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Microtubule-associated protein tau is a substrate of ATP/Mg^{2+}-dependent proteasome protease system

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Summary. Deposition of hyperphosphorylated microtubule-associated protein tau is a recognized pathological process in Alzheimer's disease (AD) brain, however, the mechanism leading to tau accumulation is still not understood. In the present study, we found that different forms of tau, including phosphorylated tau (PHF-1) and non-phosphorylated tau (Tau-1) as well as total tau (Tau-5) in rat brain cortex extract, were degraded when it was co-incubated with ATP and $MgCl_2$ at 33°C *in vitro*, and non-phosphorylated tau at Tau-1 epitope was more accessible to the ATP/Mg^{2+}-depended proteolysis. With the increase of ATP and $MgCl_2$ concentration from 5 mM to 20 mM, increased degradation of tau was observed. ATP/Mg^{2+}-induced degradation of tau was blocked by lactacystin, a specific proteasome inhibitor and was enhanced by sodium dodecyl sulphate (SDS), a commonly used *in vitro* proteasome activator, and poly-ubiquitinated tau with high molecular weight was detected in the presence of lactacystin. Hyperphosphorylated tau isolated from AD brain (AD p-tau) was also partially degraded when it was incubated with rat brain cortex extract in the present of ATP/Mg^{2+}, and the degradation of AD p-tau was also enhanced by SDS and was inhibited by lactacystin. This study has demonstrated that tau, both phosphorylated and non-phosphorylated, is a substrate of ATP/Mg^{2+}-depended proteasome. To our knowledge, this is the first report providing direct evidence that tau is degraded by 26S proteasome in an ubiquitin- and ATP-dependent manner.

Keywords: Alzheimer's disease, tau, ubiquitin, ATP, proteasome.

Introduction

Alzheimer's disease (AD) is the most common form of senile dementia. Two typical histological hallmarks of AD are extracellular senile plaques, composed of amyloid beta peptide (Aβ), and intracellular neurofibrillary tangles, composed of abnormally hyperphosphorylated tau (AD P-tau). Tau is a microtubule-associated

protein, which promotes microtubule assembly from tubulin subunits and stabilizes the microtubules under normal conditions. In AD brain, tau becomes abnormally hyperphosphorylated and the abnormally hyperphosphorylated tau is polymerized into paired helical filaments (PHF). The recent discovery of mutations in tau gene in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) provides unequivocal evidence that dysfunction of tau alone may be sufficient to cause neurodegeneration and dementia (Hutton et al., 1998; Goedert et al., 2000).

It is reported previously that ubiquitin is a component of paired helical filament in AD brain (Mori et al., 1987; Grundke-Iqbal et al., 1988), paired helical filament contains ubiquitinated tau (Morishima-Kawashima et al., 1993), and the level of ubiquitin in CSF and brain of AD patients is significantly higher than that of non-neurological and neurological controls (Kudo et al., 1994). With formation of the neurofibrillary tangle, tau in paired helical filaments is increasingly ubiquitinated (Iqbal et al., 1998). Recent *in vitro* studies show that phosphorylation of tau is the signal for tau ubiquitination (Shimura et al., 2004).

Ubiquitin is a highly conserved protein composed of 76-amino acid residues and participates in proteasome-mediated proteolysis. By a relay reaction involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase family (E3), ubiquitin conjugates to the substrates. Branched multi-ubiquitin chains are formed by sequential addition of mono-ubiquitin to the lysine residue of the substrate-bound ubiquitin. The multi-ubiquitin-chain serves as a targeting signal recognized by 26S proteasome, then the ubiquitinated proteins are degraded by 26S proteasome in an ATP-dependent manner. The 26S proteasome is a multimeric complex assembled from two subcomplexes, the two ATPase-containing 19S regulatory particles and the 20S proteasome, which constitutes its proteolytic core. 20S proteasome degrades only poorly folded or unfolded proteins in an ubiquitin- and ATP-independent manner in eukaryocytes, whereas 26S ubiquitin-proteasomal system generally participates in degrading damaged and folded proteins (Goldberg, 2003).

Why ubiquitin is co-deposited with neurofibrillary tangles in AD brain and why tau is ubiquitinated? Can ubiquitinated tau be degraded by the ubiquitin-proteasomal system? In the present study, we try to understand this matter by incubating the brain cortex extract, which contains all the components of the ubiquitin/proteasome system, with ATP/Mg^{2+} in the presence or absence of specific activator or inhibitor of the protease. We have found that tau is a substrate of ATP-depended proteasomal protease.

Materials and methods

Antibodies and chemicals

Monoclonal antibodies (mAb) Tau-5 (1:200), which reacts with total tau, and polyclonal Ubiquitin Ab-1 (1:100) were obtained from Lab Vision Corporation (Fremont, CA, USA). mAb PHF-1 (1:500) against tau phosphorylated at Ser396/404, and Tau-1 (1:30000) against tau not phosphorylated at Ser199/202 were gifts from Dr. Davies (Albert Einstein College of Medicine, Bronx, NY, USA) and Dr. Binder (Northwestern University, Chicago, Illinois, USA), respectively. 111e (1:5000) to total tau and hyperphosphorylated tau isolated from AD brain (AD P-tau) were

gifts from Drs. Iqbal and Grundke-Iqbal (Alonso et al., 1995). Immunopure immobilized protein G, goat anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibodies, and chemiluminescent substrate kit were obtained from Pierce Chemical Company (Rockford, IL, USA). Adenosine 5′-triphosphate disodium salt (ATP disodium salt) and lactacystin were purchased from Sigma (St. Louis, MO, USA).

Preparation of rat brain cortex extract

Sprague-Dawley rats (Grade II, weight 100-150 g) were supplied by Experimental Animal Central of Tongji Medical College. Rat brain cortex was isolated and homogenized at 4°C using a Teflon glass homogenizer in 20 vol of buffer containing 50 mM Tris-HCl, pH 7.4, 0.8 M NaCl, 3 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1.0 mM phenylmethyl sulfonyl fluoride (PMSF), 2 μg/ml aprotinin, and 1 μg/ml pepstatin. The homogenates were centrifuged at 10,000 g for 15 min at 4°C, and the resulting supernatants were the sources of endogenous tau and the enzymes of ubiquitin-proteosomal system. The concentration of protein in the extracts was measured by BCA Protein Assay kit according to manufacture's instruction (Pierce, Rockford, IL, USA) (Bennecib et al., 2000), and equal mounts of protein were analyzed by Western blot.

Degradation of endogenous tau

The reaction was carried out in a total volume of 50 μl of the brain extract at 33°C without or with 2.5 mM, 5 mM, 10 mM, or 20 mM ATP and $MgCl_2$. After 3 h of incubation, the reaction was terminated by addition of one-third volume of sample buffer containing 200 mM Tris-HCl, pH 7.6, 8% SDS, 40% glycerol, 40 mM DTT, 0.025% bromophenol blue and boiled for 5 min. When needed, proteasome activity was inhibited by addition of lactacystin or parallel degradation assays were performed in the presence of 0.01%, 0.05%, 0.1% SDS, a treatment known to activate 20S proteasome activity *in vitro* (Akopian et al., 1997). Degradation of AD P-tau was carried out by addition of 1.5 μg AD P-tau into 30 μl of the brain cortex extract, and incubated with ATP/Mg^{2+} alone or with ATP/Mg^{2+} plus lactacystin, or ATP/Mg^{2+} plus 0.1% SDS at 33°C for 3 h. Final protein of 24 μg brain cortex extract and 0.3 μg AD P-tau (1/5 of the total volume) were used for Western blot.

Immunoprecipitation

For immunoprecipitation, 50 μl of brain cortex extract (about 200 μg total proteins) was incubated with 20 mM ATP/$MgCl_2$ and 10 μM lactacystin at 33°C for 3 h. Then ubiquitinated tau was immunoprecipitated with primary Tau-5 antibody at 4°C overnight. The brain cortex extract without the above treatments was used as a control. After overnight incubation with Tau-5 antibody, protein G agarose was added to each sample and incubated at room temperature for 2 h. The immunoprecipitates were collected by centrifugation and washed four times with the washing buffer (50 mM Tris-HCl, pH 7.4, 0.8 M NaCl, 3 mM EGTA). The agarose beads were resuspended in 30 μl of sample buffer containing 50 mM Tris-HCl, pH 7.6, 2% SDS, 10% glycerol, 10 mM DTT, 0.00625% bromophenol blue and boiled for 5 min to release the proteins. After 3 min of centrifugation, the supernatants were separated and analyzed by immunoblotting with various antibodies.

Western blot

The degradation of tau was determined by Western blot as described previously (Bennecib et al., 2000). Briefly, the samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electronically transferred to nitrocellulose membrane. Blots were probed with various primary antibodies as shown in figure legends, developed with peroxidase-conjugated anti-mouse or anti-rabbit second antibodies, visualized by enhanced chemiluminescent substrate kit and exposed to CL-XPosure film (Pierce, Rockford, IL, USA).

Results

ATP/Mg^{2+} stimulates tau proteolysis in extract of rat brain cerebral cortex

To detect the effect of proteasome on tau degradation, we first incubated the rat brain cerebral cortex extract with Mg^{2+} alone or ATP/Mg^{2+} at 33°C for up to 3 h according to the characteristic ATP/Mg^{2+} dependence of the two major subtype proteasomes (Akopian et al., 1997). The activity of calpain in the crude extract was excluded by depletion of calcium in the extract with 3 mM of EGTA, and the non-specific lysosomal proteolysis was eliminated by protease inhibitors PMSF, pepstin and aprotinin as well as by the control brain extract incubated at 33°C in the absence of ATP/Mg^{2+}. It was observed that in the presence of both ATP and Mg^{2+}, the immunoreactivity of full length tau (detected by Tau-5 recognizing total tau) was diminished, and at the same time, the low molecular weight degradation fragments of tau were increased in a concentration-dependent manner of ATP/Mg^{2+}. The most significant degradation of tau was observed in the presence of 20 mM of ATP/Mg^{2+}, and no significant degradation of tau was observed when brain cortex extract was incubated with $MgCl_2$ alone (Fig. 1A)

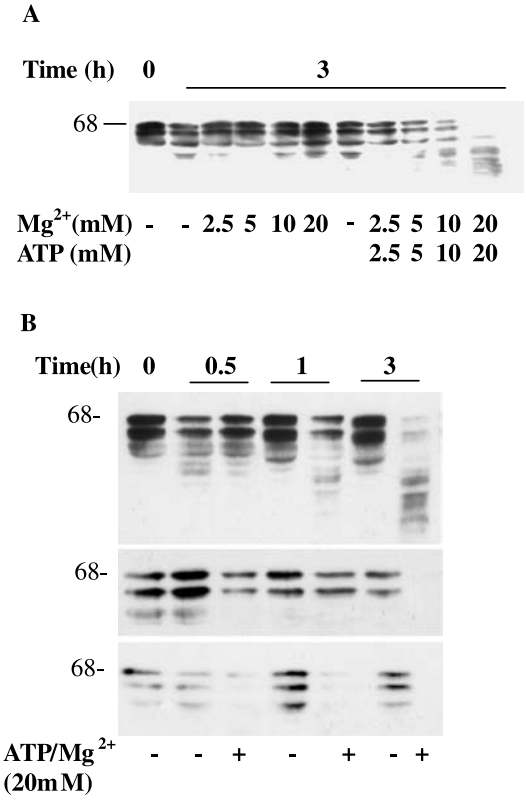

Fig. 1. ATP/Mg^{2+} stimulates endogenous tau proteolysis in the rat brain cortex extract. The brain extract was incubated with $MgCl_2$ alone or $MgCl_2$ and ATP at 33°C for different time period as indicated. The degradation of tau was detected by Western blot with total tau antibody Tau-5 (**A** and **B**, upper panel), or phosphorylation-dependent tau antibodies PHF-1 (**B**, middle panel) and Tau-1 (**B**, lower panel)

or ATP alone (data not shown). Thus, in the following experiments, ATP and Mg^{2+} were administrated together at the same concentration. When the concentration of ATP/Mg^{2+} was raised to 80 mM, no tau staining was detected (data not shown). By using 20 mM of ATP/Mg^{2+}, we further detected the degradation of Ser-396/404-phosphorylated tau (PHF-1) and Ser-199/202-non-phosphorylated tau (Tau-1) in the presence of 20 nM calyculin A, an inhibitor of protein phosphatase (PP)-2A and PP-1. It was found that the Tau-1 immunoreactivity was almost fully blanked at half an hour of ATP/Mg^{2+} incubation. A time-depended degradation of PHF-1 epitope phosphorylated-tau was also observed, and the immunoreactivity was vanished at 3 h after the incubation (Fig. 1B). These results suggest that an ATP/Mg^{2+}-depended protease effectively degrades both

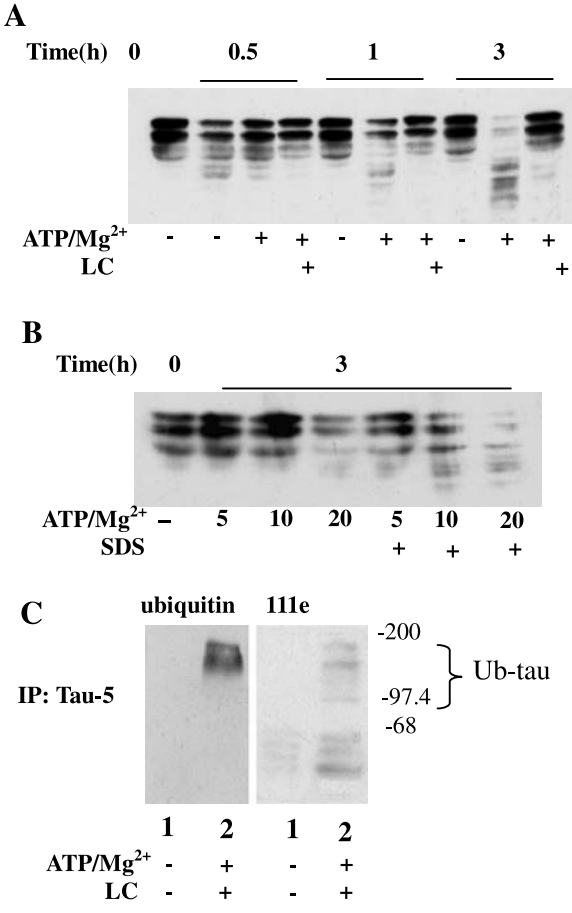

Fig. 2. Effect of lactacystin (LC) and SDS on endogenous tau degradation. **A** Brain extract was incubated with 20 mM ATP/Mg^{2+} in the presence or absence of 10 μM LC at 33°C for time point as indicated and degradation of tau was detected by Western blot with Tau-5. **B** Brain extract was incubated with ATP/Mg^{2+} alone or with ATP/Mg^{2+} plus 0.1% SDS at 33°C for 3 h, and degradation of tau was detected by Western blot with Tau-5. **C** Brain extract was incubated in the presence or absence of 20 mM ATP/Mg^{2+} alone or with 20 mM ATP/Mg^{2+} plus 10 μM lactacystin at 33°C for 3 h. Tau was immunoprecipitated with Tau-5 and the blots were probed with Ubiquitin Ab-1 and 111e

phosphorylated and non-phosphorylated tau and non-phosphorylated tau is more accessible to the proteolytic system.

Effect of lactacystin and SDS on ATP/Mg^{2+}-stimulated tau degradation

To confirm the effect of proteasome on ATP/Mg^{2+}-induced tau degradation, we incubated the brain extract with 20 mM ATP/Mg^{2+} in the presence or absence of 10 μM lactacystin, a specific inhibitor of proteasome (Dick et al., 1997), at 33°C for various time periods. It was found that in the presence of 20 mM ATP/Mg^{2+}, a time depended increased degradation of tau was observed, and addition of 10 μM lactacystin completely blocked the ATP/Mg^{2+}-stimulated tau degradation (Fig. 2A). Then, we investigated whether treatment of 0.01%, 0.05%, 0.1% SDS (w/v), a routinely utilized *in vitro* proteasome activator (David et al., 2002), accelerated the degradation of tau. We incubated the brain extract with ATP/Mg^{2+} alone or with ATP/Mg^{2+} and SDS at 33°C for 3 h. More degraded fragments were formed by the treatment of 0.1% SDS than those of ATP/Mg^{2+} alone treated controls (Fig. 2B). We next tested whether tau was ubiquitinated in the process of degradation. Brain cortex extract was incubated with 20 mM ATP/Mg^{2+} and 10 μM lactacystin at 33°C for 3 h. Then tau was immunoprecipitated with primary Tau-5 antibody. The precipitates were then probed with Ubiquitin Ab-1 and 111e. We observed high molecular weight polyubiquitinated tau co-stained by 111e and Ubiquitin Ab-1 (Fig. 2C). These data further confirms that it is proteasome responsible for the ATP/Mg^{2+}-induced tau degradation.

Effect of ATP/Mg^{2+}-dependent proteasome on degradation of AD P-tau

To investigate further if AD P-tau isolated from AD brain is degraded by the proteasome, we incubated 1.5 μg of AD P-tau with brain extract in the presence of 20 mM ATP/Mg^{2+} alone, or ATP/Mg^{2+} plus 0.1% of SDS or ATP/Mg^{2+} plus 10 μM or 20 μM lactacystin at 33°C for 3 h and tau degradation was

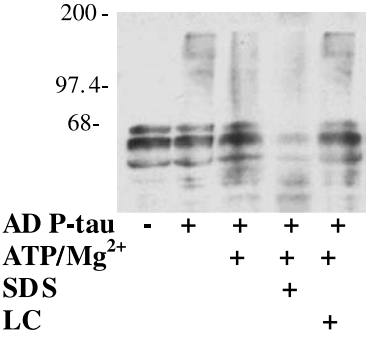

Fig. 3. Effect of ATP/Mg^{2+} and SDS on degradation of AD P-tau. AD P-tau was added to the rat brain extract, then incubated with ATP/Mg^{2+} alone, or ATP/Mg^{2+} plus 0.1% SDS, ATP/Mg^{2+} plus 20 μM lactacystin (LC) at 33°C for 3 h as indicated. Degradation of AD P-tau was detected by Western blot with Tau-5

detected by Western blot with Tau-5. In the absence of ATP/Mg^{2+}, we detected smeared staining bands of AD P-tau in a molecular weight ranged from 97 kDa to 200 kDa. AD P-tau was readily degraded after incubated with ATP/Mg^{2+} at 33°C for 3 h. The addition of 0.1% SDS accelerated and of 20 μM lactacystin fully blocked the degradation of AD P-tau, respectively (Fig. 3).

Discussion

Abnormal accumulation of microtubule associated protein tau is a recognized pathological process in AD brain. However, the underlying mechanism of tau deposition is still not quite understood. Increasing evidence suggests that in addition to hyperphosphorylation, a deficit in proteolytic system may also contribute to tau aggregation (Goldbaum et al., 2003). Ubiquitin-proteasomal system is a major proteolytic system in cleaning out unfolded or damaged proteins in an ATP-depended manner (Goldberg, 2003). Does this system also work on tau? To answer this question, we employed proteasomal system dependent reagents ATP/Mg^{2+}, and lactacystin (a specific activator of proteasome, Dick et al., 1997) as well as 0.1% SDS (the *in vitro* activator of proteasome, David et al., 2002) to co-incubate with crude brain extract, which contains a complete set of ubiquitin-proteasomal system. The proteolytic effect of calpain, a calcium-depended protease, was eliminated by depleting calcium in the reaction. We found by using this system that ATP/Mg^{2+}-dependent proteasomal system degraded not only endogenous normal rat brain tau, including Tau-1 epitope non-phosphorylated tau and PHF-1 sites phosphorylated tau, but also AD P-tau *in vitro*, and the high molecular weight polyubiquitinated tau was detected in the presence of specific proteasome inhibitor. Was tau degraded by 20S proteasome or by 26S proteasome? According to the previous report that 26S proteasome is an ATP/Mg^{2+}-dependent protease, whereas 20S proteasome degrades the substrate in an ATP-independent manner and it is activated by Mg^{2+} alone (Akopian et al., 1997), our results suggest that tau is degraded by 26S but not 20S proteasome. It was also reported that 20S proteasome degraded purified recombinant unfolded tau; however, this ubiquitin- and ATP-independent tau degradation was blocked by SDS-induced conformational change of tau (David et al., 2002). The results presented in this study demonstrated for the first time that the native rat brain tau was degraded by 26S proteasome, which suggested that the conformation of native rat brain tau might be more accessible to be degraded by 26S proteasome than by 20S proteasome. Taken together these data and the previous related reports, we propose that a deficit in ATP-dependent proteasomal system may contribute to tau aggregation and neurofibrillary tangle formation in AD brain.

Ubiquitinated tau was found to be associated with neurofibrillary tangles in AD brain (Morishima-Kawashima et al., 1993). Then, why is this tau not degraded by proteasome during development of AD? It was reported previously that deficits in proteasome activity were observed in AD brains (Keller et al., 2000). The significantly decreased proteasome activity was mainly localized in regions showing severe degenerative alteration in AD brain (Braak et al., 1991), such as in the hippocampus and parahippocampal gyrus, superior and middle temporal gyri, and inferior parietal lobule. Recent *in vitro* studies have

suggested that proteasome dysfunction may result from the inhibitory binding of PHF-tau to proteasome, because the proteasome activity in AD brain is strongly correlated with the amount of co-precipitated PHF-tau, and incubation of proteasome with AD brain isolated PHF-tau or *in vitro* assembled PHF-tau results in inhibition of proteasomal activity (Keck et al., 2003). Overproduction of Aβ may also damage the proteasome system, because co-culture of astrocytes and neurons with $A\beta_{1-42}$ and $A\beta_{25-35}$ results in inhibition of 26S proteasome activity (Lopez Salon et al., 2003). Aβ itself can bind to the catalytic core of 20S proteasome and inhibit its activity (Gregori et al., 1995). These results strongly suggest that a vicious circle between damage of proteasome system and tau hyperphosphorylation/Aβ deposition must occur in AD brain, that is tau hyperphosphorylation and Aβ overproduction inactivate proteasome activity, and the decreased proteasome activity cause an inefficient tau and Aβ degradation and thus formation of neurofibrillary tangles and senile plaques.

Deficit in ubiquitination process of tau protein may also contribute to its insufficient proteolysis. It is reported that polyubiquitin-tau complex is in the minority of the ubiquitinated tau in neurofibrillary tangles, whereas monoubiquitinated form of tau seems to predominate in AD brain (Morishima-Kawashima et al., 1993). And the monoubiquitinated form of proteins do not constitute degradation signal for the 26S proteasome (Thrower et al., 2000). Additionally, the gene mutation in ubiquitin may also contribute to its altered conjugating properties towards the target substrates (Varshavsky et al., 1997), and to the inhibition of proteasome activity (Lam et al., 2000). For instance, mutated ubiquitin-B^{+1} (UBB^{+1}) was detected in the most vulnerable brain regions of AD patients, such as pyramidal neurons in CA1 region of the hippocampus and neurons in the subiculum and cortex (Braak et al., 1991; van Leeuwen et al., 1998a, b).

In summary, we have reported here that tau and AD P-tau are degraded by ATP-dependent proteasome *in vitro*. Further study will provide new insight into the degradation of tau by the ATP-dependent proteasome *in vivo*.

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