Glycogen Synthase Kinase 3β Induces Caspase-cleaved Tau Aggregation in Situ*

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Tau is a substrate of caspasess, and caspase-cleaved tau has been detected in Alzheimer’s disease brain but not in control brain. Furthermore, in vitro studies have revealed that caspase-cleaved tau is more fibrillogenic than full-length tau. Considering these previous findings, the purpose of this study was to determine how the caspase cleavage of tau affected tau function and aggregation in a cell model system. The effects of glycogen synthase kinase 3β (GSK3β), a well established tau kinase, on these processes also were examined. Tau or tau that had been truncated at Asp-421 to mimic caspase cleavage (Tau-D421) was transfected into cells with or without GSK3β, and phosphorylation, microtubule binding, and tau aggregation were examined. Tau-D421 was not as efficiently phosphorylated by GSK3β as full-length tau. Tau-D421 efficiently bound microtubules, and in contrast to the full-length tau, co-expression with GSK3β did not result in a reduction in the ability of Tau-D421 to bind microtubules. In the absence of GSK3β, neither Tau-D421 nor full-length tau formed Sarkosyl-insoluble inclusions. However, in the presence of GSK3β, Tau-D421, but not full-length tau, was present in the Sarkosyl-insoluble fraction and formed thioflavin-S-positive inclusions in the cell. Nonetheless, co-expression of GSK3β and Tau-D421 did not result in an enhancement of cell death. These data suggest that a combination of phosphorylation events and caspase activation contribute to the tau oligomerization process in Alzheimer’s disease, with GSK3β-mediated tau phosphorylation preceding caspase cleavage.

Alzheimer’s disease is a progressive neurodegenerative disorder characterized by neuronal cell loss, extracellular amyloid plaques, and intracellular neurofibrillary tangles. The relationship between these three hallmarks in the progression of Alzheimer’s disease is not entirely clear; however, increased levels of Aβ42, which forms the amyloid plaques, is likely an initiating event (1, 2). Interestingly, treatment of cells with Aβ can result in the activation of caspasess and apoptosis (3–5), and there is evidence for caspase activation in Alzheimer’s disease brain (6–8). Although there is not a general consensus concerning the role of apoptosis in Alzheimer’s disease (9, 10), increased levels of caspase-cleaved proteins are present in Alzheimer’s disease brain (8, 11–13).

In addition to Aβ deposition, the accumulation of polymeric filaments of tau as intracellular neurofibrillary tangles is an essential feature of Alzheimer’s disease brain. As a microtubule-associated protein, tau plays an essential role in maintaining microtubule stability; however, in Alzheimer’s disease brain tau is aberrantly phosphorylated, and this results in an impairment of the normal functions of tau. Intriguingly, neurons from tau knockout mice are resistant to Aβ-induced toxicity, suggesting that tau plays a fundamental role in the pathogenic events that occur in Alzheimer’s disease brain (14). In addition to impairing tau function, the phosphorylation of key sites on tau may also result in an increase in the fibrillogenic properties of tau, which may represent a toxic gain of function (15). Although the protein kinases that phosphorylate tau in vivo have not been unequivocally identified, a protein kinase that is likely to play a key role in regulating the phosphorylation state of tau is glycogen synthase kinase 3β (GSK3β).1 In cell culture models there is clear evidence that tau is a substrate of GSK3β (16, 17), and in mouse models increased expression of GSK3β results in increased tau phosphorylation (18, 19). Furthermore, there is evidence for increased activation of GSK3β in Alzheimer’s disease brain (20–22).

Recent studies demonstrate that caspase-cleaved tau is present in Alzheimer’s disease but not control brain (8, 11). Additionally in in vitro assays, caspase-cleaved tau (i.e. tau that had been truncated at Asp-421) is more fibrillogenic than full-length tau (11). Considering these findings, the focus of the study was on determining how caspase cleavage of tau affects its ability to bind microtubules and aggregate in situ. Furthermore, the modulation of these processes by GSK3β was also examined. In these studies tau with exons 2, 3, and 10 (T4L) and tau without exons 2 and 3 (T4) were truncated at Asp-421 to mimic caspase cleavage (T4L-D421 and T4-D421, respectively). Intriguingly, both T4L-D421 and T4-D421 were not phosphorylated as efficiently by GSK3β as the full-length tau constructs both in situ and in vitro. The full-length and Asp-421-truncated tau constructs interacted with the cytoskeleton and bound microtubules to the same extent. In contrast, co-expression of GSK3β with full-length tau constructs resulted in the expected decrease in tau ability to bind microtubules, whereas the ability of T4L-D421 and T4-D421 to bind microtubules was not affected by the presence of GSK3β. When either full-length or Asp-421-truncated tau was expressed alone, no tau was detected in the Sarkosyl-insoluble fraction. However, when GSK3β was co-expressed with either T4L-D421 or T4-D421, there was a robust increase in the presence of tau in the

1 The abbreviations used are: GSK3β, glycogen synthase kinase 3β; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

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Sarkosyl-insoluble fraction, and the tau in this fraction was phosphorylated. Furthermore, inclusions that were tau and thioflavin-S-positive were detected in cells transfected with GSK3β and T4L-D421 or T4-D421. Even in the presence of GSK3β, full-length tau was not detected in the Sarkosyl-insoluble fraction. In this model system expression of T4L-D421 or T4-D421 tau did not result in an increase in cell death. These data demonstrate for the first time that a combination of phosphorylation events and caspase cleavage results in formation of Sarkosyl-insoluble tau aggregates in an in situ model system.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells were grown in F-12 medium supplemented with 5% fetal bovine serum (Hyclone), 2 mM L-glutamine (In Vitrogen), 10 units/ml penicillin (In Vitrogen), and 100 units/ml streptomycin (In Vitrogen). Cells were used at a confluency of 50–80% for all experiments.

Plasmid Constructs—Preparation of the full-length tau construct containing exons 2, 3, and 10 (T4L) and the tau construct that does not contain exon 2 and 3 (T4) have been described previously (23, 24). To generate the T4L-D421 and T4-D421 constructs, T4 and T4L in pcDNA3.1 (−)/His were amplified as temporal was described using Turbo DNA polymerase (Stratagene, La Jolla, CA) with forward primer 5′-GGC GCC TCC GGC ATG GCT GAG CCC GAG GTC TCC-3′ and reverse primer 5′-CTG CTC TAG AGC ATG AGT CTA CCA TGT CGA TGC TGC GAG TGG-3′ to remove the last 20 amino acids of tau. Amplified fragments were digested with BamHI and XbaI and ligated into the same sites of pcDNA3.1 (+). The integrity of T4L-D421 and T4-D421 was confirmed by sequence analysis. Hemagglutinin-GSK3β-S9A was constructed in pcDNA3.1 (+) as described (23) and is referred to as GSK3β throughout the text.

Transient Transfections—T4L, T4L-D421, T4, or T4-D421 with or without GSK3β was transiently transfected into Chinese hamster ovary cells using FuGene-6 (Roche Applied Science) transfection reagent according to the manufacturer’s protocol. Thirty-three hours after transfection, the cells were washed and collected and processed as described below for the different assays.

Immunoblotting—Cells were collected in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, 0.2 mM sodium vanadate, 0.5% Nonidet P-40 containing 1 mM phenylmethylsulfonyl fluoride, 0.1 mM okadaic acid, and a 10 µg/ml concentration each of aprotinin, leupeptin, and pepstatin. Lysates were sonicated on ice and centrifuged, and protein concentrations in the supernatants were determined using a bicinchoninic acid assay (Pierce). Samples were diluted with 2× SDS stop buffer (2% SDS, 5 mM EDTA, 5 mM EDTA, 25 mM dithiothreitol, 10% glycerol, 0.01% bromphenol blue, and 0.25% Triton X-100) and incubated for 8 min at 37 °C, and the pellet (cytoskeletal-insoluble) and supernatant (soluble) fractions were separated by centrifugation (15 min at 15,000 × g and 25 °C). Samples were diluted with 2× SDS stop buffer and incubated in a boiling water bath for 5 min. Equal amounts of pellet and supernatant fractions were separated on 10% SDS-polyacrylamide gels, blotted, and probed with the Tau5/5A6 antibodies.

Immunocytochemistry—These procedures were modified from previously described protocols (24). The cells were transiently transfected individually with each tau construct in the absence or presence of GSK3β using FuGene-6 (Roche Applied Science) transfection reagent. Forty-eight hours after transfection, the cells were rinsed with PBS and fixed at room temperature for 1 h in fixation buffer (2% paraformaldehyde, 0.2% glutaraldehyde, 1 mM MgCl2, 1 mM EGTA, 30% sucrose, and 1% Sarkosyl (Sigma). The samples were rinsed with PBS and incubated in a boiling water bath for 5 min. All further incubations were carried out at 4 °C. The cells were then washed extensively in PBS before being incubated in a thioflavin S solution (0.005%) (Sigma) for 10 min. The cells were then washed 3 times in 70% ethanol and once in water before mounting (33).

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Instruments), digitally stored, and displayed using the accompanying software.

Caspase Cleavage Assay—Chinese hamster ovary cells were transiently transfected with T4L alone or T4L and GSK3β/H9252 and harvested in lysis buffer followed by incubation at 85 °C for 15 min to inactivate endogenous caspases. Protein concentrations were determined as described above, and 60 μg of total cell lysate was incubated in a final volume of 200 μl in a reaction mixture containing active recombinant caspase-3 (Calbiochem) (200 ng/ml), 20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (w/v) CHAPS, and 10% sucrose, pH 7.2. Incubations were carried out for 0, 5, 30, and 60 min at room temperature, and the reactions were stopped by the addition of 2 μl stop buffer and incubation for 10 min in a boiling water bath. Aliquots from each sample were separated by electrophoresis on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with the Tau5/5A6 antibodies.

Lactate Dehydrogenase Assay—The release of the intracellular enzyme lactate dehydrogenase into the media was used as a quantitative measurement of cell viability. The media and cell lysate samples were collected 48 h after transfection of the tau constructs and/or GSK3β to measure levels of cell viability. The measurement of lactate dehydrogenase was carried out as described previously (34).

RESULTS

Truncation of Tau at Asp-421 Tau Attenuates GSK3β-mediated Phosphorylation—To mimic caspase cleavage, tau constructs truncated at Asp-421 (i.e. the last 20 amino acids were deleted) were made from T4L (plus exons 2, 3, and 10) and T4 (minus exons 2 and 3, plus exon 10) (11) and are referred to as T4L-D421 and T4-D421, respectively. To determine the expression level of all tau constructs was similar, and GSK3β expression levels were also the same in all the transiently transfected cells. Representative immunoblots with Tau5/5A6 (Total tau) showed that overexpression of GSK3β reduced the electrophoretic mobility of T4L and T4 compared with the mobility observed in the absence of GSK3β. A, phosphorylation of full-length tau (T4L or T4) by GSK3β resulted in a robust increase in PHF-1 (Ser-396/404) and AT180 (Thr-231) immunoreactivity compared to when tau was expressed alone. In contrast, co-expression of GSK3β with the Asp-421-truncated tau constructs (T4L-D or T4-D) resulted in minimal increases in PHF1 and AT180 immunoreactivity. The actin blots demonstrate that equal amounts of protein were loaded in each lane. B, expression of GSK3β also resulted in a robust increase in the phosphorylation of full-length tau (T4L or T4), but not the Asp-421-truncated tau constructs (T4L-D or T4-D), at the Tau-1 epitope (Tau-1 recognizes a dephosphorylated epitope so decreased immunoreactivity indicates increased phosphorylation), Thr-205, and Ser-199. 12E8 (Ser-262/356) immunoreactivity was the same for all constructs in the absence (−) or presence (+) of GSK3β. The actin blots shows that same amount of protein was loaded in each lane.

GSK3β Induces Caspase-cleaved Tau Aggregation

![Image of immunoblots and autoradiograph showing caspase-cleaved tau aggregation]

A. Asp-421-truncated tau constructs are not phosphorylated by GSK3β as efficiently as full-length tau constructs. A and B, cells were transiently transfected with T4L, T4L-D421 (T4L-D), T4, or T4-D421 (T4-D) alone or in combination with GSK3β. The expression level of all tau constructs was similar, and GSK3β expression levels were also the same in all the transiently transfected cells. Representative immunoblots with Tau5/5A6 (Total tau) showed that overexpression of GSK3β reduced the electrophoretic mobility of T4L and T4 compared with the mobility observed in the absence of GSK3β. A, phosphorylation of full-length tau (T4L or T4) by GSK3β resulted in a robust increase in PHF-1 (Ser-396/404) and AT180 (Thr-231) immunoreactivity compared to when tau was expressed alone. In contrast, co-expression of GSK3β with the Asp-421-truncated tau constructs (T4L-D or T4-D) resulted in minimal increases in PHF1 and AT180 immunoreactivity. The actin blots demonstrate that equal amounts of protein were loaded in each lane. B, expression of GSK3β also resulted in a robust increase in the phosphorylation of full-length tau (T4L or T4), but not the Asp-421-truncated tau constructs (T4L-D or T4-D), at the Tau-1 epitope (Tau-1 recognizes a dephosphorylated epitope so decreased immunoreactivity indicates increased phosphorylation), Thr-205, and Ser-199. 12E8 (Ser-262/356) immunoreactivity was the same for all constructs in the absence (−) or presence (+) of GSK3β. The actin blots shows that same amount of protein was loaded in each lane. C, representative autoradiograph (of four separate experiments) showing the phosphorylation of recombinant T4L and T4L-D by recombinant GSK3β in an in vitro kinase assay. The tau constructs were incubated with GSK3β for the times indicated. The data show that T4L is phosphorylated by GSK3β much more efficiently than T4L-D.
D421 decreased only slightly in the presence of GSK3β (Fig. 1A, Total tau). The extent of phosphorylation of T4L and T4 constructs by GSK3β at both the PHF1 and AT180 epitopes was substantially greater than that of T4L-D421 or T4-D421 (Fig. 1A). This was not unexpected for the PHF1 epitope (Ser-396/404) (28) because it is in relative close proximity to the truncation site (Asp-421), and, therefore, the ability of GSK3β to phosphorylate the site and/or the ability of the antibody to bind could be affected. However, it was unexpected for the AT180 epitope (Thr-231) (27), which is quite distal from the C terminus. In all cases GSK3β was expressed at similar levels (Fig. 1A). To further analyze the differential phosphorylation of the full-length and Asp-421-truncated tau constructs by GSK3β, immunoblot analyses were carried out with additional phospho-tau antibodies. T4L-D421 and T4-D421 were phosphorylated less efficiently than T4L or T4 by GSK3β at Thr-205 and Ser-199 (Fig. 1B). Reactivity with the 12E8 antibody was the same in the absence or presence of GSK3β for all constructs, which was expected as Ser-262 is not phosphorylated by GSK3β (36) (Fig. 1B).

To further evaluate the phosphorylation of tau and the Asp-421-truncated tau constructs by GSK3β, an in vitro phosphorylation assay was carried out. Recombinant T4L and T4L-D421 were incubated with recombinant GSK3β in the presence of [γ-32P]ATP, and the extent of phosphorylation was analyzed. The representative autoradiograph shown in Fig. 1C clearly demonstrates that in vitro T4L was robustly phosphorylated by GSK3β, whereas under identical conditions T4L-D421 was phosphorylated by GSK3β to a much lesser extent. These data show that removal of the C terminus of tau at D421 diminishes the ability of tau to be phosphorylated by GSK3β.

Phosphorylation of T4L-D421 and T4-D421 by GSK3β Does Not Result in a Decrease in Cytoskeletal Association.—To investigate how truncation of tau at Asp-421 affects the ability of tau to bind microtubules, a microtubule binding assay was used. Supernatants from cells transfected with each tau construct alone or in combination with GSK3β were incubated with taxol-stabilized microtubules, and the amount of tau bound to the microtubules in the pellet and the amount of tau that remained unbound were measured. In the absence of GSK3β all the tau constructs showed equivalent microtubule binding. As expected, phosphorylation of full-length tau (T4L or T4) by GSK3β reduced the affinity of tau for microtubules and increased the amount of tau in the supernatant (unbound fraction) (Fig. 3). Interestingly, incubation of the Asp-421-truncated forms, T4L-D421 or T4-D421, with GSK3β did not result in a similar increase in the amount of tau in the unbound fractions (Fig. 3). These data in conjunction with the results above indicate that the Asp-421-truncated tau is not phosphorylated as efficiently by GSK3β as the full-length forms (especially at the AT180 epitope, which plays a key role in regulating tau interaction with microtubules (23, 24)), and thus, expression of GSK3β does not effectively attenuate the interaction of T4L-D421 and T4-D421 with either the cytoskeleton in general or microtubules in particular.

Fig. 2. GSK3β does not result in an increase in the presence of T4L-D421 or T4-D421 in the soluble fraction. Cells were transiently transfected with the tau constructs alone or in combination with GSK3β. Cell lysates were separated into the insoluble cytoskeletal (I) and soluble (S) fractions and immunoblotted with the phospho-independent tau antibodies Tau5/5A6 (Total tau). In the absence of GSK3β, the majority of tau was found in the insoluble cytoskeletal fraction for all the tau constructs. However, phosphorylation of full-length tau (T4L or T4) by GSK3β resulted in an increase of tau in the soluble fraction. In contrast, phosphorylation of the Asp-421-truncated forms of tau (T4L-D421 or T4-D421) by GSK3β did not result in an alteration of the distribution of tau between the insoluble and soluble fractions. The actin blots are shown as a control for protein loading.

Phosphorylation of T4L-D421 and T4-D421 by GSK3β Does Not Result in a Decrease in Microtubule Binding.—To investigate how truncation of tau at Asp-421 affects the ability of tau to bind microtubules, a microtubule binding assay was used. Supernatants from cells transfected with each tau construct alone or in combination with GSK3β were incubated with taxol-stabilized microtubules, and the amount of tau bound to the microtubules in the pellet and the amount of tau that remained unbound were measured. In the absence of GSK3β all the tau constructs showed equivalent microtubule binding. As expected, phosphorylation of full-length tau (T4L or T4) by GSK3β reduced the affinity of tau for microtubules and increased the amount of tau in the supernatant (unbound fraction) (Fig. 3). Interestingly, incubation of the Asp-421-truncated forms, T4L-D421 or T4-D421, with GSK3β did not result in a similar increase in the amount of tau in the unbound fractions (Fig. 3). These data in conjunction with the results above indicate that the Asp-421-truncated tau is not phosphorylated as efficiently by GSK3β as the full-length forms (especially at the AT180 epitope, which plays a key role in regulating tau interaction with microtubules (23, 24)), and thus, expression of GSK3β does not effectively attenuate the interaction of T4L-D421 and T4-D421 with either the cytoskeleton in general or microtubules in particular.

GSK3β Causes Asp-421-truncated Tau, but Not Full-length Tau, to Form Sarkosyl-insoluble Aggregates.—Given the in vitro data demonstrating that tau truncated at Asp-421 is more fibrillogenic than full-length tau (11), the partitioning of the tau constructs into the Sarkosyl-insoluble fraction was investigated. Lysates were prepared from cells expressing either the tau constructs alone or in combination with GSK3β and then separated into soluble, Sarkosyl-soluble, and Sarkosyl-insoluble fractions (Fig. 4A). In the absence of GSK3β none of the tau constructs localized to the Sarkosyl-insoluble fractions. Intriguingly, co-expression of GSK3β with either T4L-D421 or T4-D421, but not the full-length tau constructs, resulted in the presence of tau in the Sarkosyl-insoluble fractions (Fig. 4A). These data suggest that a combination of phosphorylation and caspase cleavage greatly facilitate tau aggregation. To determine whether the tau that is present in the Sarkosyl-insol-
ducible fractions is phosphorylated, Sarkosyl-insoluble fractions were prepared from cells transfected with GSK3β and T4L or T4L-D421 and probed with antibodies to phospho-independent epitopes (Tau5/5A6) or with antibodies that recognize phospho-Ser-396/404 (PHF1), phospho-Ser-199, phospho-Thr-231, or phospho-Ser-262 (12E8) (Fig. 4B). These data demonstrate that although T4L-D421 is phosphorylated less efficiently than T4L, the T4L-D421 that aggregates is phosphorylated at both the PHF1 and Ser-199 epitope. No 12E8 immunoreactivity was present in the Sarkosyl-insoluble fraction, but this was not unexpected as it has been reported that phosphorylation of Ser-262 inhibits tau polymerization (37). In addition, the presence of phospho-Thr-231 was also not detected in the Sarkosyl-insoluble fractions (Fig. 4B).

**Co-transfection with GSK3β Results in the Formation of Thioflavin-S-positive Inclusions Containing T4L-D421**—To determine whether GSK3β induces aggregation of full-length or Asp-421-truncated tau, cells were transiently transfected with T4L or T4L-D421 constructs in the absence or presence of GSK3β. Cells were immunostained with an antibody that recognizes total tau and counterstained with thioflavin-S. Thioflavin-S binding is a known indicator of insoluble protein aggregates with β-sheet structure (38), and thioflavin-S readily stains the intracellular neurofibrillary tangles in Alzheimer’s disease brain (39). Thioflavin-S-positive inclusions were not observed in cells transfected with either the full-length or Asp-421-truncated tau constructs alone (Fig. 5). Co-expression of GSK3β with the full-length tau constructs also did not result in any detectable thioflavin-S-positive inclusions (Fig. 5). These data suggest that in this model increased phosphorylation alone is not sufficient to induce aggregation. In contrast, co-expression of GSK3β with T4L-D421 resulted in the presence of thioflavin-S-positive inclusions that were also tau-positive (Fig. 5). These results suggest that a combination of phosphorylation events and caspase cleavage facilitate the formation of tau aggregates with a defined structure. Although data for only the T4L and T4L-D421 tau constructs is shown, identical results were obtained with the T4 and T4-D421 constructs (data not shown).

**T4L and T4L Phosphorylated by GSK3β Are Equivalent Substrates of Active Caspase-3**—To determine whether phosphorylation by GSK3β affects the ability of tau to be cleaved by active caspase-3, lysates were prepared from cells that were transfected with T4L or T4L and GSK3β and incubated with active caspase 3. The results of these studies (Fig. 6) demonstrate that phosphorylation by GSK3β has no effect on the ability of tau to be cleaved by caspase 3.

**Asp-421-truncated Tau Does Not Selectively Increase Cell Death**—It had been reported previously that expression of a tau cleavage product generated by caspase-3 (which is equivalent to T4-D421 or T4L-D421 in this study) induced cell death (40). Therefore, the effects of T4-D421 or T4L-D421 alone or in combination with GSK3β (which resulted in the formation of tau aggregates) on cell viability were measured. Cells were transfected with the indicated constructs, and 48 h after trans-
FIG. 5. GSK3β expression results in the formation of thioflavin-S-positive inclusions by T4L-D421. T4L or T4L-D421 was expressed in the absence or presence of GSK3β. Cells were fixed immunostained for total tau (red) and also stained with thioflavin-S (green). When tau and thioflavin-S staining co-localize, the merged image is yellow/orange. No specific thioflavin-S staining was observed in cells transfected with the tau constructs alone or when T4L was transfected with GSK3β. In contrast, co-expression of T4L-D421 and GSK3β resulted in thioflavin-S-positive inclusions that were also immunostained with the tau antibody.

FIG. 6. T4L and T4L phosphorylated by GSK3β are cleaved by active caspase-3 at the same rate and to the same extent. Lysates from cells transfected with either T4L alone or T4L and GSK3β (T4L/GSK3β) were incubated with active caspase-3 for the times indicated and subsequently immunoblotted for total tau levels with Tau5/5A6. These data demonstrate that T4L and phosphorylated T4L are equivalent substrates of caspase-3.

FIG. 7. The Asp-421-truncated tau constructs do not selectively increase cell death. Cells were either not transfected or transiently transfected with the indicated constructs. To normalize the amount of cDNA transfected into the cells, some cells were transfected with the vector (Vec). Forty-eight hours after transfection lactate dehydrogenase (LDH) release was measured as an indicator of cell death and calculated as percent of the total lactate dehydrogenase (media + lysate). These results demonstrate that expression of T4L-D421 (T4L-D4) or T4-D421 (T4-D4) alone does not significantly increase cell death when compared with cells transfected with the full-length tau constructs alone. Co-transfection of 50% with each of the tau constructs resulted in a slight but significant increase in cell death when compared with the result obtained when cells were transfected with each tau construct alone or GSK3β alone. However, there was no selective effect of the Asp-421-truncated tau constructs on cell death. Data are presented as the mean ± S.E. for three separate experiments carried out in triplicate. * p < 0.05 when compared with cells transfected with the tau constructs alone.

Discussion

This study demonstrates for the first time that a combination of phosphorylation events and caspase cleavage results in tau aggregation and partitioning into the Sarkosyl-insoluble fraction. In addition, these data indicate that phosphorylation of tau by GSK3β likely precedes caspase cleavage, because Asp-421-truncated tau is not efficiently phosphorylated by GSK3β and phosphorylation of tau by GSK3β does not inhibit cleavage by caspase-3. It is also important to note that the truncated tau-containing inclusions that form in the cells stain with thioflavin-S, indicating that the aggregates have some ordered structure, which given previous studies is likely filamentous. These are important new findings that add to our understanding of the processes that may contribute to the pathological changes in tau that occur in Alzheimer’s disease brain.

Caspases are a large family of cysteine proteases that cleave after specific Asp residues (DXXD motifs) and play a central role in the apoptotic cascade both upstream (“initiator caspases”) and downstream (“executioner caspases”) in the signaling pathways (41). In addition to their well established role in apoptosis there is increasing evidence that caspases have non-apoptotic functions as well (42). Tau is a caspase substrate and is cleaved at a single site, Asp-421/Ser-422, in the C terminus (43). Initial studies were carried out with caspase-3, which is an executioner caspase (40, 43, 44), but subsequent studies have shown that caspases 1, 6, 7, and 8 also cleave tau in vitro (11). Therefore, tau is a substrate of caspases that act both upstream and downstream in the apoptotic cascade, at least in vitro.

Although the role of apoptosis in Alzheimer’s disease pathogenesis is controversial (4, 9, 10), there is evidence for increased caspase activation in Alzheimer’s disease brain. The increased presence of the active forms of caspases 3, 8, and 9 has been observed in Alzheimer’s brain relative to controls (6–8). In addition, Alzheimer’s disease brains, but not control brains, stained with an antibody that selectively recognizes caspase-cleaved spectrin (fodrin) (12). Of more relevance to the presence study, the presence of caspase-cleaved tau has been detected in Alzheimer’s disease brain using antibodies that specifically recognize tau truncated at Asp-421 (8, 11). Staining with these antibodies revealed that caspase-cleaved tau is localized primarily to the intracellular neurofibrillary tangles and dystrophic neurites. No significant staining was observed in control brain (8, 11). Furthermore, treatment of primary
cortical neurons with Aβ resulted in the rapid cleavage of tau at Asp-421, and in vivo tau truncated at Asp-421 is significantly more fibrillogenic than full-length tau (11). Taken together these studies indicate that cleavage of tau by caspases may be an important event in the pathogenesis of Alzheimer’s disease.

Although caspase cleavage of tau may be a contributing event to the pathogenic events that occur in Alzheimer’s disease, increases in tau phosphorylation are clearly also involved. It is well established that tau from Alzheimer’s disease brain is hyperphosphorylated and functionally impaired (for reviews, see Refs. 16, 17, and 45), that the phosphorylation of specific sites on tau correlates with the severity of the neuropathology, and that increases in tau phosphorylation occurs before the presence of marked neuronal pathology in Alzheimer’s disease brain (46). Therefore, it is important to consider the combined effects of both the caspase cleavage of tau and increases in tau phosphorylation in the pathogenic processes that occur in Alzheimer’s disease brain.

In the present study, both T4L-D421 and T4-D421 were phosphorylated by GSK3β, albeit to a much lesser extent than the full-length tau constructs both in vitro and in situ. In the case of the PHF1 epitope it is not unreasonable to assume that removal of the last 20 amino acids would effect GSK3β-mediated phosphorylation of Ser-396/404 given the close proximity of this epitope to the cleavage site (Asp-421). However, the finding that in general the Asp-421-truncated tau constructs were inefficiently phosphorylated by GSK3β compared with the full-length tau constructs was unexpected. Nonetheless, it is clear that removal of the C-terminal of tau affects the conformation of the molecule (11, 15) and, therefore, could negatively impact the ability of GSK3β to bind and efficiently phosphorylate tau. Given the fact that tau in Alzheimer’s disease brain is more phosphorylated than tau in control brain and phosphorylation by GSK3β does not negatively impact the ability of tau to be cleaved by caspase 3, these findings strongly indicate that in Alzheimer’s disease brain-increased tau phosphorylation may preclude cleavage of tau by caspases.

Interestingly, there were no gross differences in the ability of full-length tau and Asp-421-truncated tau to associate with the cytoskeleton and bind microtubules. However, co-transfection with GSK3β did not attenuate the cytoskeletal association and microtubule binding of T4L-D421 and T4-D421 compared with full-length tau, which showed decreased cytoskeletal and microtubule binding in the presence of GSK3β. This is likely due to the fact that the Asp-421-truncated tau constructs were not as efficiently phosphorylated by GSK3β as the full-length tau constructs, especially at the AT180 epitope, which plays a key role in regulating tau-microtubule interactions (23, 24). These findings also indicate that although removal of the C terminus of tau increases the fibrillogenic nature of tau (11, 15) and perhaps attenuates the ability of GSK3β to bind and phosphorylate tau, this cleavage event does not directly affect the ability of tau to bind microtubules. Given these findings it can be suggested that removal of the last 20 amino acids of tau results in a specific conformational change that does not affect all aspects of tau function.

Although GSK3β did not affect the microtubule binding ability of the T4L-D421 and T4-D421, this protein kinase significantly increased the ability of these constructs to aggregate. Although there was no Sarkosyl-insoluble tau in the cells when T4L-D421 or T4-D421 was expressed alone, co-expression with GSK3β resulted in the pronounced presence of tau in the Sarkosyl-insoluble fraction, and this aggregated tau was thioflavin-S-positive, indicating that it was likely filamentous. In contrast, full-length tau was never observed in the Sarkosyl-insoluble fraction, even in the presence of GSK3β. The finding that GSK3β stimulated the aggregation of the Asp-421-truncated tau constructs was intriguing given the fact that GSK3β did not phosphorylate these constructs as efficiently as the full-length constructs. These data suggest that perhaps only a few phosphorylated truncated tau molecules are needed to “seed” the formation of the aggregates, and indeed the Asp-421-truncated tau that was Sarkosyl-insoluble was phosphorylated at specific epitopes. Alternatively, given the fact that GSK3β phosphorylates many other substrates (47), it could be stimulating the aggregation of the Asp-421-truncated tau indirectly by phosphorylating other targets, which modulate the process of tau polymerization. However, given these findings it can be suggested that if tau were more extensively phosphorylated before caspase cleavage, the aggregation potential may be enhanced to an even greater extent than in the present study.

Previous studies suggest that overexpression of Asp-421-truncated tau constructs increases cell death compared with full-length tau constructs (40, 43). In contrast to these previous reports, we found that Asp-421-truncated tau did not cause an increase in cell death relative to the full-length tau constructs in any situation. The reason for these differences may be the cell lines used or tau expression levels. In addition, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction, trypan blue exclusion (40), or analyses of DNA fragmentation using an ApoTag kit (43) were used to measure cell viability in the previous studies, whereas in the present study lactate dehydrogenase release was used as a measure of cell death. In addition, the ability of Asp-421-truncated tau to potentiate the cell death process may require the input of an additional stressor. Clearly the ability of caspase-cleaved tau to facilitate cell death needs to be investigated further. Nonetheless it is clear that both GSK3β expression and Asp-421 truncation of tau was required for the formation of Sarkosyl-insoluble aggregates. Overall these studies indicate that a combination of phosphorylation and caspase cleavage contribute to the tau pathology in Alzheimer’s disease.

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