

Differential sensitivity to proteolysis by brain calpain of adult human tau, fetal human tau and PHF-tau

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Received 28 March 1995; revised version received 18 May 1995

Abstract Reduced turn-over of tau by calpains is a possible mechanism to facilitate the incorporation into paired helical filaments (PHFs) in Alzheimer's disease. The present study shows that the differently phosphorylated fetal tau isoforms are all rapidly proteolysed to an equal extent by human brain m-calpain. This result argues against the hypothesis that this type of fetal phosphorylation is involved in reducing tau turn-over by calpain in Alzheimer's disease. Adult and fetal tau fragments *in vitro* generated by m-calpain, but not trypsin, cathepsin D or chymotrypsin resemble the post-mortem *in situ* degradation patterns, suggesting a possible role for calpains in tau metabolism *in vivo*. Tau incorporated into PHFs was considerably more resistant to proteolysis by calpain which can help to explain the persistence of these structures in Alzheimer's disease.

Key words: Alzheimer; tau; Calpain; Cathepsin D; Paired helical filament; Microtubule; Neurofibrillary tangle; Phosphorylation

1. Introduction

Dysregulation of Ca²⁺ homeostasis is considered a possibly important factor in neuronal degeneration but little is known about the precise mechanisms involved. The pathway may include dysregulation of proteolysis by the calpain system. Abnormally high levels of activated isoforms of μ -calpain [1] and reduced endogenous calpain inhibitor (calpastatin) [2,3] levels have been measured in the brains of patients with Alzheimer disease. Intracellular neurofibrillary tangles are a neuropathological hallmark in Alzheimer's disease and the presence of these lesions shows a high correlation with the development of clinical symptoms of dementia [4,5]. Major progress is made in the biochemical characterization of the paired helical filaments (PHFs), the ultrastructural components of the neurofibrillary degeneration. It is now generally accepted that the major protein component in these structures is the microtubule-associated protein tau in a state of hyperphosphorylation (for review see [6–9]). Antibodies produced against phosphorylation-dependent epitopes on PHF-tau [10–12] and mass spectroscopy on isolated peptides [13,14] have shown that fetal and PHF tau isoforms share many phosphorylated sites. Previous studies showed that tau is a substrate for the Ca²⁺-dependent neutral

protease [15] and the sensitivity to calpain of differently phosphorylated tau isoforms has been shown to vary [16].

In this study we investigated the hypothesis that fetal type phosphorylation reduces the turn-over rate of tau by calpain and might, therefore, represent a mechanism for the accumulation of tau in PHFs. We also studied the degradation pattern generated *in vitro* by m-calpain from fetal and adult tau isoforms in comparison to proteolytic tau fragments present in post-mortem brain in an effort to provide more evidence for the possible involvement of calpains in tau metabolism. Finally, we examined the differential sensitivity to calpains in mixtures of fetal and adult tau with PHF-tau.

2. Materials and methods

2.1. Protein preparations

The affinity-purified human tau and the polyclonal anti-tau antiserum (JM) was prepared as described elsewhere [17], except that the tau-1 monoclonal antibody was used for the preparation of the affinity column. Heat-stable fetal (22 weeks of gestation) brain extract (0.15 g/ml) for use in the calpain assay was prepared in Tris/saline, pH 7.4, containing 1% Triton X-100 and 1 mM EGTA. For the preparation of enriched PHF-tau fractions, cerebral cortex from post-mortem Alzheimer brain was homogenized (2 g/20 ml) in 10 mM Tris, pH 7.4, 0.8 M NaCl, 1 mM EGTA, 10% sucrose and centrifuged at 27,000 $\times g$. The supernatant was incubated for 2 h at 37°C with addition of 1% mercaptoethanol and 1% *N*-lauroylsarcosine or 1% Triton X-100 and centrifuged at 100,000 $\times g$ for 120 min. The pellet was then rinsed and resuspended in Tris/saline, pH 7.4. Human m-calpain was purified from postmortem cerebral cortex by a procedure involving chromatography on DEAE-cellulose, phenyl-Sepharose, Ultrogel AcA-44, and DEAE-Biogel A as described [18]. Human post-mortem normal and AD brains were provided by the McLean Hospital Brain Tissue Center.

2.2. Electrophoresis and blotting

All aliquots were run on 10% SDS-PAGE minigels and blotted to nitrocellulose. Tau proteins were detected using the polyclonal rabbit anti-tau antiserum (JM) at a dilution of 1/2000. Densitometric analysis was performed using an Color OneScanner (Apple) and Scan Analysis Software.

2.3. Protease assays

For proteolysis with calpain, trypsin and chymotrypsin, the tau preparations were incubated at 30°C in a buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 mM DTT and 5 mM CaCl₂. Cathepsin D was used in 0.1 M NaAc, 1 M NaCl at pH 3.8. The calpain/tau molar ratio was 1:10 when heat-stable tau extracts were examined or tau and PHF-tau degradation were compared and 1:100 when only purified adult tau was used. Cathepsin D was used at a tau/enzyme ratio of 1:20 and trypsin and chymotrypsin at a ratio of 1:100. Proteolysis was stopped by adding an equal volume of 2 $\times$ SDS-Laemmli Sample buffer [19] to aliquots of the reaction mixture removed taken at the different time points and boiling for 5 min.

Cathepsin D (human liver) was purchased from Athens Research and Technology Inc., Athens, GA. Trypsin (bovine pancreas) and chymotrypsin (bovine pancreas, Type VII) were purchased from Sigma, St. Louis, MO.

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Abbreviations: PHF, paired helical filament; MAP, microtubule associated protein; AD, Alzheimer disease.

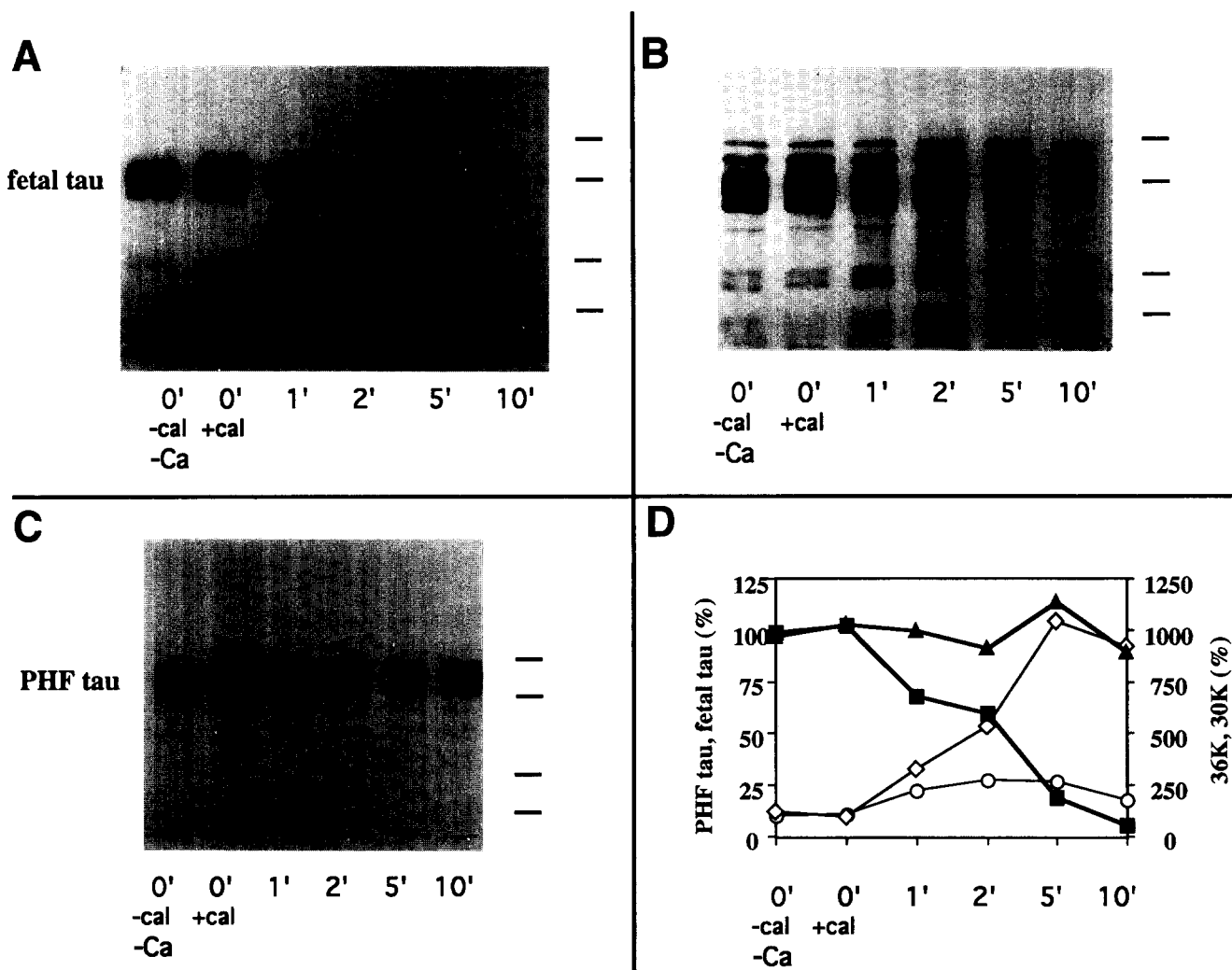


Fig. 1. Tau immunoblot analysis showing the different sensitivity of fetal tau isoforms and PHF-tau to degradation by m-calpain. (A) Heat-stable fetal brain extract. (B) Mixture of heat-stable fetal brain extract and PHFs. (C) PHFs. The indications for molecular weight markers are 64 kDa, 50 kDa, 36 kDa and 30 kDa. Panel D shows the densitometric analyses of tau immunoblots of mixtures of heat-stable fetal brain extract and PHF as shown in C. Intact proteins (fetal tau (■), PHF-tau (▲)) and breakdown products (30 kDa (◇), 36 kDa (○)) were plotted as a percentage of their starting concentration (fetal tau and PHF-tau values on the left y-axis; 30 kDa and 36 kDa values on the right y-axis). The data in the graph represent the mean values obtained from duplicate experiments. Notice the decrease for fetal tau to 5% of its starting value and an increase to over 1000% for the 30 kDa breakdown products.

3. Results

Tau isoforms in heat-stable extracts from fetal brain at 22 weeks of gestation were rapidly degraded by m-calpain (Fig. 1A). The polyclonal antibody that was used for immunodetection of tau reacts with tau isoforms independent of their state of phosphorylation and shows how the multiple tau bands, representing different phosphorylation states of the single tau isoform expressed in fetal brain, were hydrolysed by m-calpain at a similar rate. Several major degradation products generated by m-calpain could be identified, especially at short incubation times (Fig. 1A). The proteolytic fragments, which included those with apparent molecular weights of approximately 30 and 36 kDa, were already present at lower concentrations in the heat-treated extracts before the addition of calpain or Ca^{2+} . Because these heat-treated brain extracts were prepared in the

presence of EGTA sufficient to block endogenous Ca^{2+} -mediated proteolysis during the preparation of the sample, the results indicate that calpain is also active on tau in situ. We next examined the activity of m-calpain toward tau isoforms in a mixture containing an enriched PHF preparation and heat-treated fetal brain extract (Fig. 1B). At a calpain concentration resulting in a rapid degradation of fetal tau isoforms, the PHF-tau in the mixture (Fig. 1B) or by itself (Fig. 1C) was almost completely protected from degradation. To rule out the possibility that *N*-lauroylsarcosine was responsible for protecting PHF from degradation, we also used PHFs that were prepared with Triton X-100 and found the same results. Triton X-100 was also present in the heat-stable extracts and did not inhibit the degradation of fetal tau. Densitometric analysis of the tau band intensities, expressed as percentages of the initial concentrations of tau in untreated extracts, demonstrates that the

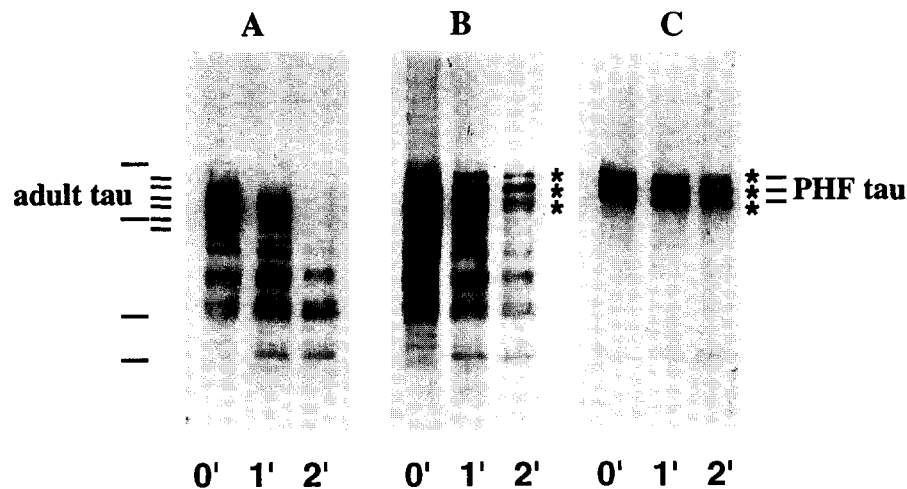


Fig. 2. Tau immunoblot analysis showing the different sensitivity of adult tau isoforms and PHF-tau to degradation by m-calpain. (A) Adult tau. (B) Mixture of adult tau and PHFs. (C) PHFs. The indications for molecular weight markers are 64 kDa, 50 kDa, 36 kDa and 30 kDa.

30 kDa and 36 kDa tau breakdown products were increased 10 and 2.5 times, respectively, after 5 min incubation (Fig. 1D). In the same experiment the values for full-size fetal tau were decreased to 5% of their original density, while the values for the PHF-tau bands were unchanged.

A similar m-calpain experiment performed on a mixture of PHFs and purified adult human tau is shown in Fig. 2. Compared to soluble adult tau, the PHFs were considerably more resistant to proteolysis by calpains as observed in the earlier experiments. Several breakdown product, generated by m-calpain from adult tau, including bands at 30K and 36K, were similar in size to those detected before calcium was added to the incubation mixture.

In Fig. 3 the pattern of human adult tau degradation generated by m-calpain was compared to that of other common proteases to investigate the specificity of the calpain in generating fragments pre-existing in post-mortem brain samples. As shown in Fig. 3B and 3C, respectively, purified tau was rapidly degraded by trypsin and chymotrypsin but the complex mixture of breakdown products generated by each protease did not include persisting limited proteolytic fragments such as those seen after calpain digestion. Cathepsin D generated two major tau degradation bands (Fig. 3D) but these differed from the tau breakdown products generated by calpain and the ones present in unincubated tau preparations.

4. Discussion

Our results indicate that fetal-type phosphorylation is not a significant factor in reducing the sensitivity of tau to proteolysis by human brain m-calpain. Previous reports [16] have shown that in vitro phosphorylation of bovine tau by cAMP-dependent protein kinase increased its resistant to calpain-mediated degradation. The same group recently also reported that phosphorylation of the longest human tau isoform by the proline-directed kinase ERK1 had no effect [20]. Many of the phosphorylation sites identified in PHF are proline-directed sites and were also shown to be phosphorylated, to a certain extent, in fetal tau [21]. However, several phosphorylated positions iden-

tified in PHF-tau are not flanked by a proline and have not been detected in fetal tau [13,22]. Taken together, the data indicate that decreased susceptibility of hyperphosphorylated tau to calpain may only result from site specific phosphorylation that is possibly not proline-directed and distinct from the phosphate groups present on tau in fetal brain. Among the possible candidate kinases for this process are the cAMP-dependent protein kinase, shown to render calpain resistance to tau in vitro [16] and glycogen synthase kinase β , which can phosphorylate tau in vitro at sites not flanked by a proline [23] and to an extent that makes it shift in SDS to the PHF-tau position [24–26]. Another interesting candidate is a 35/41 kDa brain kinase, described by Biernat et al. [27] that phosphorylates tau in a non-proline-directed way and strongly affects its binding to microtubules.

The low efficiency of calpain in degrading PHF's is not surprising in view of the reported high resistance of these structures to proteases [28] and phosphatases [29] and most likely reflects inaccessibility to the enzymatic activity due to steric hindrance. Alternatively other modifications of PHF-tau, such as Maillard-reaction-related events may also confer protease resistance [30].

The calpains have been suggested as an efficient system to regulate the turn-over of cytoskeletal proteins [31,32] and the microtubule associated proteins MAP1A [33], MAP2 [34] and tau [15] are all good in vitro substrates. Our results show a remarkable similarity between the tau breakdown products generated with purified human brain m-calpain in vitro and the tau degradation products present in post-mortem brain. The post-mortem calpain-like tau degradation products detected in the first lane in Figs. 2A and 3A for adult tau and in the first lane of Fig. 1A for fetal tau are indeed generated in situ since all endogenous calpain activity was inactivated during the preparation of the samples by heat treatment and by the addition of 1 mM EGTA. Although this may indicate that some calpain is activated during the postmortem interval, this event likely reflects a similar function in vivo.

Altered calcium uptake was observed in vitro in fibroblasts isolated from aged individuals and Alzheimer patients [35] and

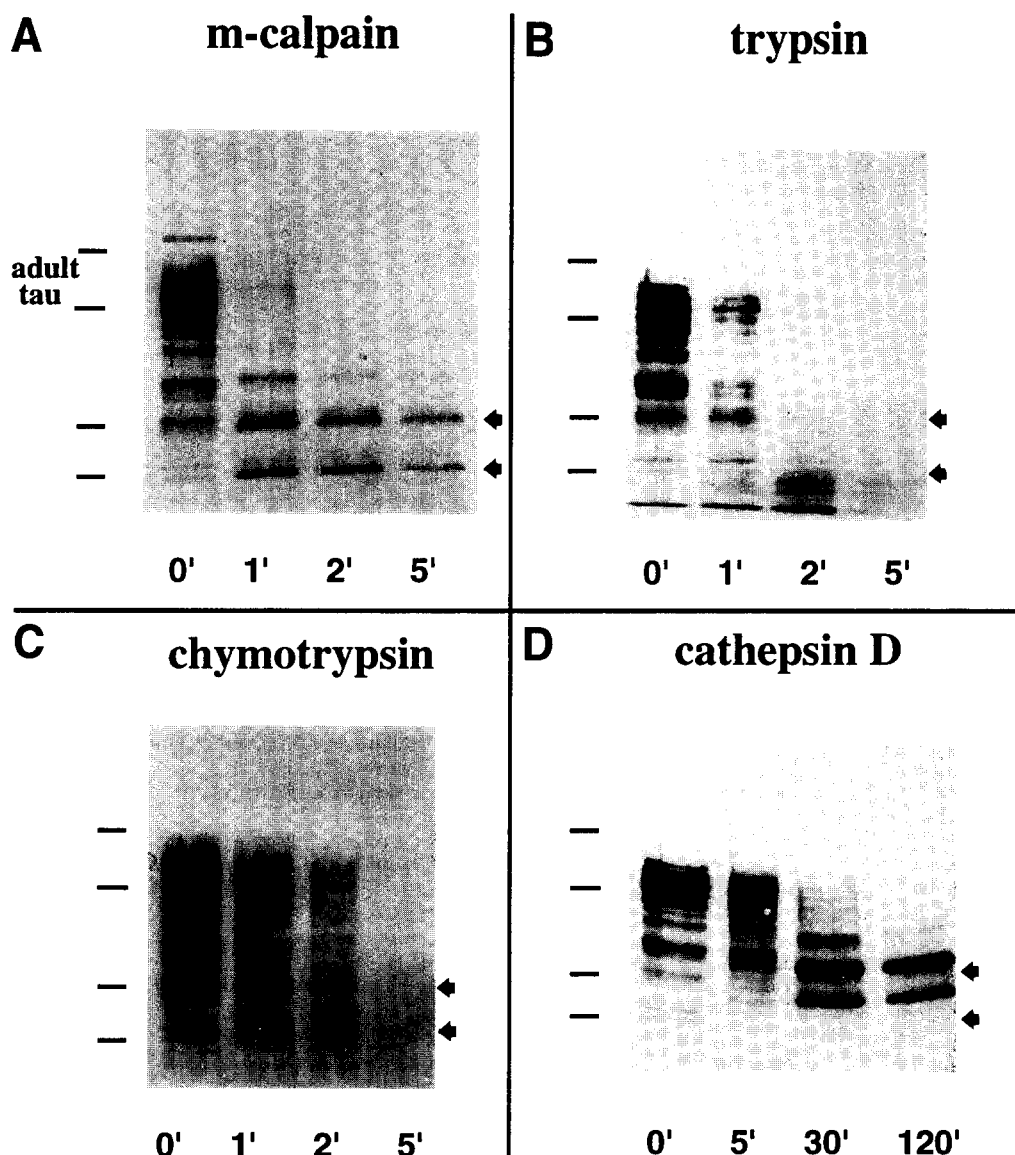


Fig. 3. Immunoblot showing the degradation of purified adult tau by different proteolytic enzymes. (A) m-calpain. (B) Trypsin. (C) Chymotrypsin. (D) Cathepsin D. The indications for molecular weight markers are 64 kDa, 50 kDa, 36 kDa and 30 kDa. Arrowheads in lane 4 of every blot indicate the corresponding positions for tau breakdown generated by m-calpain as shown in panel A.

in vivo postmitotic neurons are likely to show the highest susceptibility to an abnormal Ca^{2+} metabolism. Increased levels of intracellular Ca^{2+} will induce increased proteolysis by chronic calpain activation. Neurons may try to counteract the abnormally high turn-over of the cytoskeleton by altering its phosphorylation state. It is reasonable to expect that some of these phosphorylation events, apparently distinct from those generating the different fetal tau isoforms, could result in an unbalanced decreased turn-over of tau by calpains [36] and also reduce the microtubule binding of tau [12,28,37,38].

A recent report by Matsuo et al. [39] has forced a reevaluation of the phosphorylation state of normal tau by demonstrating that biopsy-derived adult human tau is phosphorylated at most sites thought to be abnormally phosphorylated in PHF-tau. However, the authors also mention several observations that confirm the existence of differences in the phosphorylation state of normal brain tau and PHF-tau. They find a considera-

ble quantitative difference in the degree of phosphorylation between PHF-tau and biopsy-derived brain tau and also describe the selective specificity of the monoclonal antibody AT10 (which binds to an unknown phosphorylation-dependent site on PHF-tau [40]) for PHF-tau in the absence of cross-reactivity with any of the normal biopsy- or autopsy-derived adult or fetal brain tau preparations. Considerable differences in concentrations of highly phosphorylated tau is therefore still most likely the mechanism that may favor precipitation into highly protease resistant PHFs, which then can accumulate to intolerably high levels and cause neuronal dysfunction.

Acknowledgements: We are grateful to Dr. Lester Binder for generously providing the tau-1 antibody and to the McLean Hospital Brain Tissue Center, which is supported in part by PHS Grant MH/NS 31862, for providing human post-mortem normal and AD brains. These studies were supported by NIA Grant AG05604 and NIH Grant NS24725.

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