Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease

(A microtubule assembly / microtubule-associated proteins / cytoskeletal protein pathology / dephosphorylation / paired helical filaments)

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ABSTRACT The microtubule assembly-promoting activity of different pools of tau protein isolated from Alzheimer disease (AD) and control brains and the effect of dephosphorylation on this activity were studied. Tau isolated from a 2.5% perchloric extract of AD brain had almost the same activity as that obtained from control brain, and this activity did not change significantly on dephosphorylation. Abnormally phosphorylated tau (AD P-tau) isolated from brain homogenate of AD patients had little activity, and upon dephosphorylation with alkaline phosphatase, its activity increased to approximately the same level as the acid-soluble tau. Addition of AD P-tau to a mixture of normal tau and tubulin inhibited microtubule assembly. AD P-tau bound to normal tau but not to tubulin. These studies suggest that the abnormal phosphorylation of tau might be responsible for the breakdown of microtubules in affected neurons in AD not only because the altered protein has little microtubule-promoting activity but also because it interacts with normal tau, making the latter unavailable for promoting the assembly of tubulin into microtubules.

In the brains of patients with Alzheimer disease (AD), the cytoskeleton is progressively disrupted and displaced by the appearance of bundles of paired helical filaments (PHF), which are composed mainly of hyperphosphorylated forms of tau protein (1, 2). Unlike normal tau, which contains two or three phosphate groups, the soluble hyperphosphorylated tau from AD brain (AD P-tau) contains 5–9 mol of phosphate per mol of the protein (3). Levels of tau are severalfold higher in AD than in the age-matched control brains, and this increase is in the form of the abnormally phosphorylated tau (4). Neurons with neurofibrillary tangles of PHF lack microtubules, and microtubule assembly from brain cytosol in the absence of an added polycation, DEAE-dextran, is not observed (5). The reason for this disruption might therefore be some alteration in either tau or other microtubule-associated proteins.

In AD brain, tau can be isolated from different pools: (i) a cytosolic fraction, (ii) abnormally phosphorylated tau that is not polymerized into PHF and sediments at 200,000 × g, and (iii) as a component of PHF. To understand the role of the abnormal phosphorylation of tau in microtubule disruption in AD brain, we studied the ability of the normal cytosolic tau and the AD P-tau to bind to tubulin and to promote microtubule assembly and investigated the effect of alkaline phosphatase treatment of tau on microtubule assembly. The studies described in this paper suggest that the abnormal phosphorylation of tau is a likely cause of the breakdown of the microtubule system in AD because the AD P-tau does not bind to tubulin and inhibits the in vitro assembly of normal tau and tubulin into microtubules. The altered tau inhibits microtubule assembly, probably through its binding to normal tau, making the latter unavailable for interaction with tubulin.

MATERIALS AND METHODS

Tissue Source and Preparation of Brain Cytosol. Six brains from patients with histopathologically confirmed AD diagnosis and, as a control, six Huntington disease brains obtained between 3 and 5 h postmortem were stored frozen at −75°C until used. Cytosol was obtained by centrifugation (100,000 × g for 1 h) of frontal cortex homogenate (1 g/0.5 ml) in microtubule assembly buffer (see below) containing protease inhibitors (3).

Antibodies. Monoclonal antibody (mAb) Tau-1, ascites (6) and antisemur 92e to bovine tau (7) were used at a dilution of 1:25,000 and 1:5000, respectively; mAb DM1-A (Sigma) against tubulin was used at 1:1000 dilution.

Isolation of AD P-Tau and Acid-Soluble Tau. AD P-tau was isolated by the method of Köpke et al. (3). For acid-soluble tau, AD and control brains were homogenized with an Omnimixer at 4°C in 2% (vol/vol) perchloric acid (10 ml/g of tissue) containing protease inhibitors, as described (3). The homogenates were centrifuged at 100,000 × g for 30 min. The supernatant was brought to 2.5% perchloric acid and centrifuged for another 30 min. The supernatant was concentrated to 10-fold by Amicon filtration and dialyzed against 20 mM sodium acetate (pH 5.6). After dialysis, the extract was centrifuged for 10 min at 100,000 × g, and the supernatant was subjected to carboxymethyl chromatography using Millipore MemSep CM 1010 disk. The protein sample (25–40 mg/50 ml) was loaded at a flow rate of 0.5 ml/min, and tau was eluted with 0.25 M NaCl in 20 mM sodium acetate (pH 5.6). The eluate was analyzed by absorbance at 254 nm and immuno-slot blot using antisemur 92e to tau. The tau peak was pooled and dialyzed against 5 mM Mes (pH 6.7) containing 0.05 mM EGTA. Aliquots of ~500 μl, containing 120 μg of protein, were dried in a Speed-Vac concentrator (Savant). For each assay, the lyophilized tau preparation was reconstituted in 1/10th volume of water immediately before use.

Protein Determination, Immunoblots, Radioimmuno-Slot Blot, and Dephosphorylation. Protein concentrations were estimated by the method of Bensadoun and Weinstein (8). Sample preparation and immunoblots were carried out as described (9). The levels of normal and AD P-tau were determined by the radioimmuno-slot-blot method of Khattoon et al. (4). To detect AD P-tau, the blots were pretreated with alkaline phosphatase (86 μg/ml in 0.1 M Tris, pH 8.0/1 mM phenylmethylsulfonyl fluoride) for 15 h prior to immunostaining with mAb Tau-1.

Abbreviations: AD, Alzheimer disease; AD P-tau, abnormally phosphorylated tau; PHF, paired helical filaments; mAb, monoclonal antibody.

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**Isolation of Tubulin.** Rat brain tubulin was isolated through two temperature-dependent cycles of microtubule polymerization–depolymerization (10) followed by phosphocellulose ion-exchange column chromatography (11).

**Microtubule Assembly–Disassembly Assay.** Tau (0.1–1.0 mg/ml) was mixed at 4°C with purified rat brain tubulin (2 mg/ml) and 1 mM GTP, all in polymerization buffer (100 mM Mes, pH 6.7/1 mM EGTA/1 mM MgCl2). Tau was added last to initiate the reaction. After rapid mixing, the samples were pipetted into quartz microcuvettes and equilibrated at 37°C in a thermostatically controlled Cary 1 recording spectrophotometer. Solution turbidity was continuously monitored at 350 nm. Steady-state values were determined by measuring the total OD change after a turbidity plateau was reached. For the disassembly assay, after the steady state was reached, the reaction mixture was cooled to 6°C, and the turbidity was monitored. The state of assembly of microtubules was confirmed by negative-stain electron microscopy (12).

**In vitro dephosphorylation of tau was carried out by using the following conditions: acid-soluble tau from AD and control brains and AD P-tau from AD brains were reconstituted with water to a final protein concentration of 0.1–0.2 mg/ml and then dialyzed against 0.1 M Tris (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride.** The dialyzed samples were treated with alkaline phosphatase (500 units/ml) and a mixture of protease inhibitors (10 μM leupeptin/0.31 μM aprotinin/1.46 μM pepstatin) at 37°C for 15 h. After this incubation, samples were dialyzed against 5 mM Mes (pH 6.7) containing 0.05 mM EGTA, boiled for 5 min to inactivate the alkaline phosphatase, and then centrifuged at 15,000 × g for 10 min. The phosphatase control samples were treated identically, except that alkaline phosphatase was omitted and the samples were kept at 4°C. Unless otherwise stated, all steps were carried out at 4°C.

**Dot Overlay Assay.** AD P-tau interaction was carried out as described by Kremer et al. (13). Different amounts (0.5, 1, 2, and 3 μg) of AD P-tau were dotted on nitrocellulose paper and overlaid with either normal human tau (8 μg/ml) or tubulin (10 μg/ml). All the incubations, blocking, and washing were done as described (13). Tau was detected by using Tau-1 antibody, whereas DM1-A antibody was used for tubulin.

**RESULTS**

**Microtubule Assembly–Promoting Activity of 2.5% Perchloric Acid-Soluble Tau from AD and Control Brains Is Similar.** Acid-soluble tau was isolated from six AD and six control brains. No AD P-tau was detected, and the pattern of tau isotypes was very similar in all the preparations (Fig. 1), although the yield of tau from AD brains was ~30% lower than that from the control brains (AD brains, 0.20 ± 0.004 mg/g of tissue; control brains, 0.259 ± 0.002 mg/g of tissue). The total protein composition of the tau preparations was also very similar (data not shown). The microtubule assembly-promoting activity of AD acid-soluble tau was not significantly different from that of control tau, as determined by the amount of microtubules formed at steady state and the rates of assembly and disassembly (Table 1). Furthermore, no ultrastructural differences, either in length or appearance, were detected between the microtubules obtained with the two tau preparations (Fig. 2 a and b).

**Dephosphorylation Increases the Microtubule Assembly–Promoting Activity of AD P-Tau but not That of AD Acid-Soluble Tau. In vitro** phosphorylation of tau diminishes its ability to promote the assembly of tubulin into microtubules (14). Experiments were performed to determine whether dephosphorylation of AD acid-soluble tau and AD P-tau affects this property. The amount of AD P-tau in the acid-soluble preparations was undetectable, as judged by the increase of Tau-1 immunoreactivity after dephosphorylation on Western blots (Fig. 1) and by immuno-slot-blot assay (data not shown). In contrast, the AD P-tau was labeled intensely with Tau-1 on immunoblots treated with alkaline phosphatase and was hardly detectable before dephosphorylation (Fig. 1). The dephosphorylation treatment had no effect on the microtubule assembly-promoting activity of AD acid-soluble tau, whereas it markedly increased the activity of AD P-tau, bringing it to approximately the same level as that obtained with the acid-soluble tau (Fig. 3). Before alkaline phosphatase treatment, only an occasional microtubule could be seen by electron microscopy (Fig. 2c). After the alkaline phosphatase treatment, many microtubules with no ultrastructural differences from those formed with AD acid-soluble tau were observed (Fig. 2d).

**AD Cytosolic Fraction Is Able to Promote Microtubule Assembly.** The effect of dephosphorylation of tau on microtubule assembly was also studied in brain cytosol. The concentration of normal tau in AD brain cytosols was ~65% of the corresponding value in the control cytosols (Table 2). Because it is well known that tubulin in frozen tissue loses its ability to polymerize, we added fresh rat brain tubulin to a cytosolic fraction of either AD or control brain and assayed polymerization of tubulin; the concentration of tau in both...
cytosols was adjusted to the same level by dilution with the buffer. A high background resulting from the use of cytosol did not allow a reliable measure of turbidity changes, and, therefore, the polymerization of tubulin was measured by immunoassaying the amount of the cold-disassembled protein following the assembly at 37°C for 20 min. The cytosolic fraction of AD brain was effective in promoting microtubule assembly, although this activity was ≈40% less than that of the control cytosolic fraction, as judged by the amount of tubulin in the cold-disassembled fraction obtained after the incubation (Table 2).

Dephosphorylation with alkaline phosphatase treatment dramatically increased the microtubule assembly-promoting activity of AD cytosols, but this increase was negligible in control cases (Table 2). The microtubules obtained with both preparations were of similar length and appearance (not shown).

**AD P-Tau Inhibits Microtubule Assembly and Binds to Normal Tau.** Because even after adjusting the normal tau levels to control values the AD brain cytosol was significantly less (i.e., 40%) active than the corresponding control fraction in promoting assembly of microtubules (Table 2), we investigated further if and how AD P-tau inhibited the activity of normal tau. Different concentrations of AD P-tau were added to normal tau before it was mixed with tubulin, and the assembly was determined as described above. AD P-tau inhibited microtubule assembly, and this inhibition was al-

![Fig. 2](image1.png)

**Fig. 2.** Electron micrographs showing the products of microtubule assembly negatively stained with phosphotungstic acid. Microtubule assembly was carried out from rat brain tubulin by the addition of control acid-soluble tau (a), AD acid-soluble tau (b), AD P-tau (c), or AD P-tau after dephosphorylation (d). Aliquots of each sample were taken at steady state of polymerization. Only an occasional microtubule was seen with tubulin alone (not shown) and with AD P-tau (c), and large numbers of microtubules were observed in all of the other situations above (a, b, and d). No ultrastructural differences could be seen among microtubules assembled with tubulin and normal control tau, AD cytosolic tau, or dephosphorylated AD P-tau.

![Fig. 3](image2.png)

**Fig. 3.** Effect of alkaline phosphatase treatment on AD acid-soluble and AD P-tau microtubule assembly-promoting activity. The microtubule assembly-promoting activity of AD P-tau (b) but not of AD acid-soluble tau (a) was increased after the alkaline phosphatase treatment (curve 1, after treatment; curve 2, before treatment).

Table 2. Tau levels and the effect of dephosphorylation on the microtubule-promoting activity of AD and control (Ct) brain cytosols

<table>
<thead>
<tr>
<th>Cytosol</th>
<th>N tau, (^\text{cpm/µg})</th>
<th>Assemb., (^\text{cpm} )</th>
<th>Inc. Assemb., (^%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>1910 ± 458§</td>
<td>77 ± 53(^\dagger)</td>
<td>3800 ± 1300(|)</td>
</tr>
<tr>
<td>Ct</td>
<td>2923 ± 649§</td>
<td>1.8 ± 7.5(|)</td>
<td>6330 ± 1134(|)</td>
</tr>
</tbody>
</table>

*The concentrations of normal tau (N tau) and abnormally phosphorylated tau (P-tau) were determined by radioimmunoassay (4). The values of normal tau are expressed as the amount of radioactivity bound (cpm) per µg of cytosol protein; P-tau values are expressed as a percentage of normal tau.

§, \(P < 0.01\); \(\dagger, P < 0.004\); \(\|, P < 0.006\) using the nonparametric Mann-Whitney U Test.

![Fig. 4](image3.png)

**Fig. 4.** Effect of AD P-tau on microtubule assembly. Polymerization of tubulin was determined as described in Materials and Methods, except that a mixture of normal tau and AD P-tau was used. The assembly reaction was carried out using normal tau at 0.1 mg/ml mixed with AD P-tau at 0.1 mg/ml (curve 4) or 0.2 mg/ml (curve 5). For comparison, normal tau was used in different amounts, 0.1 mg/ml (curve 3), 0.2 mg/ml (curve 2), and 0.3 mg/ml (curve 1). AD P-tau inhibited the microtubule assembly-promoting activity of normal tau (compare curves 2 and 3 with curve 4 and compare curves 1 and 3 with curve 5).
FIG. 5. Interaction of AD P-tau with normal tau and tubulin. AD P-tau was dotted on nitrocellulose strips and overlaid with tubulin (■) or normal tau (○). The nitrocellulose strips were developed either with anti-tubulin antibody DM1A (●) or with Tau-1 antibody (●). (Inset) Binding of tubulin to normal tau (N. tau). The amount of tubulin or tau bound is expressed as the relative amount of radioactivity from the radioimmunoprecipitation. Normal tau bound to AD P-tau (●), and tubulin bound to normal tau (Inset) but had only background binding to AD P-tau (■).

most total when the concentration of AD P-tau was 2 times that of normal tau (Fig. 4).

To study if the inhibition observed with AD P-tau was caused by its interaction with tubulin or normal tau, protein-binding studies were carried out by an overlay dot assay. AD P-tau was dotted on a nitrocellulose paper and overlaid either with tubulin or normal tau, followed by an incubation with anti-tubulin antibody or Tau-1 antibody. In the case of the strip overlaid with tubulin, there was no detectable binding, whereas there was a considerable binding of normal human tau to AD P-tau (Fig. 5). In these assays, tubulin was bound to normal tau when it was dotted (Fig. 5 Inset), and no binding was observed when bovine serum albumin was used as a negative control (data not shown). These studies suggest that AD P-tau inhibits the microtubule assembly, probably through its interaction with normal tau and not with tubulin.

Levels of Sedimentable Tau Correlate with Levels of AD P-Tau. Previously, we have shown that some of the tau that is nonphosphorylated at the Tau-1 epitope sediments at 200,000 × g and that the levels of this sedimentable tau are markedly higher in AD than in control brains (3). In light of our findings in the present study on the binding of AD P-tau to normal tau, we investigated whether the 27,000–200,000 × g pellet where the soluble AD P-tau sediments also contain proportionally higher levels of the nonhyperphosphorylated tau. We determined the levels of the nonhyperphosphorylated tau and AD P-tau in the 27,000–200,000 × g fraction and tau in 200,000 × g supernatants from four AD brains. We also carried out the above studies on four control brains. The levels of the nonhyperphosphorylated tau showed a direct correlation with the levels of AD P-tau in the 27,000–200,000 × g fraction, whereas the levels of tau in the 200,000 × g supernatant had an inverse correlation. Therefore, the ratio of the sedimentable nonhyperphosphorylated tau to tau in the supernatant directly and strongly correlated with the amount of AD P-tau in the 27,000–200,000 × g fraction (Fig. 6). The control brains did not contain any detectable levels of the abnormally phosphorylated tau and had only background levels of tau in the 27,000–200,000 × g fraction.

These findings are consistent with the studies in the previous section showing that AD P-tau binds to normal tau.

FIG. 6. Relationship of the ratio of sedimentable nonhyperphosphorylated tau/supernatant tau (s.nP-tau/sup.tau) to the levels of AD P-tau. The levels of tau were determined in the 200,000 × g supernatant (sup.tau) and 27,000–200,000 × g pellet (s.nP-tau plus AD P-tau) from brain homogenates of four AD (●) and four control (○) patients by radioimmuno-slot-blot assay or with or without alkaline phosphatase treatment. AD P-tau was calculated from the increase in immunoreactivity after dephosphorylation (see Materials and Methods). The AD P-tau values are expressed as cpm of radioactivity bound per microgram of protein; ratios of sedimentable nonhyperphosphorylated tau to supernatant tau were obtained from the means of triplicate assays of these pools of tau determined at two different concentrations. The levels of the nonhyperphosphorylated tau correlate directly with the levels of AD P-tau in the 27,000–200,000 × g fraction (Spearman R = 0.824; P < 0.012), and levels of supernatant tau in the 200,000 × g supernatant correlate inversely with AD P-tau (Spearman R = 0.748; P < 0.032). The ratio of sedimentable nonhyperphosphorylated tau to supernatant tau shows a highly significant (Spearman R = 0.913; P < 0.002) direct correlation with the AD P-tau levels.

DISCUSSION

One of the most characteristic brain lesions of AD is the formation of PHF of abnormally phosphorylated tau in the affected neurons. In these neurons with neurofibrillar tangles of PHF, the cytoskeleton is markedly disrupted, and microtubules are rarely seen (15). Previously, we have shown that tubulin is assembly competent, and yet there is a deficiency in the assembly of microtubules from brain cytosol of AD patients. We have linked this lack of microtubule assembly to the abnormal phosphorylation of tau and its polymerization into PHF (5). In the present study, we have investigated a cause and a mechanism of this breakdown of the microtubule system. We have found that (i) the abnormally phosphorylated tau is functionally inactive in binding to tubulin and stimulating the assembly of microtubules, (ii) the microtubule assembly-promoting activity of the abnormal tau is restored by dephosphorylation, (iii) levels of normal functional tau in brain cytosol of AD patients are >50% lower than those in non-AD control patients, (iv) the abnormally phosphorylated tau inhibits tau-promoted assembly of tubulin into microtubules, and (v) the abnormal tau binds to normal tau and not to tubulin, suggesting that it inhibits the assembly by interacting with normal tau.

In AD brains, the levels of tau are severalfold higher than in age-matched control brains, and this increase is in the form of the abnormally phosphorylated protein (4). However, in the present study, when tau was isolated from AD brains with 2.5% HClO4 extraction, only normally phosphorylated tau was obtained. AD P-tau is probably denatured by 2.5% HClO4 treatment and is not extracted. This finding is in agreement with our previous observations (3). Although the yield of the acid-soluble tau from AD brains was ~70% of that of the control brains, the microtubule-promoting activity of this tau was not significantly different from that of control
tau, both in the total amount of microtubules formed and the rates of assembly and disassembly.

On the other hand, AD P-tau isolated from AD brains showed minimal, if any, microtubule-promoting activity. When this tau was dephosphorylated, the activity increased to approximately the same level as occurs with the acid-soluble tau. These results suggest that the abnormal phosphorylation of tau diminishes its microtubule-promoting activity, which can be recovered after dephosphorylation. Dephosphorylation of AD brain cytosol with alkaline phosphatase led to an increase in the microtubule-promoting activity, suggesting that AD P-tau in the extract also could be reactivated by dephosphorylation. However, in a brain cytosolic extract, tau is not the only protein that can promote microtubule assembly; microtubule-associated protein 2 might also be present, and its activity is also modulated by its degree of phosphorylation. Thus, the increase in microtubule assembly obtained with the dephosphorylated cytosol cannot rule out the involvement of proteins in addition to tau. Recovery of tau activity by dephosphorylation was also obtained with PHF tau by Iqbal et al. (16) and Bramblett et al. (17); the latter study, however, employed the binding of tau to taxol-stabilized microtubules and not the microtubule-assembly-promoting activity.

Because AD P-tau has minimal microtubule-promoting activity, this protein did not contribute to the assembly-promoting activity found when AD cytosolic extracts were used, although AD P-tau is present there in considerable amounts. Furthermore, we suspected that the altered protein could be inhibiting the assembly because the levels of microtubules formed with AD extracts were lower than those formed with control extracts. The putative inhibitory effect of AD P-tau was confirmed in a system of purified tubulin and normal tau in which AD P-tau inhibited the tau-promoted assembly of tubulin. This inhibitory effect of AD P-tau might be the reason for the low level of polymerization found with AD cytosolic extract.

We studied the mechanism by which AD P-tau might be inhibiting the microtubule assembly, testing the interactions between AD P-tau, normal tau, and tubulin. We found that AD P-tau was able to bind normal tau but not tubulin. These results indicate that the inhibition of microtubule assembly might be caused by an interaction of AD P-tau with normal tau in the putative system. It is also possible that the inhibition seen in the assembly with the AD cytosolic extracts is the result of an interaction of AD P-tau with normal tau. This possibility is supported by the findings of Iqbal et al. (5), who were able to see polymerization of tubulin in AD extracts when they replaced tau with DEAE-dextran, showing that in AD brains tubulin is not compromised and is able to polymerize.

It has been reported that normal tau is able to aggregate (18, 19). Whether tau self-associates in the presence of tubulin is not known, but if this is the case, our findings suggest that this association does not interfere with the ability to promote microtubule assembly, because increments in the amount of tau produce increments in the assembly of tubulin. It is possible that the abnormal phosphorylation of tau increases the affinity for normal tau, competing with tubulin for tau and making the latter unable to promote microtubule assembly.

Other findings that support the possibility of an interaction of AD P-tau with normal tau was the amount of the latter protein in the 27,000–200,000 χ g pellets. It was reported that in AD brains nonhyperphosphorylated tau is found in the 27,000–200,000 χ g pellet (3). In the present study, we found that the amount of the tau found in those pellets correlated with the amount of sedimented AD P-tau and with a reduction of normal tau in the 200,000 χ g supernatants. This result supported the finding that AD P-tau could be interacting with normal tau, making it sedimentable. However, the composition of this sediment is unknown, and we cannot rule out the possibility that tau in this fraction is aggregated with other proteins or membranous components.

In conclusion, the present study suggests that the abnormal phosphorylation of tau probably causes microtubule disruption by decreasing the levels of functional tau in two ways: (i) directly, by diminishing its microtubule-promoting activity and (ii) indirectly, by binding to normal tau and making it unavailable for promoting microtubule assembly. Dephosphorylation restores this tau functional deficit. A disruption of the microtubule system may lead to a breakdown of axoplasmic transport and consequently a retrograde degeneration of affected neurons. It appears that avoiding the hyperphosphorylation of tau can result in the prevention of microtubule disruption in neurons with neurofibrillary degeneration in AD.

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