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Involvement of endoplasmic reticulum stress in tauopathy

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ABSTRACT

Tauopathy is a pathological condition with an abnormal intracellular accumulation of tau protein in neurons and glias, which is a feature of Alzheimer's disease (AD) as well as frontotemporal lobar degenerations (FTLD). Recent reports showed that tauopathy occupies an important position for pathological process of dementia generally. Previously, we reported that endoplasmic reticulum (ER) stress has an influence on the onset of AD. In addition, some reports on brain autopsy findings suggest that ER stress is associated with AD and tauopathy. However, the mechanism underlying the association between ER stress and tauopathy is still unknown. Here, we show that ER stress, induced by glucose deprivation or chemicals, increases total endogenous tau protein in cultured neurons and primary cultured neurons. Under ER stress, no significant differences were observed in the transcription of tau, and no differences were observed in the translation of tau with or without the 5′-untranslated region (5′UTR) of tau. In contrast, the degradation rate of tau was decreased by 20% under ER stress. ER stress reduced the binding between tau and carboxyl terminus of Hsc70-interacting protein (CHIP), ubiquitin E3 ligase for tau. These results suggest that ER stress increases total tau protein and its mechanism is due to the decrease in the binding between tau and CHIP, which delays the degradation of tau protein through the ubiquitin–proteasome pathway. This mechanism may provide clue to treatment for tauopathy.

1. Introduction

One of the hypotheses of the etiology of AD suggests that neurodegeneration starts from the accumulation of amyloid proteins that leads to the formation of neurofibrillary tangles containing tau protein [1]. This hypothesis, called the "amyloid cascade hypothesis," is currently widely accepted. On the basis of this hypothesis, new treatment strategies targeting amyloid β (Aβ), i.e., amyloid vaccines and γ-secretase inhibitors, have been developed and evaluated; however, anticipated results have yet to be obtained. At present, alternative hypotheses, other than the amyloid cascade hypothesis, are being sought and are gaining increasing interest as the basis for the development of new AD treatments.

In the cause of AD, accumulation of abnormally phosphorylated tau is as equally important as the accumulation of amyloid. In other words, tauopathy is one of the components comprising the etiology of AD. The discovery of frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) revealed that abnormal tau itself is directly linked to neurodegeneration, which has recently attracted considerable interest [2].

The importance of tau in the etiology of AD has been indicated in some studies. When expression of tau was inhibited by crossing amyloid precursor protein-overexpressing mice with tau knockout mice, the dementia phenotype manifesting as abnormal space perception, abnormal behavior, etc., was not observed, even though the level of amyloid accumulation was unchanged [3]. It suggests that tau is an essential element in the progression of AD. Moreover, a recent new finding on tau accumulation in AD etiology suggested that the tau oligomer level was significantly increased in the cortex of early-stage AD patients [4]. The study of tau transgenic mice (Tg4510 strain) and wild-type mice overexpressing wild-type 4-repeat tau showed that caspase activation occurs before the tangle formation, suggesting that soluble tau may be more toxic than fibrillar tau [5]. These findings imply that soluble tau oligomers may have toxic effects and cause neurodegeneration before forming neurofibrillary tangles, indicating that an increase in tau protein itself may be an important factor in the early stage of AD. Another study shows that aggregated extracellular tau is taken up by cells and induces the intracellular accumulation of tau, and it transfers between co-cultured cells. It suggests that tau aggregates may propagate from the outside to the inside and spread tauopathy [6]. Therefore some researchers recently think tauopathy can be a new target in the development

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of AD treatments. Thus, research on the expression/degradation of tau is important.

Previously, we reported that mutant presenilin-1 inhibits endoplasmic reticulum (ER) stress responses and makes neurons more vulnerable to ER stress. Namely, ER stress is involved in neurodegenerative diseases, including AD [7–10]. Other researchers have reported that ER stress is activated in the hippocampus of AD patients [11]. Recently, activation of ER stress and increased levels of phosphorylated tau were observed in the hippocampus of patients with tauopathy, suggesting that ER stress may be related to tauopaty [12].

These studies suggest that ER stress is associated with AD and tauopathy. However, the pathological mechanism is still largely unknown. Thus, in the present study, the expression and degradation of total tau protein under ER stress were evaluated with the objective of identifying the mechanism of tauopathy.

2. Materials and methods

2.1. Plasmid construction

Human tau gene contained the published 320 nucleotides of the 5′UTR and 1239 nucleotides of the coding region (GenBank accession numbers NM_001123067). -5′UTR: The human tau coding region (1239 bps) was only inserted into pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA, USA). +5′UTR: The human tau 5′UTR (320 bps) was inserted into pcDNA3.1D/V5-His-TOPO (Invitrogen), and the human tau cording region was inserted at the back of the human tau 5′UTR, using Takara BKL Kit (Takara, Shiga, Japan). These were performed according to the manufacturer's instructions.

2.2. Cell cultures and transfections

SH-SY5Y cells and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/ F12 (Gibco, Grand Island, NY, USA) and in DMEM (Gibco) respectively, supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Leneza, KS, USA) and 1% penicillin streptomycin (Invitrogen). For glucose-deprivation experiments, cells were incubated in DMEM containing D-glucose (4500 mg/l) (Gibco) or DMEM without glucose (Gibco) supplemented with 10% FBS (JRH Biosciences) and 1% penicillin streptomycin (Invitrogen). Tunicamycin (Sigma–aldrich, St. Louis, MO, USA) or dithiothreitol (DTT) (Sigma–aldrich) were added to the cell media to induce ER stress. MG132 (Calbiochem, San Diego, CA, USA) for proteasome inhibition was added to the cell media. Transient transfections in HEK293 cells were performed using Lipofectamin LTX (Invitrogen) according to the manufacturer's instructions.

2.3. Neuronal cultures

Primary cultured neurons were prepared from E18.5 mice cerebral cortex. The mice were wild type E18.5 mice (PKR-like endoplasmic reticulum kinase (PERK) +/+) and PERK knockout E18.5 mice (PERK −/−). The cells were cultured for 7 days in DMEM (Gibco) supplemented with 10% FBS (JRH Biosciences) and then used for tunicamycin treatment.

2.4. Cell lysis and immunoblotting

Cells were lysed in 1× RIPA buffer (Pierce, Rockford, IL, USA) with protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Roche). Cleared cell extracts were obtained by centrifugation for 10 min at 31,000×*g* at 4 °C. Protein levels were determined by BCA method (Pierce). Equal amounts of protein were boiled in SDS sample buffer and separated using SDS-polyacrylamide gels (BIO CRAFT, Tokyo, Japan), then transferred to polyvinylidene fluoride (PVDF) transfer membrane (Immobilon-P; Millipore, Billerica, MA, USA). After bloking with 5% ECL Blocking Agent (GE Healthcare, Buckinghamshire, UK), membranes were incubated overnight with primary antibody at 4 °C. After incubation with horseradish peroxidase-conjugated second antibody (Promega, Madison, WI, USA), membranes were developed with enhanced chemiluminescence (ECL; GE Healthcare). Primary antibodies used included anti-Tau-5 (Calbiochem), anti- phosphorylation of eukaryotic translation initiation factor 2 subunit α (eIF2α-P) (Cell Signaling, Danvers, MA, USA), anti-KDEL (for immunoglobulin heavy-chain binding protein (BiP)) (Stressgen, Victoria, BC, Canada), anti-TAR DNA-binding protein 43 kDa (TDP-43) (Proteintech, Chicago, IL, USA), anti-Ubiquitin (Zymed, South San Francisco, CA, USA) and anti-GAPDH (Thermo, Fremont, CA, USA).

2.5. Immnoprecipitation

HEK293 cells were transiently transfected with the plasmid containig the human tau coding region (−5′UTR). After overnight recovery, cells were incubated for 24 h in DMEM with glucose (4500 mg/l) or DMEM without glucose. Cleared cell extracts were immunoprecipitated with Protein G Sepharose (Sigma–aldrich) and 2.5 μg Anti-Tau-5 (Calbiochem). Immunoprecipitates were boiled in 2× SDS sample buffer and separated using SDS-polyacrylamide gels (BIO CRAFT). Primary antibody used for immunoblotting included anti-Tau (DakoCytomation, Denmark), anti-CHIP (Santa Cruz, Santa Cruz, CA, USA).

2.6. Pulse-chase

Before pulse-labeling, SH-SY5Y cells were incubated in methionine/cystine/glutamine-deficient DMEM (Gibco) added with cystine (Sigma–aldrich) and glutamine (Invitrogen) for 40 min. Cells were pulse-labeled with 4 ml of the same media supplemented with 0.28 mCi ^{35}S-methionine/cystine (Perkin Elmer Japan, Kanagawa, Japan) for 20 min. Cells were washed once and chased with either DMEM with glucose (4500 mg/l) (Gibco) or DMEM without glucose (Gibco). At 24 h after labeling, cells were lysed in 1× RIPA with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche) and immunoprecipitated with Protein G Sepharose (Sigma–aldrich) and 2 μg anti-Tau-5 (Calbiochem). Immunoprecipitates were separated using 4–20% SDS-polyacrylamide gels (Invitrogen). Gels were fixed, dried and exposed to films (Kodak, Rochester, NY, USA).

2.7. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

SH-SY5Y cells were cultured in DMED/F12 (Gibco) with 1 μg/ml tunicamycin for indicated time. Total RNAs were extracted from cells by Trizol (Invitrogen) according to the manufacture's recommendations. For RT-PCR, 2 μg of total RNA from each sample was used for first-strand cDNA synthesis using Prime Script II (Takara). PCR was performed with the cDNA, 0.5 mM downstream primer, 0.5 mM upstream primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 unit of Taq polymerase (Takara) for 25 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C. The PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. The primers used for amplification were as follows: human tau; 5′-TCATGAAGGGCCTAAACCAC-3′ and 5′-CACCCTCCTCAGTCTTCCTG-3′, β-actin; 5′-GTTTGAGACCTTCAACACC-3′ and 5′-GTGGTGGTGAAGCTGTAG-3′, X-box binding protein 1

(XBP1); 5′-TAAGACAGCGCTTGGGGATGG-3′ and 5′-CAGAATCCATGGGGAGATGTT-3′.

2.8. Statistical analysis

Statistical comparisons were made using a Student's t-test. $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. ER stress increases tau protein

The change in total tau protein under ER stress was evaluated. Glucose deprivation induces ER stress. SH-SY5Y cells were subjected to glucose deprivation in order to evaluate the change in the amount of total tau protein under ER stress. Compared with the control (d-glucose: 4500 mg/L), the level of ER stress markers, i.e., phosphorylation of eIF2α and BiP, were increased in both 24 h incubation and 48 h incubation cultures with glucose deprivation. Under these conditions, a significant increase was observed in the amount of total tau protein, which was assessed using anti-TAU-5 (Fig. 1A, and B). When SH-SY5Y cells were treated with ER stress inducers tunicamycin or DTT, the expression of total tau protein was also increased, indicating that ER stress, in general, increases the amount of total tau protein (Fig. 1C). Recently, various causative proteins were found to accumulate in neurodegenerative diseases, e.g., FTLD. One of such proteins is TDP-43. We evaluated the relationship between TDP-43 and ER stress. In both 24 h incubation and 48 h incubation cultures with glucose deprivation, the amount of total tau protein was increased, but the amount of TDP-43 did not change (Fig. 1D).

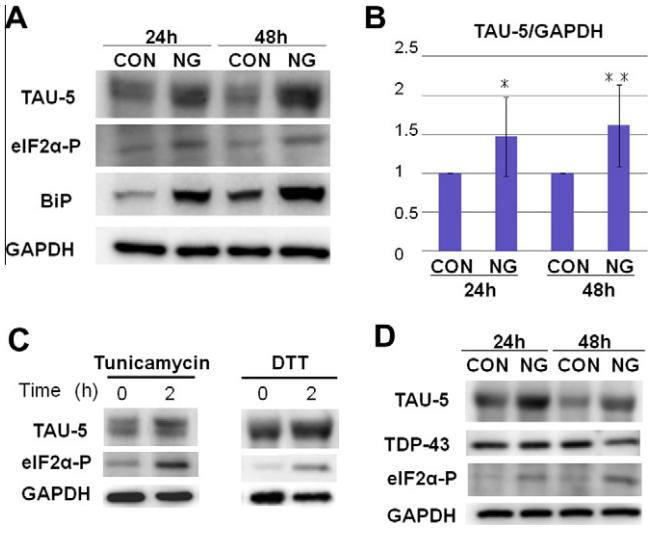

Fig. 1. ER stress increases tau protein levels. (A) SH-SY5Y cells were incubated for 24 or 48 h in either normal DMEM with glucose (4500 mg/l; CON) or DMEM without glucose (NG). The representative immunoblots show that total tau (TAU-5) protein levels were elevated in response to glucose deprivation, which causes ER stress. ER stress were monitored by the elevated levels of phosphorylation of eIF2α (eIF2α-P) and BiP. (B) The densitometric scanning of bands of tau in normarlized to GAPDH, shows that tau protein levels were significantly elevated in response to glucose deprivation. (*: $p < 0.05$; **: $p < 0.05$, $n = 8$). (C) SH-SY5Y cells were treated with 1 μg/ml tunicamycin or 1 mM DTT for the indicated period. Tau protein levels were also elevated in parallel with eIF2α-P during ER stress. (D) SH-SY5Y cells were incubated for 24 or 48 h in either normal DMEM with glucose (4500 mg/l; CON) or DMEM without glucose (NG). The representative immunoblot shows that the levels of TDP-43 protein, another key molecule of FTLD, were not changed nor decreased in response to glucose deprivation-induced ER stress, whereas tau protein levels were increased.

3.2. Mechanism of ER stress-induced increase in tau protein

We investigated whether the ER stress-induced increase in tau protein is due to an increased production of tau protein, i.e., the transcription or translation of tau. SH-SY5Y cells were incubated with tunicamycin for 1–3 h, and RNA isolated from these cells was subjected to RT-PCR using XBP1 and tau probes. While confirming the presence of ER stress on the basis of XBP1 splicing, the change in tau mRNA was evaluated. The result showed that no increase in tau mRNA was observed under ER stress, indicating that the transcription of tau did not increase (Fig. 2A). Under ER stress, activation of PKR-like endoplasmic reticulum kinase (PERK) causes phosphorylation of eIF2α, which inhibits the translation of most proteins. However, due to the unique sequence of the 5′UTR, it is known that translation of some molecules, e.g., activation transcription factor 4 (ATF4), is induced by the activation of PERK [16]. In order to investigate whether the increase in tau is due to such induction of translation, the expression of tau protein was compared between cultures in the presence and absence of the tau 5′UTR. HEK293 cells were transiently transfected with either a tau (+5′UTR) or tau (−5′UTR) construct, and subjected to ER stress induced by glucose deprivation. Compared with the control, the expression of total tau protein was increased in both (+5′UTR) and (−5′UTR) cultures with glucose deprivation. No significant difference was observed between the (+5′UTR) and (−5′UTR) cultures in the ER stress-induced increase in total tau protein (Fig. 2B). Next, the change in total tau protein under ER stress was evaluated using primary cultured neurons prepared from the cerebral cortex of E18.5 PERK knock-out (PERK−/−) and wild-type (PERK+/+) mice fetuses. In the primary cultured neurons, an increased level of tau was observed in both the PERK knock-out and wild-type mice, suggesting that this phenomenon is not related to the PERK-mediated induction of translation (Fig. 2C). These findings indicate that the increase in total tau protein is not due to increased transcription or translation.

In order to investigate whether the ER stress-induced increase in total tau protein is caused by decreased degradation, pulse-chase experiments with labeled tau were performed. SH-SY5Y cells were pulse-labeled with ^{35}S-methionine/cystine for 20 min, and cultured for 24 h in the absence of glucose. Compared with the control, the degradation rate of total tau protein was decreased in glucose-deprived cultures (Fig. 3A). Densitometry analysis showed that, compared with the control culture, the rate of metabolism was reduced by approximately 20% in glucose-deprived cultures after a 24 h incubation (Fig. 3B). This result suggests that the level of tau protein increases due to a delay in its degradation.

With regard to the mechanism of the delay in total tau protein metabolism, the proteasome system (the major degradation pathway of tau protein) was evaluated. When SH-SY5Y cells were treated with the reversible proteasome inhibitor MG132, total tau protein was increased (Fig. 4A). CHIP, ubiquitin E3 ligase for tau, binds to tau and is thought to promote the degradation of tau by its ubiquitination through ubiquitin–proteasome system. The relationship between CHIP and total tau protein was investigated. To evaluate the change in the binding between tau and CHIP under ER stress, HEK293 cells were transiently transfected with the tau (−5′UTR) construct and subjected to glucose deprivation. Immunoprecipitation with anti-TAU-5 was performed. Compared with the control culture, the binding between tau and CHIP was decreased in glucose-deprived cultures (Fig. 4B). The molecular weight of CHIP monomers is approximately 35 kD; however, a significant difference was observed at approximately 70 kD, which is probably attributable to CHIP dimers. These findings imply that, under glucose deprivation-induced ER stress, the binding between tau and CHIP decreases and the degradation of tau through ubiquitin–proteasome system is inhibited, resulting in an increase in total tau protein.

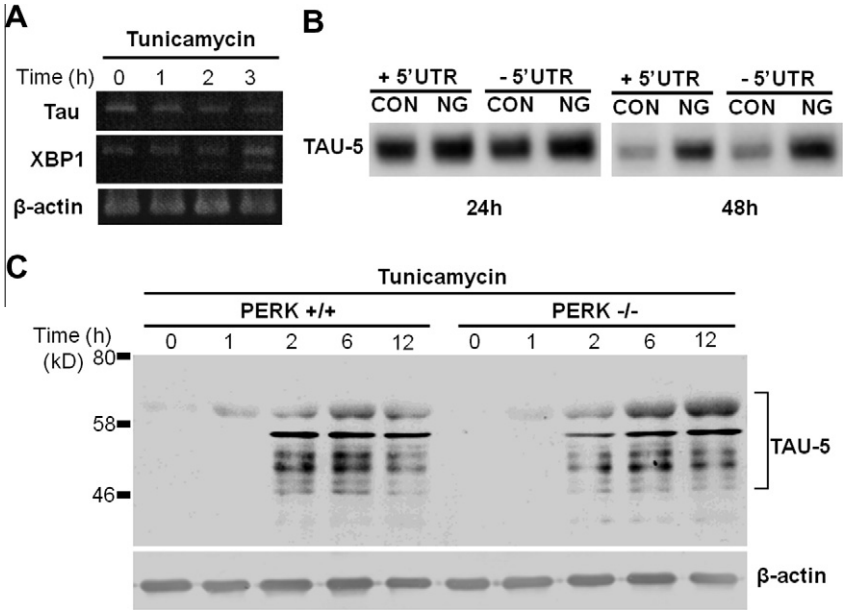

Fig. 2. ER stress does not increase tau gene transcription nor translation. (A) SH-SY5Y cells were treated with 1 μg/ml tunicamycin for the indicated period. Levels of endogenous tau and XBP1 mRNAs were detected by RT-PCR. The level of tau mRNAs did not increase in response to ER stress, whereas spliced transcripts of XBP1 existed showing ER stress. (B) HEK293 cells were transiently transfected with pcDNA3.1 vector containing the entire human tau coding region plus the tau 5′UTR (+5′UTR) or the vector containing only the human tau coding region (−5′UTR). After overnight recovery, the cells were incubated for 24 or 48 h in normal DMEM with glucose (4500 mg/l; CON) or DMEM without glucose (NG). The immunoblot shows that tau protein levels were elevated in response to glucose deprivation with or without 5′UTR both in 24 and in 48 h. (C) Primary cultured neurons were prepared from E18.5 mice (PERK+/+ and PERK−/−) cerebral cortex. The cells were cultured for 7 days and then treated with 1 μg/ml tunicamycin for the indicated period. Tau protein levels were elevated after a two hours incubation in both PERK+/+ and PERK−/− neurons.

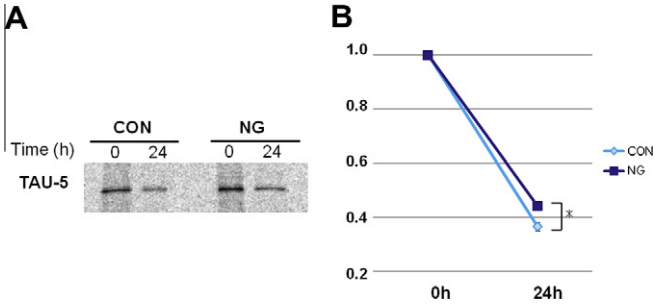

Fig. 3. Glucose deprivation affects degradation of tau protein. (A) SH-SY5Y cells were pulse-labeled in media containing ^{35}S-methionin/cystine and chased for 24 h in either normal DMEM with glucose (4500 mg/l; CON) or DMEM without glucose (NG). The representative autoradiography shows that glucose deprivation reduces the rate of degradation of tau protein. (B) The densitometric scanning of bands of tau, normarlized to 0 h tau, shows that glucose deprivation causes the significant delay of degradation of tau by 20% (NG/CON = 1.2) (*: $p < 0.05$, $n = 4$).

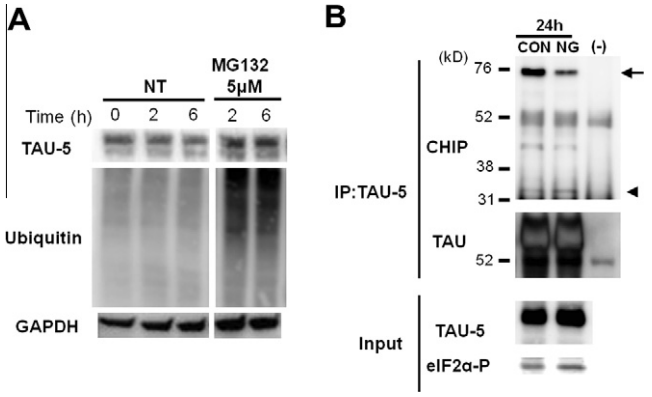

Fig. 4. Decrease in CHIP binding to tau under ER stress. (A) SH-SY5Y cells were incubated for 2 or 6 h in either with or without MG132 (5 μM), proteasome inhibitor. The representative immunoblot shows that tau protein levels were elevated in response to proteasome inhibition. (NT: without MG132). (B) HEK293 cells were transiently transfected with pcDNA3.1 vector containing the human tau coding region. After overnight recovery, the cells were incubated for 24 h in normal DMEM with glucose (4500 mg/l; CON) or without glucose (NG). The cell lysates and only lysis buffer (−) were immunoprecipitated with anti-TAU-5 antibody. The immunoblot shows that the binding of CHIP to tau, represented as arrow, decreases in response to glucose deprivation. The arrow and the arrowhead show the dimer and the monomer of CHIP respectively. The Input shows the increase of tau protein levels and phosphorylated eIF2α (eIF2α-P) in response to glucose deprivation.

4. Discussion

Previously, an association between ER stress and tauopathy (including AD) has been suggested in brain autopsy studies [11,12]. However, the mechanism of tauopathy is still largely unknown. The results of the present study show that ER stress causes an increase in the level of tau protein and that the mechanism of such an increase is due to the decrease in the binding between tau and CHIP, which causes a delay in the degradation of tau through the ubiquitin–proteasome system.

The increase in total tau protein was observed in both glucose deprivation-induced ER stress and ER stress induced by tunicamycin or DTT, showing that ER stress in general causes an increase in total tau protein. Since glucose deprivation can occur in the central nervous system in clinical settings, it is noteworthy that glucose deprivation causes ER stress and increases tau protein levels, which may lead to tauopathy. In the present study, glucose deprivation-induced ER stress caused an increase in total tau protein, but did not cause an increase in TDP-43 (one of the accumulative proteins associated with FTLD). This suggests that ER stress specifically causes an increase in tau protein; in other words, ER stress is specifically involved in tauopathy.

We examined whether the increase in tau is due to increased tau production (i.e., increased transcription or translation) or inhibition of tau degradation. The result of RT-PCR analysis showed

that the level of tau mRNA did not change under ER stress. This implies that the level of tau transcription remains unchanged. Under ER stress, activation of PERK causes phosphorylation of eIF2α, which inhibits the translation of most proteins. However, the level of translation of some molecules is somewhat increased by the activation of PERK [13,14]. The mechanism that results in increased translation of ATF4 depends on the 5′UTR sequence [15,16]. We studied whether the tau 5′UTR has an effect on increasing the translation of tau. However, no significant difference was observed in the translation of tau with or without the 5′UTR of tau. Moreover, in primary cultured neurons, an increased level of total tau was observed in both PERK knock-out and wild-type mice, suggesting that this phenomenon is not related to the PERK-mediated induction of translation. These findings indicate that the increase in total tau protein is not due to increased transcription or translation. In other words, the mechanism that causes an increase in total tau protein involves not an increase in tau production, but, conversely, an inhibition of tau degradation.

The pulse-chase experiment showed that the ER stress-induced increase in total tau protein is due to a delay in tau degradation. When the proteasome system, the major metabolic pathway of tau, is inhibited, the level of total tau protein increases, which is consistent with previously reported findings [17]. Because the ER stress-induced metabolic delay may be due to the inhibition of ubiquitination, we studied CHIP, the ubiquitin E3 ligase for tau [18,19]. The result showed that ER stress affects the binding between tau and CHIP dimer, suggesting that the ubiquitination of tau is inhibited. This is consistent with the previously reported findings that activation of CHIP requires dimerization [20].

The possible mechanism that explains why the binding between tau and CHIP is affected under ER stress could be as follows. ER stress increases the amount of unfolded proteins, and CHIP is mobilized to degrade these unfolded proteins, and then the degradation of tau is relatively decreased. In this model, unfolded proteins and tau compete as a substrate for CHIP under ER stress. A similar mechanism for decreased binding is suggested for old astrocytes specifically induced substance (OASIS) and E3 ligase 3-hydroxy-3-methylglutaryl CoA reductase degradation 1 (HRD1) under ER stress [21].

With regard to the relationship between ER stress and tauopathy, some reports on brain autopsy samples have suggested that ER stress is involved in tauopathies, including AD [11,12]. However, since these were brain autopsy studies, it is unclear whether ER stress caused tauopathy or tauopathy caused ER stress. We demonstrated that ER stress causes an increase in the level of total endogenous tau protein in cultured neuronal cells and primary cultured neurons. This finding suggests that ER stress is placed upstream of the increase in tau protein level, in other words, ER stress may cause tauopathy.

In conclusion, the present study showed that ER stress causes an increase in the expression of total tau protein and clarified the mechanism underlying such increase in total tau protein. A recently published study indicates that Aβ42 induces ER stress and activates c-Jun N-terminal kinase-3, which results in an increase in Aβ42 [22], implying that ER stress is an important factor in the etiology of AD. Moreover, it is reported that mutant tau, overexpressed specifically in the entorhinal cortex of mice, acts as an initiator that causes aggregation or propagation of the mutant tau [23]. This suggests that increased levels of neuronal tau are an important factor in the progression of tauopathy. These findings suggest that the management of ER stress is an important element for the prevention and treatment of tauopathies, including AD.

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