

Suppression of Autophagy and Activation of Glycogen Synthase Kinase 3 β Facilitate the Aggregate Formation of Tau

Song-In Kim¹, Won-Ki Lee¹, Sang-Soo Kang¹, Sue-Young Lee¹, Myeong-Ja Jeong¹, Hee Jae Lee¹, Sung-Soo Kim¹, Gall V. W. Johnson², and Wanjoo Chun¹

¹Department of Pharmacology, College of Medicine, Kangwon National University, Chuncheon 200-701, Korea, ²Department of Anesthesiology, University of Rochester, Rochester, NY, 14642, USA

Neurofibrillary tangle (NFT) is a characteristic hallmark of Alzheimer's disease. GSK3 β has been reported to play a major role in the NFT formation of tau. Dysfunction of autophagy might facilitate the aggregate formation of tau. The present study examined the role of GSK3 β -mediated phosphorylation of tau species on their autophagic degradation. We transfected wild type tau (T4), caspase-3-cleaved tau at Asp421 (T4C3), or pseudophosphorylated tau at Ser396/Ser404 (T4-2EC) in the presence of active or enzyme-inactive GSK3 β . Trehalose and 3-methyladenine (3-MA) were used to enhance or inhibit autophagic activity, respectively. All tau species showed increased accumulation with 3-MA treatment whereas reduced with trehalose, indicating that tau undergoes autophagic degradation. However, T4C3 and T4-2EC showed abundant formation of oligomers than T4. Active GSK3 β in the presence of 3-MA resulted in significantly increased formation of insoluble tau aggregates. These results indicate that GSK3 β -mediated phosphorylation and compromised autophagic activity significantly contribute to tau aggregation.

Key Words: Tau, Autophagy, Glycogen synthase kinase 3 β , Trehalose, Neurofibrillary tangles

INTRODUCTION

Intracellular accumulation of the microtubule-associated protein tau as the NFTs is one of the characteristic features of several diseases known as tauopathies, which include AD and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). NFTs are mainly composed of paired helical filaments (PHFs), which are formed from abnormally hyperphosphorylated tau [1]. There is increasing evidence that inappropriate phosphorylation of tau, which leads to tau dysfunction, results in decreased cell viability. Indeed, in all neurodegenerative diseases in which tau pathology has evidence that glycogen synthase kinase 3 β (GSK3 β) may play a major role in regulating tau phosphorylation in pathological conditions [2]. Increased expression of GSK3 β results in the increased phosphorylation of tau at numerous sites including PHF-1 epitope [3]. Furthermore, there is evidence for abnormally increased activation of GSK3 β in AD brain [4].

Along with aberrant phosphorylation, caspase cleavage of tau has been reported to play an important role in the aggregation of tau [5]. Although tau is present predominantly as an intact and full-length form in AD brain, tau truncated at Asp421 has been found in AD brain. Caspase-

cleaved tau, albeit not a large amount, may play a significant role in the formation of NFTs, given that truncated tau is more fibrillogenic than wild type tau [5] and contributes to the nucleation-dependent filament formation of tau [6]. Although the role of NFTs as a toxic mediator in neuronal dysfunction and death in AD is still not clear, the tau aggregation is closely related with the pathology of AD [7].

Autophagy has been reported to play important roles in many diseases such as cancer [8] and neurodegenerative diseases [9]. Soluble, misfolded proteins in the cytosol are normally degraded by the ubiquitin-proteasome system. However, when the levels of such proteins overload the ubiquitin-proteasome system, they may form toxic oligomers or large aggregates that cause neurodegeneration [10]. Therefore, dysfunction of autophagic pathway might contribute to the pathology of various neurodegenerative disorders including AD and Parkinson's disease (PD) [9]. Mice with neuronally confined autophagy-gene knockouts develop intraneuronal aggregates and neurodegeneration [11]. It has been reported that alterations of autophagic degradation pathway are present in AD. For instance, the number of lysosomal-endosomal compartments is increased in vulnerable neuronal populations of AD brains [12]. The expression of cathepsins is increased and concentrated in senile plaques [13].

The present study was undertaken to determine the role of autophagic degradation pathway in the accumulation

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Corresponding to: Wanjoo Chun, Department of Pharmacology, College of Medicine, Kangwon National University, Hyoja-2-dong, Chuncheon 200-701, Korea. (Tel) 82-33-250-8853, (Fax) 82-33-255-8809, (E-mail) wchun@kangwon.ac.kr

ABBREVIATIONS: AD, Alzheimer's disease; NFT, neurofibrillary tangle; FTDP-17, parkinsonism linked to chromosome 17; PHFs, paired helical filaments; GSK3 β , glycogen synthase kinase 3 β .

and its consequent aggregation of post-translationally modified tau. To achieve this goal wild type or abnormal tau species were expressed in the presence of constitutively active GSK3 β or kinase-dead GSK3 β in the milieu of modified autophagic activity, and the propensity of intracellular accumulation and aggregation of tau was examined. The present study demonstrates that constitutively active GSK3 β and 3-MA-induced inhibition of autophagy resulted in increased intracellular accumulation and aggregation of tau and that wild type tau phosphorylated by GSK3 β is resistant to autophagic degradation. These results strongly indicate that compromised autophagic activity significantly facilitates the accumulation and aggregate formation of post-translationally modified tau.

METHODS

Plasmid constructs

All plasmid constructs (Fig. 1) used in this study have been previously described [14,15]. T4 contains human tau with four microtubule-binding repeats but without exons 2 and 3. T4C3, the last 20 amino acids deleted, mimics caspase-cleaved tau and T4-2EC, pseudophosphorylated at serine 396 and 404E residues, mimics phosphorylation at PHF-1 epitope. All the mutations were confirmed by DNA sequencing analysis. GSK3 β -S9A-HA, which is a constitutively active form of GSK3 β , was constructed in pcDNA3.1(-) and kinase dead-GSK3 β -HA construct, in which lysine residues at 85 and 86 were mutated to alanines, was a generous gift of G. Meares at the University of Alabama at Birmingham, AL, USA.

Cell culture

Chinese hamster ovary (CHO) cells were grown in D-MEM/F-12 (Gibco, Dulbecco's Modified Eagle's Medium: Mixture F-12 (1 : 1)) supplemented with 5% fetal bovine serum (Gibco) and 2 mM L-glutamine (Gibco), 10 units/ml penicillin (Gibco), 10 μ g/ml streptomycin at 37°C in a humidified incubator with 5% CO₂.

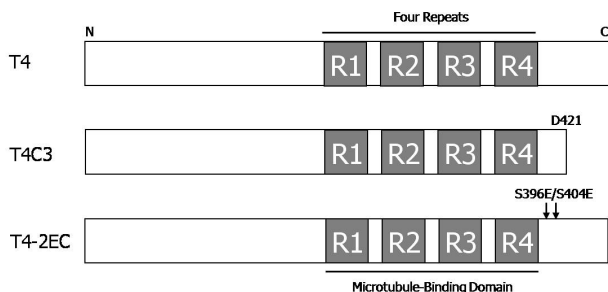


Fig. 1. Plasmid constructs of human tau. All types of human tau constructs are contained four microtubule-binding repeats but without exons 2 and 3. T4 is wild type of tau in pcDNA3.1(-). T4C3 is mimicking caspase-cleaved tau form which was deleted the last 20 amino acids at D421 in pcDNA3.1(+). Mutant constructs T4-2EC (S396E/S404E) is generated by mutating the serine 396 and 404 sites to glutamic acid to mimic phosphorylation.

Transient transfections and drug treatments

Tau constructs alone or with either GK3 β -S9A or GSK3 β -kinase-dead were transiently transfected into CHO cells using LT-1 (Mirus) transfection reagent according to the manufacturer's protocol. After transfection, the cells were treated 5 mM 3-MA (Sigma) or 200 mM trehalose (Sigma). Twenty-four hrs after drug treatments, cells were washed with ice-cold PBS and then harvested and processed for below assays.

Immunoblotting

Cell lysates were sonicated briefly and centrifuged. Protein concentrations in the supernatants were determined using the bicinchoninic acid assay (Sigma). Equal amounts of proteins were diluted with 2X protein loading buffer (0.25 M Tris-HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, and 10% glycerol with bromophenol blue as the tracking dye), incubated in a boiling water bath for 5 min, separated on 8~15% SDS-polyacrylamide gels, transferred to PVDF membrane (GE Healthcare), and blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 30 min at room temperature, and probed with the indicated antibodies in same buffer overnight at 4°C. The membranes were then washed with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 2 hrs at room temperature. The membranes were rinsed with TBST. The blots were visualized with enhanced chemiluminescence (GE Healthcare) and exposed on film (Kodak). Gel images were scanned and quantified using Image Quant software (Molecular Dynamics, Sunnyvale, California, USA). For fair comparison, total protein levels in each β -actin levels were used as loading control. The following antibodies were used in this study; anti-5A6 for total tau (phosphorylation-independent); anti-LC 3 antibody (MBL) for autophagic activity; Anti-GSK3 β antibody (Cell Signaling); anti- β -actin (Sigma).

Sarkosyl fractionation assay

The assay was carried out as described previously [15]. CHO cells were co-transfected with one of tau constructs and with or without either active GSK3 β or kd-GSK3 β . Twenty four hours after transfection, the cells were treated 5 mM 3-MA or 200 mM trehalose for 24 hrs, then rinsed with ice-cold PBS, collected by spinning at 13,000 rpm for 10 min at 4°C, resuspended in RAB buffer (100 mmol/l Mes, 1 mmol/l EGTA, 0.5 mmol/l MgSO₄, 750 mmol/l NaCl, and 20 mmol/l NaF) containing protease inhibitors; and then homogenized with 30 strokes using a tissue grinder. The homogenates were centrifuged at 13,000 rpm for 20 min and the supernatants were collected as RAB fraction. The pellets were resuspended with RIPA buffer (50 mmol/l Tris-HCl at pH 8.0, 150 mmol/l NaCl, 1% Nonidet P-40, 5 mmol/l EDTA, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors, sonicated briefly and then incubated on ice for 30 min. The homogenates were centrifuged at 13,000 rpm for 20 min and the supernatants were collected as RIPA fraction. The pellets were resuspended with RAB buffer containing 1% N-lauroylsarcosine (sarkosyl) in Mes buffer (20 mmol/l Mes at pH 6.8, 80

mmol/l NaCl, 1 mmol/l MgCl₂, 2 mmol/l EGTA, 10 mmol/l NaH₂PO₄, and 20 mmol/l NaF) with protease inhibitors, vortexed for 30 min at 15~25°C, incubated overnight at 4°C, and then centrifuged at 13,000 rpm for 30 min at 15~25°C. The supernatants (sarkosyl-soluble fraction) were collected and diluted with protein loading buffer, and the pellets (sarkosyl-insoluble fraction) were resuspended in 2X protein loading buffer and incubated in a boiling water bath for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis, transferred, and blotted with 5A6 antibody.

Immunocytochemistry

These procedures were modified from previously described protocols [16]. The cells were transiently transfected individually with each tau construct in the absence

or presence of active GSK3 β . Twenty four hours after transfection cells were treated 5 mM 3-MA. Two days after transfection the cells were rinsed with PBS and fixed at room temperature for 1 hr in fixation buffer (3% paraformaldehyde, 0.2% glutaraldehyde, 1 mM MgCl₂, 1 mM EGTA, 30% (v/v) glycerol in 70 mM PIPES, pH 6.8). Cells were washed 3 times with PBS then permeabilized with 0.2% Triton X-100 in PBS for 2 min. After rinsing with PBS, cells were blocked with 4% bovine serum albumin in PBS to reduce background before staining. The cells were incubated for 1.5 hrs with a 5A6 antibody diluted in 0.4% bovine serum albumin. After rinsing with PBS, the cells were incubated with Texas Red-conjugated goat anti-mouse IgG (Jackson Laboratories). Cells were then washed extensively in PBS before being incubated in a thioflavin-S solution (Sigma) (0.005%) for 10 min. The cells were then washed in 70% ethanol and water before mounting [17].

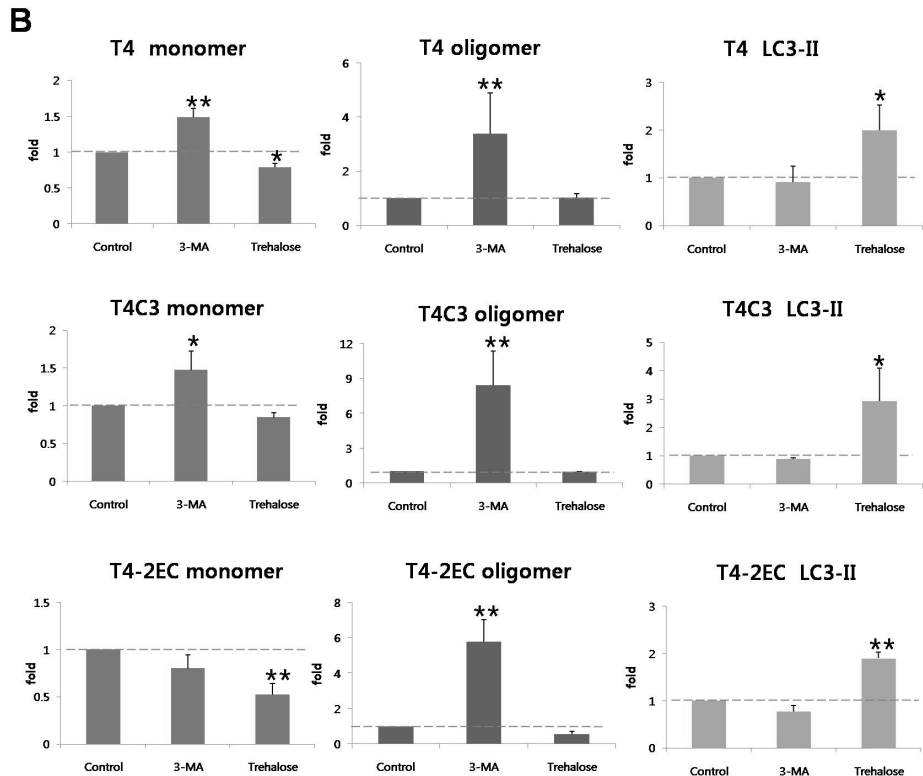
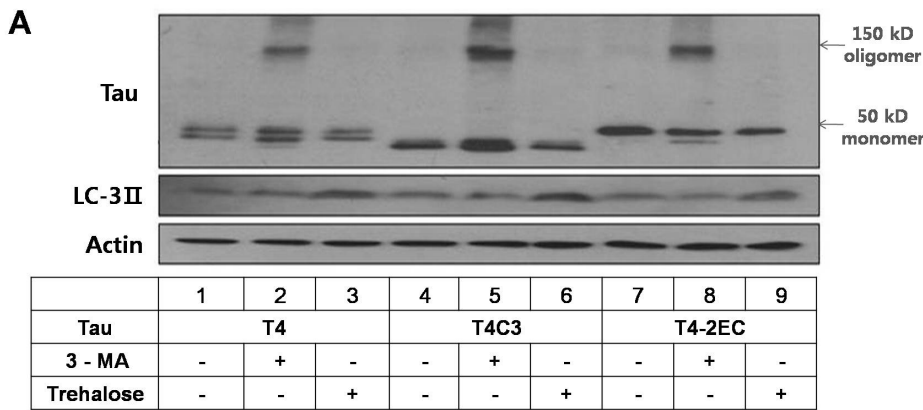


Fig. 2. Tau is degraded via the autophagic pathway. Representative immunoblot (A) and quantitative analysis (B) of intracellular tau levels. Tau constructs were transiently transfected into CHO cells. One day after transfection, 3-MA (5 mM) or trehalose (200 mM) was added to the medium immunoblotted and incubated for 24 hrs. Lysates were immunoblotted with tau (5A6), LC3, or β -actin antibodies. (B) The intensity of monomers and oligomers was quantitated by a densitometer and calculated as a fold to untreated control of each tau. Results are plotted as mean \pm SD from three independent experiments (n=3). 3-MA treatment caused the significant accumulation of oligomers in all tau species. Trehalose treatment exhibited decreased intracellular tau levels. Increased LC3-II levels indicate facilitated autophagic activity with trehalose treatment. Arrows indicate monomeric (~50 kDa) and oligomeric (~150 kDa) tau, respectively. *p < 0.05, **p < 0.01 to each control.

Cells images were captured with OLYMPUS Fluoview FV1000 Confocal Microscope and processed with Olympus FluoView software.

Statistical analysis

All data were analyzed by ANOVA using the statistical software SPSS (version 7.5). Values were expressed as mean±SE and considered significantly different when $p < 0.05$.

RESULTS

Tau is degraded via autophagic degradation pathway

To determine the role of autophagy in tau degradation, tau levels were measured in the presence of 3-MA, autophagic inhibitor [18] or trehalose, autophagic inducer [19]. In all types of tau species, intracellular levels of tau were significantly increased with 3-MA treatment (Fig. 2). Especially, oligomeric form of tau, which is presumed to precede the

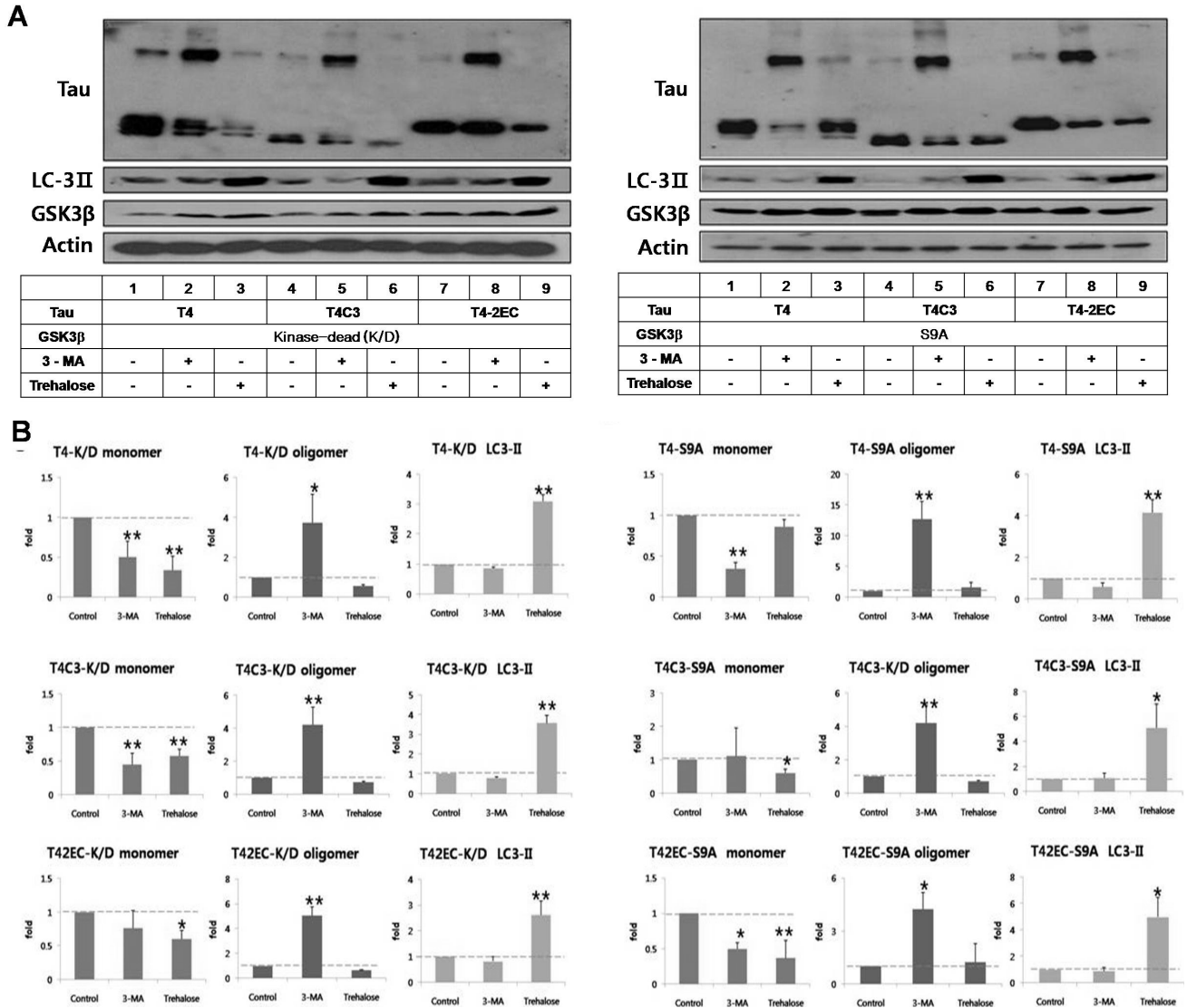


Fig. 3. GSK3β-phosphorylated T4 is less efficient in trehalose-facilitated autophagic degradation. (A) Representative immunoblots of tau levels with enzyme-inactive GSK3β (kinase-dead (k/D), left) or constitutively active GSK3β (S9A, right), respectively. Tau and each GSK3β constructs were transiently co-transfected into CHO cells. One day after transfection, 3-MA (5 mM) or trehalose (200 mM) was added to the medium and incubated for 24 hrs. Lysates were immunoblotted with tau (5A6), LC3, GSK3β, or β-actin antibodies. (B) Quantitative analysis of tau levels. The intensity of monomers and oligomers was quantitated and calculated as a fold to untreated control of each tau. Results are plotted as mean±SD from three independent experiments (n=3). In the presence of kinase-dead GSK3β, 3-MA treatment resulted in the shift of monomeric tau to oligomeric tau in all tau species and trehalose treatment showed significantly decreased intracellular tau level of all tau species. In the presence of active GSK3β, 3-MA treatment resulted in the increased accumulation of oligomeric tau in all tau species. Trehalose treatment resulted in facilitated clearance of T4C3 and T4-2EC. However, phosphorylated T4 by GSK3β was not efficiently degraded, which indicates phosphorylation hinders autophagic degradation. LC3-II levels indicate facilitated autophagic activity with trehalose treatment. Arrows indicate monomeric (~50 kDa) and oligomeric (~150 kDa) tau, respectively. *p<0.05, **p<0.01 to each control.

aggregate formation, was observed with 3-MA treatment in all tau species (Fig. 2). T4C3 showed highest amount of oligomers compared to other tau species, albeit not significant. To the contrary, trehalose treatment exhibited significantly decreased level of tau in all tau species. In addition, no oligomers were observed with trehalose treatment (Fig. 2). Facilitation of autophagic degradation with trehalose was confirmed with increased level of LC3-II, an autophagic degradation marker. The fact that modulation of autophagic activity directly affects the intracellular level of tau clearly demonstrates that tau is degraded via autophagy pathway.

GSK3 β -induced phosphorylation of T4 is resistant to trehalose-facilitated autophagic degradation

To determine the role of GSK3 β -mediated phosphorylation in autophagic degradation of tau, intracellular level of tau was examined in the presence of enzyme-inactive (kinase-dead) or constitutively active GSK3 β (S9A) (Fig. 3). As shown in Fig. 2A & C, intracellular level of each tau species in the presence of kinase-dead GSK3 β was similar to tau-only expression in Fig. 1. However, level of wild type tau (T4) was not attenuated with trehalose treatment in the presence of active GSK3 β , suggesting that GSK3 β -mediated phosphorylation might interfere autophagic degradation of tau (Fig. 3). Interestingly, attenuated clearance of tau was not observed in abnormal tau species, T4C3 and T4-2EC, even in the presence of active GSK3 β . This phenomenon might be due to the fact that wild type tau is

more efficiently phosphorylated than abnormal tau species by GSK3 β as previously [20]. 3-MA treatment significantly increased the formation of oligomers in all tau species.

Active GSK3 β and 3-MA treatment result in the formation of thioflavin-S-positive inclusions

To determine whether intracellular accumulation of tau proceeds to the aggregate formation, thioflavin-S staining was carried out. Thioflavin-S binding is a known indicator of insoluble protein aggregates with β -pleated sheet structure and thioflavin-S readily stains the intracellular neurofibrillary tangles in AD brain [21]. Thioflavin-S-positive inclusions were not observed in T4- or T4-2EC- expressing cells (Fig. 4). However, T4C3 expressing cells showed thioflavin-S-positive inclusions even in basal condition (Fig. 4). These data suggest that T4C3 is more fibrillogenic than T4 and T4-2EC. Expression of kinase-dead GSK3 β did not affect the formation of thioflavin-S-positive inclusions in all tau species (data not shown).

Expression of constitutively active GSK3 β resulted in the formation of thioflavin-S-positive inclusions in all tau species. Autophagic inhibition with 3-MA also exhibited the formation of thioflavin-S positive inclusions in all tau species. Immunostaining with tau antibody showed that thioflavin-S-positive inclusions were composed of tau. 3-MA treatment further increased GSK3 β -induced thioflavin-S-positive inclusions in all tau species (Fig. 4), suggesting that GSK3 β -mediated phosphorylation and autophagic inhibition facilitate the formation of tau aggregates in a coop-

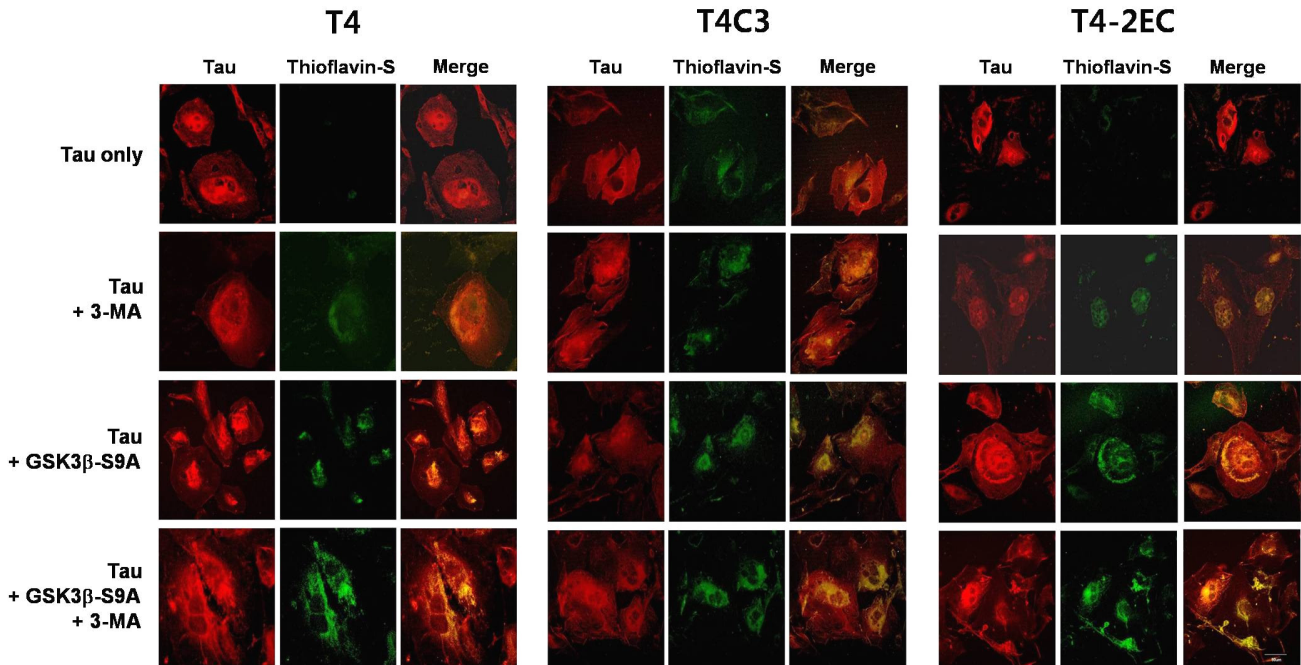


Fig. 4. Active GSK3 β and 3-MA treatment result in the formation of thioflavin-S-positive inclusions of tau in a co-operative manner. Each tau and active GSK3 β or enzyme-inactive GSK3 β constructs were transiently co-transfected into CHO cells on coverslips. One day after transfection, 3-MA (5 mM) or trehalose (200 mM) was added to the medium and incubated for 24 hrs. Cells fixed with 3% PFA were immunostained with an antibody that recognizes total tau (red) and counterstained with thioflavin-S (green). When tau and thioflavin-S staining co-localize, the merged image is yellow/orange. 3-MA treatment or active GSK3 β exhibited thioflavin-S-positive tau inclusion in all tau species. Inhibition of autophagic activity and GSK3 β -induced phosphorylation facilitated aggregate formation of tau in a co-operative manner. The scale bar shows 50 μ m.

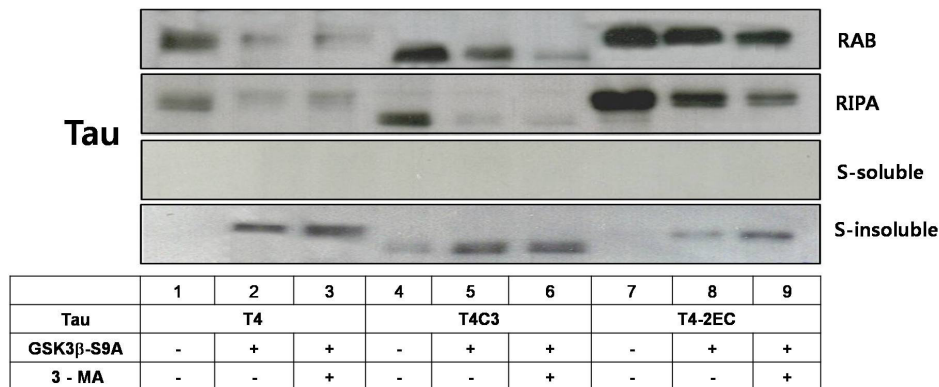


Fig. 5. Co-operative facilitation of sarcosyl-insoluble aggregation with active GSK3 β and 3-MA treatment. Cells were transiently transfected with each tau construct in the presence of constitutively active GSK3 β or kinase-dead GSK3 β . One day after transfection, the cells were treated with 3-MA for 24 hrs. Cell lysates were homogenized and then fractionated into RAB, RIPA, Sarkosyl-soluble, and Sarkosyl-insoluble fractions. After sarcosyl fractionation assay, each fraction was separated by SDS-PAGE and immunoblotted with total tau antibody (5A6). Even in the absence of active GSK3 β , partitioning into the sarcosyl-insoluble fraction was observed in T4C3, an aggregation-prone tau species, whereas not observed in T4 and T4-2EC. In the presence of active GSK3 β , sarcosyl-insoluble aggregates were observed in all tau species and 3-MA treatment resulted in the increased amount of sarcosyl-insoluble aggregates.

erative manner. In addition, expression of kinase-dead GSK3 β exhibited negligible effect on the 3-MA-induced facilitation of thioflavin-S-positive inclusions in all tau species (data not shown), suggesting that the enzyme activity of GSK3 β may be necessary to affect the propensity of inclusion formation of tau.

3-MA potentiates GSK3 β -mediated formation of Sarkosyl-insoluble aggregates

To determine whether thioflavin-S-positive tau inclusions eventually form insoluble aggregates, the presence of tau-positive Sarkosyl-insoluble materials was examined. Tau levels were decreased with active GSK3 β and 3-MA treatment at RAB and RIPA fractions. However, tau-positive Sarkosyl-insoluble materials were observed with active GSK3 β and further increased with concurrent 3-MA treatment (Fig. 5). These results suggest that GSK3 β -mediated phosphorylation and 3-MA-induced inhibition of autophagy shift the intracellular pool of tau toward more fibrillogenic forms, favoring aggregate formation of tau.

DISCUSSION

The present study demonstrates that inhibition of autophagic degradation pathway results in the accumulation and subsequent aggregation of tau and that GSK3 β -mediated phosphorylation of wild type tau significantly interfered autophagic degradation of tau. Intracellular buildup of oligomeric tau species may be the initial step in the aggregation process of tau given that the aggregation of tau may be concentration-dependent [22].

The underlying mechanism of tau degradation is still not clear. It has been reported that tau is not normally degraded by the proteasome given the fact that tau degradation by proteasome requires polyubiquitination of tau but it is difficult to observe polyubiquitinated tau in cells [23]. It has been suggested that autophagy might play a key role in the degradation of protein aggregates in age-related neu-

rodegenerative diseases [24]. Activation of autophagic activity such as proliferation of lysosomes and increased expression of lysosomal hydrolases, was observed during the progression of neurodegenerative disorders [25], suggesting that compensatory activation of autophagy was made to counteract the accumulation of insoluble proteins. In the present study, data showed that autophagic activity regulates turnover of tau. Inhibition of autophagic activity resulted in the accumulation of tau in all tau species whereas activation of autophagy facilitated the clearance of tau species. In accordance with a previous report that autophagic inhibition with chloroquine resulted in the formation of oligomers of wild type tau [25], inhibition of autophagic degradation with 3-MA exhibited the formation of tau oligomers, which are presumed to precede the formation of insoluble aggregates. However, no differential accumulation pattern of oligomers was observed among tau species.

It has been suggested that post-translational modifications of tau such as aberrant phosphorylation contribute to the formation of NFTs. Several studies have provided evidence that phosphorylation of key sites on tau has a strong impact on the normal function of tau and likely contributes to its pathological role including the tendency of aggregate formation [26,27]. It has been also reported that phosphorylation of tau impedes turnover of tau, resulting in the intracellular accumulation of phosphorylated tau [28]. In this study, the phosphorylation of T4 by GSK3 β resulted in significantly increased intracellular level of tau, indicating that phosphorylation of T4 by GSK3 β causes the intracellular accumulation, which is supported by a previous report that phosphorylated tau is less efficiently degraded [28]. Present data demonstrate that extensive phosphorylation of wild type tau by active GSK3 β makes tau less efficient in the autophagic degradation. Given that presence of active GSK3 β does not affect the autophagic degradation of other tau species, which are less efficiently phosphorylated tau forms by GSK3 β , phosphorylation is considered to be the key mediator that interferes autophagic degradation of tau. However, further studies are necessary to understand the mechanism by which phosphorylation of tau

interferes autophagic activity of wild type tau. Although GSK3 β -mediated phosphorylation of wild type tau caused an accumulation of phosphorylated tau including phosphorylation at the PHF-1 epitope, which can form aggregates *in vitro* [29], aggregation of tau was observed *in situ*. These data suggest that phosphorylation may not be sufficient to induce the formation of insoluble aggregates and other factors be necessary to facilitate aggregate formation of full length tau *in situ*. Interestingly, aggregation of T4-2EC was observed in the presence of active GSK3 β , although T4-2EC is not an efficient substrate of GSK3 β . In addition, T4-2EC showed increased sarkosyl-insoluble inclusions with the presence of active GSK3 β . The aggregation of T4-2EC by active GSK3 β might be due to further phosphorylation at other sites by active GSK3 β . However, it is still possible that GSK3 β may phosphorylate other intracellular mediators, which in turn facilitate the aggregate formation of tau, given the numerous roles of GSK3 β in cells [2]. Inhibition of autophagic activity facilitated aggregate formation of T4-2EC.

Caspase cleavage of tau has been reported to facilitate aggregate formation of tau [6,30], given the fact that tau is a substrate for caspase-3 and its cleaved product truncated at Asp421 aggregates more efficiently [30]. The present data demonstrated that T4C3 formed increased sarkosyl insoluble aggregates in the presence of active GSK3 β . Although T4C3 was not efficiently phosphorylated by GSK3 β compared to wild type tau [16], minimally phosphorylated T4C3 by GSK3 β may act as a nucleation seed for the remaining unphosphorylated T4C3. Autophagic activation facilitated the degradation of T4C3 whereas 3-MA-induced inhibition of autophagy resulted in the accumulation of oligomers. However, presence of active GSK3 β does not affect autophagic degradation of T4C3, which is presumably due to less efficient phosphorylation status of T4C3.

Taken together the present study demonstrates that inhibition of autophagic degradation pathway causes the intracellular accumulation and subsequent inclusions of tau. Furthermore, GSK3 β -mediated phosphorylation of wild type tau significantly interferes autophagic degradation of tau. Therefore, the results strongly suggest that compromised autophagic activity might be a key factor in the aggregation of tau, which is posttranslationally modified.

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