE causes biochemical changes of TDP-43 that resemble the aberrant alterations of this protein observed in sporadic ALS.

Under oxidative stress conditions induced by H_2O_2, TDP-43 has been reported to localize to cytoplasmic stress granules [20]. In contrast, only a small proportion of HNE-induced cytoplasmic TDP-43 co-localized with TIAR, a marker of stress granules (Fig. 1D). Stress granules are transient structure that are known to disappear 2–3 h after the removal of stressors [21,22]. Because most of the HNE-induced cytoplasmic TDP-43 did not co-localize with stress granules, we hypothesized that the presence of cytoplasmic TDP-43 would persist over the 2- to 3-h timeframe of stress granules. To examine the persistence of aberrant cytosolic TDP-43 induced by HNE, we tested whether the effects of HNE treatment on insolubilization and phosphorylation of TDP-43 were observed 24 h after removal of the stress. COS-7 cells were first incubated with 100 μM HNE for 60 min and then the solution containing HNE was removed, and cells were incubated in culture medium for 24 h. The level of TDP-43 in the insoluble fraction of cells was still elevated in HNE treated cells compared with untreated cells, even 24 h after removal of HNE (Fig. 2A and B). The level of TDP-43 in the soluble fraction of HNE-treated cells was still lowered (Fig. 2A and B), and insoluble TDP-43 was phosphorylated in HNE-treated cells, while soluble TDP-43 in HNE treated cells were not phosphorylated (Fig. 2A). These data indicate that the effects of HNE treatment on insolubilization and phosphorylation of TDP-43 are persistent. We next examined the persistence of HNE effects on intracellular localization of TDP-43. We observed that a small proportion of TDP-43 was localized to cytoplasm in HNE-treated cells 24 h after removal of HNE (Fig. 2C). These persistent alterations of TDP-43 are consistent with TDP-43 pathology observed in sporadic ALS.

Next, we investigated the mechanism underlying insolubilization of TDP-43 by HNE treatment. Because HNE can directly modify cellular proteins, we analyzed whether HNE modifies TDP-43. COS-7 cells overexpressing FLAG-tagged TDP-43 were treated with HNE and then immunoprecipitated with an anti-FLAG antibody followed by immunoblotting using an anti-HNE antibody. We found that TDP-43 was directly modified with HNE (Fig. 3A). HNE preferentially targets cysteine sulfhydryl groups [23] and TDP-43 possesses six cysteine residues (Cys-39, Cys-50, Cys-173, Cys-175, Cys-198, and Cys-244). We therefore constructed mutant TDP-43 vectors in which each cysteine residue was individually substituted with serine, to determine which cysteine is responsible for HNE-

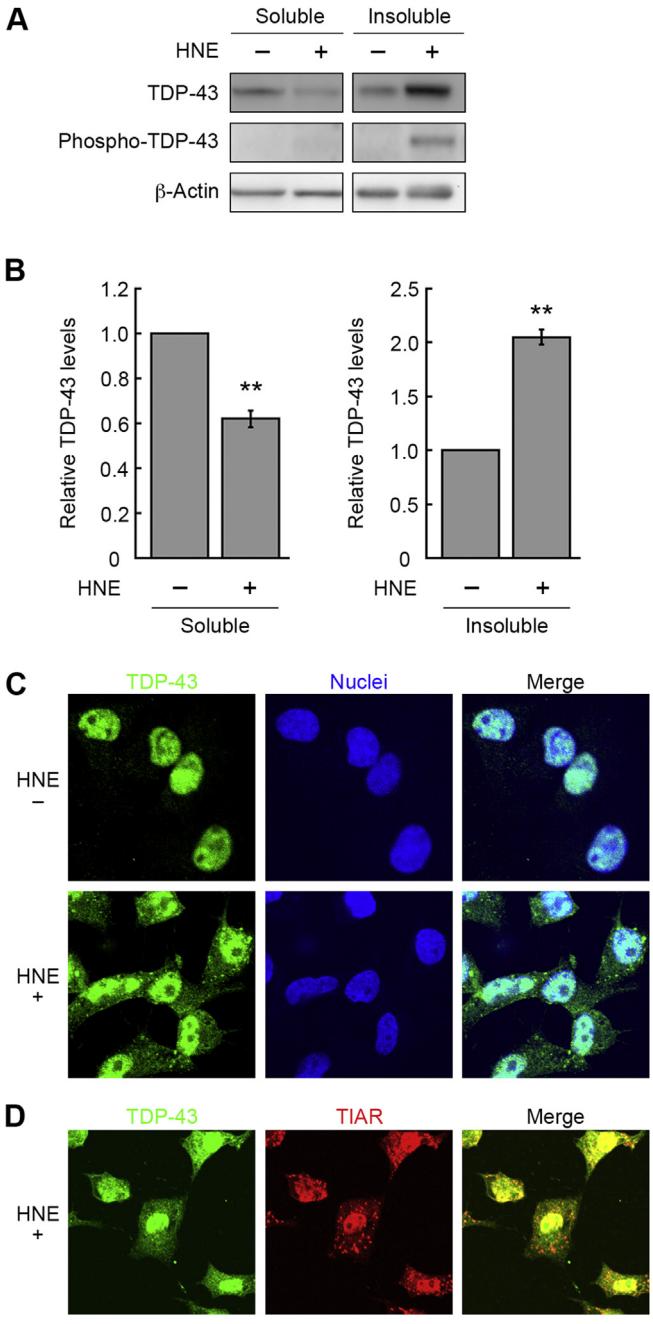

Fig. 1. Effects of HNE treatment on solubility, phosphorylation and intracellular localization of TDP-43. (A and B) COS-7 cells were treated with or without 100 μM HNE for 60 min, and Triton X-100-soluble and -insoluble fractions were prepared from each sample. The levels of phosphorylated TDP-43 and the total levels of TDP-43 in each fraction were analyzed by immunoblotting (A). The relative levels of TDP-43 in soluble and insoluble fractions were quantified by densitometry and then normalized to β-actin (B). Data are presented as fold changes compared with controls (mean ± SEM, n = 3). $^{**}p < 0.01$ (Student's t-test). (C and D) COS-7 cells were treated with or without 100 μM HNE for 60 min, and then TDP-43 localization was analyzed by immunocytochemistry. Nuclei were visualized by DAPI staining (blue). TIAR was used as a marker of stress granules (D).

induced insolubilization of TDP-43. Insolubility of C39S, C50S, and C244S mutants was markedly increased by HNE treatment, while substitution of Cys173, Cys175, or Cys198 dramatically reduced HNE-induced insolubilization of TDP-43 (Fig. 3B, C and Supplementary Fig. S1). We also constructed multiple cysteine TDP-43 mutants, 2CS (C173/175S), 3CS-1 (C173/175/198S), 3CS-2 (C173/175/244S), and 4CS (C173/175/198/244S), and tested the effects of

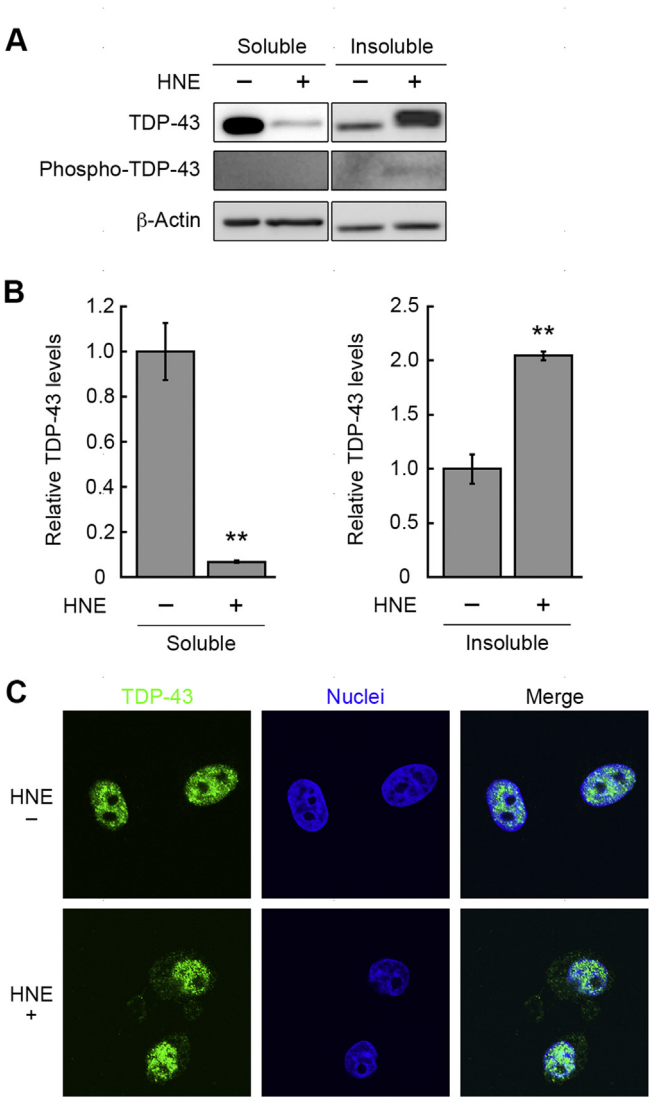

Fig. 2. Effects of HNE treatment on TDP-43 24 h after washout of HNE. (A and B) COS-7 cells were treated with or without 100 μM HNE for 60 min and incubated in culture medium for 24 h. Triton X-100-soluble and -insoluble fractions were prepared from each sample. The levels of phosphorylated TDP-43 and the total levels of TDP-43 in each fraction were analyzed by immunoblotting (A). The relative levels of TDP-43 in soluble and insoluble fractions were quantified by densitometry and then normalized to the levels of β-actin (B). Data are presented as fold changes compared with controls (mean ± SEM, n = 3). $^{**}p < 0.01$ (Student's t-test). (C) COS-7 cells were treated with or without 100 μM HNE for 60 min and incubated in culture medium for 24 h. TDP-43 localization was analyzed by immunocytochemistry.

mutations on HNE-induced insolubilization of TDP-43. Insolubility of 2CS and 3CS-2 was slightly increased by HNE treatment, while insolubility of 3CS-1 and 4CS was not significantly increased by HNE treatment (Fig. 3B, C and Supplementary Fig. S1). These results indicate that Cys173, Cys175, and Cys198 are responsible for HNE-induced insolubilization of TDP-43.

4. Discussion

HNE is known to be elevated in serum and cerebrospinal fluid samples from sporadic ALS patients and this study focused on the relationship between HNE and TDP-43. We found that HNE treatment of cells induces aggregation, phosphorylation, and mislocalization of TDP-43, which are similar to the properties of TDP-43 observed in the pathology of sporadic ALS. HNE-induced

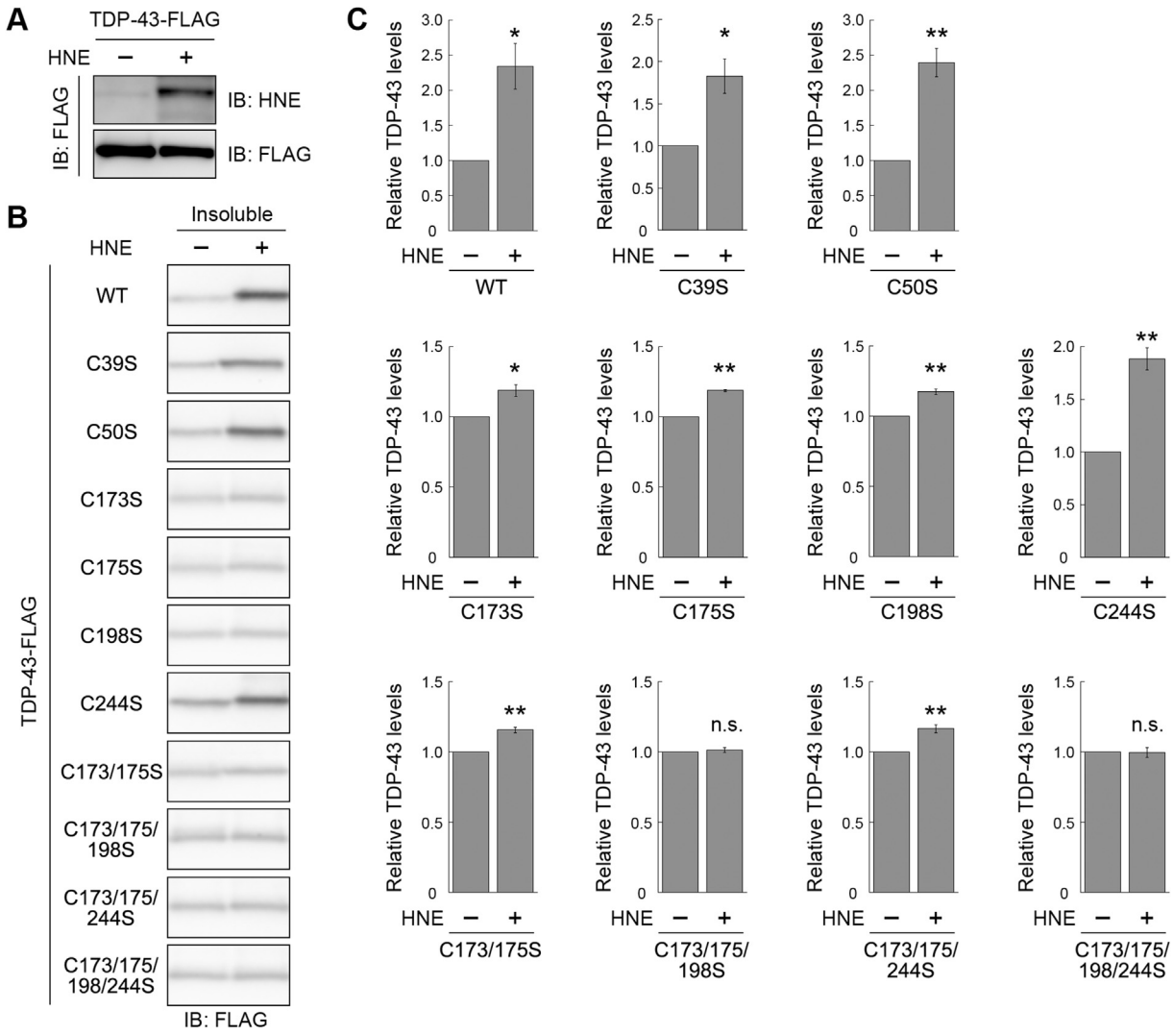

Fig. 3. Mechanism underlying HNE-induced insolubilization of TDP-43. (A) COS-7 cells overexpressing TDP-43-FLAG (wild type) were treated with or without 100 μM HNE for 60 min. Cells were then subjected to immunoprecipitation (IP) with an anti-FLAG antibody followed by immunoblotting using anti-HNE and anti-FLAG antibodies. (B and C) The indicated TDP-43-FLAG variants were overexpressed in COS-7 cells, treated with or without 100 μM HNE for 60 min, and Triton X-100-insoluble fractions were prepared from each sample. The levels of TDP-43 were analyzed by immunoblotting using anti-FLAG (B) and anti-β-actin (Supplementary Fig. S1) antibodies. The relative levels of TDP-43 in insoluble fractions were quantified by densitometry and then normalized to the levels of β-actin (C). Data are presented as fold changes compared with controls (mean ± SEM, n = 3). *$p < 0.05$, **$p < 0.01$, n.s., not significant (Student's t-test).

aggregation and phosphorylation of TDP-43 were persistent at least 24 h after removal of stress treatment. Moreover, we demonstrated that C173/C175/C198 cysteine residues in TDP-43 are responsible for the insolubilization of TDP-43 induced by HNE.

Recently, several studies reported that various oxidative stressors, such as ethacrynic acid, H_2O_2, and sodium arsenite cause insolubilization, aberrant intracellular localization, and TDP-43 phosphorylation, which resemble the features of TDP-43 in the pathology of sporadic ALS [20,24–27]. Although elevation of the levels of HNE in serum and cerebrospinal fluid of sporadic ALS patients were reported, ethacrynic acid and sodium arsenite are artificial oxidative stressors, and to our knowledge, there is no report showing that H_2O_2 levels are increased in sporadic ALS patients. Taken together, our results suggest that HNE is one of the key mediators of oxidative stress and is involved in the TDP-43 pathology observed in sporadic ALS, and that upregulation of HNE could be risk factor for ALS.

In oxidative stress conditions induced by HNE, TDP-43 localized diffusely in the cytosoplasm and only a small proportion of TDP-43 localized to stress granules. Therefore, the mechanism of cytoplasmic TDP-43 accumulation by HNE treatment is probably independent of stress granule formation. The diffuse cytosolic localization of TDP-43 by HNE treatment is similar to translocation of TDP-43 by ethacrynic acid treatment causing glutathione depletion [24], or by long-term/sub-lethal stress induced by arsenite [28]. In contrast, under oxidative stress conditions induced by H_2O_2, mislocalized TDP-43 has been reported to be mainly localized to cytoplasmic stress granules [20].

We have shown that HNE-induced insolubilization of TDP-43 is retained even 24 h after removal of HNE, indicating that insolublization of TDP-43 by HNE is a persistent change. These persistent alterations of TDP-43 are consistent with TDP-43 pathology observed in sporadic ALS. However, only a small proportion of TDP-43 retained cytoplasmic localization 24 h after removal of HNE. Whether insoluble TDP-43 induced by oxidative stress including HNE contributes to cytoplasmic aggregation of TDP-43 in the pathology of sporadic ALS is an important issue to be resolved.

We showed that HNE stress reduces the levels of soluble TDP-43 and this downregulation is persistent for at least 24 h after its removal. Because TDP-43 re-distributes from the nucleus to the cytosol in motor neurons of sporadic ALS patients, aggregation and loss of function of TDP-43 are thought to be related to the pathogenesis of sporadic ALS. Knockdown of TDP-43 in cultured cells was reported to induce cell death [29,30]. Mice in which TDP-43 was knocked out specifically in postnatal motor neurons exhibited an age-dependent progressive degeneration of motor neurons [31]. Thus, HNE-induced reduction of soluble TDP-43 may be involved in the TDP-43 pathology of sporadic ALS.

It has been reported that H_2O_2 or arsenite treatment induces intra-molecular disulfide bond formation of TDP-43, and four cysteine residues (Cys-173, Cys-175, Cys-198, and Cys-244) are required for this disulfide bond formation [20]. It is interesting that Cys-173, Cys-175, and Cys-198 are also required for HNE-induced insolubilization of TDP-43. Although H_2O_2 or arsenite can induce disulfide bonds of 2CS (C173/175S) mutant TDP-43 comparable to wild-type levels [20], substitutions of Cys173/175 dramatically reduced HNE-induced insolubilization of TDP-43 (Fig. 3B and C). Thus, mechanisms of HNE-induced insolubilization of TDP-43 are distinct from that of intra-molecular disulfide bond formation. Rather, modification of Cys-173, Cys-175, and Cys-198 by HNE may be involved in HNE-induced insolubilization of TDP-43.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.027.

Transparency document

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