Effect of Calpain on The Degradation of Tau in Rat Brain Cortex Extracts*

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Abstract Calpain is a calcium-activated protease and there are two ubiquitously distributed mammalian calpains, namely calpain 1 (μ-calpain and CAPN1) and calpain 2 (m-calpain and CAPN2). Calpains regulate the function of many proteins by limited proteolysis. To determine the nature of different subtypes of calpain on degradation of microtubule-associated protein tau, the rat brain cortex extracts were incubated with 0.2 mmol/L, 1 mmol/L, 3 mmol/L and 5 mmol/L of $CaCl_2$ for 15 min at 37℃. The findings were that Ca^{2+} treatment at concentration 1 ~ 5 mmol/L led to significant proteolysis of tau protein and this degradation was blocked by calpain inhibitor, calpeptin. In addition, when the extracts containing 1 mmol/L $CaCl_2$ were treated with μ-calpain inhibitor (0.05 μmol/L of calpastatin) or m-calpain inhibitor (100 μmol/L calpain inhibitor Ⅳ) or both, the Ca^{2+}-induced degradation of tau protein was decreased to 8.6%, 92.5% and 97.8%, respectively. These data suggest that both μ-calpain and m-calpain in brain cortex extracts are activated by Ca^{2+} and both of them degrade tau protein, although, m-calpain plays a more important role in proteolysis of tau.

Key words calpain, tau, calcium, Alzheimer's disease

Alzheimer's disease (AD), the most common cause of dementias, is pathologically characterized by the deposition of amyloid-β peptides (Aβ) as senile plaques (SP) and by the occurrence of neurofibrillary tangles (NFTs) composed primarily of arrays of paired helical filaments (PHF). PHF are anomalous structures generated by self-aggregation of hyperphosphorylated forms of tau protein[1,2]. Tau belongs to the family of microtubule-associated proteins (MAPs) with the functions of modulating microtubule assembly, dynamic behavior and spatial organization. However, the phosphorylation alters the ability of tau to interact with tubulin and affects microtubule polymerization and stabilization. Although the molecular factors involved in aggregation of hyperphosphorylated tau are unknown, proteases have long been considered to be critical to this process.

Calpain, the first documented calcium-activated protease in mammalian cells, was reported over 30 years ago[3]. It was subsequently demonstrated that there are two ubiquitously distributed mammalian calpains: calpain 1 (μ-calpain) and calpain 2 (m-calpain)[4]. Calpains regulate the function of various proteins by limited proteolysis. In particular, they modulate processes that govern the function and metabolism of proteins key to the pathogenesis of AD, and calpain activated by calcium can degrade tau in PC12 cell[5]. However, the effect of calpain on the degradation of tau protein in rat brain cortex extracts, especially the nature of different subtypes of calpain in tau degradation is still unknown at the moment.

In the present study, the particular question was studied by co-incubating rat brain cortex extracts with Ca^{2+} as well as by using the inhibitors of μ- and m-type of calpains.

1 Materials and methods

1.1 Antibodies and chemicals

Mouse monoclonal antibody tau Ab-2 against total tau was purchased from NeoMarkers (Fremont CA, USA). Bicinchoninic acid (BCA) protein detection kit and 5-bromo-4-chloro-3-indole phosphate (BCIP) / nitrobluetetrazolium (NBT) kit were obtained from Pierce Chemical Company (Rockford, IL, USA). Goat anti-mouse alkaline phosphatase-conjugated secondary antibodies and other chemicals in analytical grade purity were from Sigma Chemical Company (St Louis, MO, USA).

1.2 Preparation of rat brain extracts

The brain cortex of Wistar rats (weighing 180 ~ 250 g) was used for the study. 50 mg of the wet tissue was homogenized in 0.2 ml of lysis buffer (20 mmol/L Tris·HCl, pH 7.2, 0.5% NP-40, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 10 mmol/L NaF, 1 mmol/L $Na_3VO_4 \cdot 12H_2O$, 1 mmol/L EGTA, 1 mmol/L PMSF, 5 mg/L Aprotinin, 2 mg/L Pepstatin) on ice. The homogenates were vortexed thoroughly and then centrifugated at 10 000 *g* at 4℃ for 15 min. The resulting supernatant was used as the crude extracts[6,7].

1.3 Determination of Ca^{2+}-induced tau degradation

To stimulate calpain, the crude extracts were

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incubated with different concentrations of $CaCl_2$ for various time periods at 37℃. To confirm the effect of calpain on tau proteolysis, the crude extracts containing 1 mmol/L $CaCl_2$ were treated with 552 μmol/L calpeptin (total calpain inhibitor)[8] at 37℃ for 15 min. To differentiate the effect of μ-calpain and m-calpain on tau proteolysis, the crude extracts containing 1 mmol/L $CaCl_2$ were treated with 0.05 μmol/L calpastatin (μ-calpain inhibitor)[9] or 100 μmol/L calpain inhibitor Ⅳ (m-calpain inhibitor)[10] or both at 37℃ for 15 min. The reactions were stopped by adding 10 mmol/L of EGTA and boiling in SDS sample buffer without dithiothreitol (50 mmol/L Tris·HCl, pH 6.8, 2% SDS and 10% glycerol).

1.4 Quantitation of protein concentration

The protein level was quantitated by the BCA method using the Protein Assay Reagent from Pierce (USA) and bovine serum albumin was used as standard.

1.5 Western blot

30 μg of proteins per lane were separated on 10% SDS-PAGE and electronically transferred to the nitrocellulose membrane. Blots were probed with primary antibody tau Ab-2 (1∶200) recognizing total tau, and followed by incubating with alkaline phosphatase-conjugated goat anti-mouse antibody (1∶30 000 dilution). The protein bands were quantitatively analyzed by Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and the amount of protein was expressed as relative level of sum optical density.

1.6 Statistical analysis

Data were summarized as $(\bar{x} \pm s)$ and analyzed using SPSS 10.0 statistical software (SPSS Inc., Chicago, Illinois, USA).

2 Result

2.1 Calcium treatment resulted in tau degradation

As shown in Figure 1, the crude extracts were incubated with Ca^{2+} at indicated concentrations at 37℃ for 15 min. There were low molecular fragments about 29 ku in groups containing 1 ~ 5 mmol/L Ca^{2+}. However, these fragments were not seen in groups without or with 0.2 mmol/L Ca^{2+}. These results suggest that 1 ~ 5 mmol/L Ca^{2+} must have activated certain calcium-activated protease which is able to degrade tau protein.

When the supernatant was incubated with 1 mmol/L $CaCl_2$ for 5, 10, 15, 30 min at 37℃, the Ca^{2+}-induced degradation of tau was 41%, 72%, 93% and 96% of the control level of total tau, respectively (Figure 2), revealed a time-dependent degradation of tau induced by Ca^{2+}.

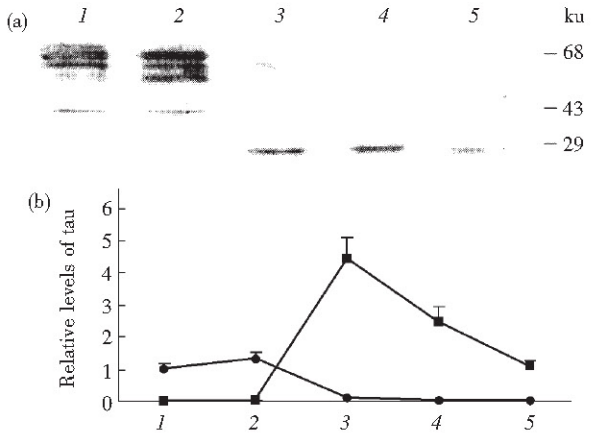

Fig. 1 Concentration dependent tau degradation induced by Ca^{2+}

(a) The rat brain extracts were centrifuged at 10 000 *g* for 15 min, and the supernatant was incubated with $CaCl_2$ for 15 min at indicated concentrations. Then the supernatant was examined by Western blot with tau Ab-2. (b) Relative levels of total tau and tau fragments shown in panel (a) were quantified by densitometry (●——●: total tau; ■——■: tau fragments). The results were expressed as $(\bar{x} \pm s)$. *1* ~ *5*: 0, 0.2, 1.0, 3.0, 5.0 mmol/L Ca^{2+} respectively.

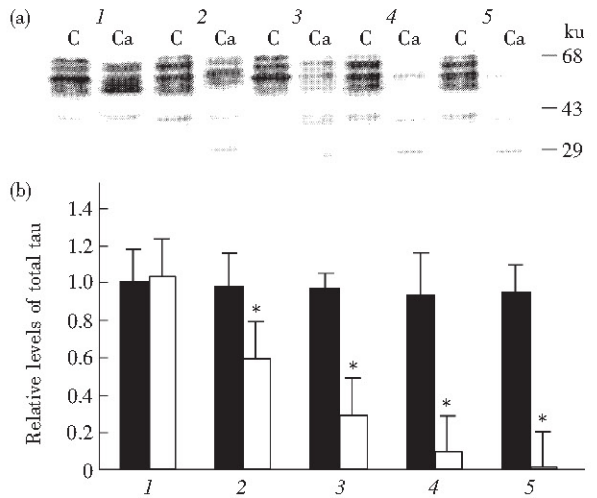

Fig. 2 Time course of Ca^{2+}-induced tau degradation

(a) The rat brain extracts were centrifuged at 10 000 *g* for 15 min, and the supernatant was incubated with 1 mmol/L $CaCl_2$ for indicated times (C: control; Ca: 1 mmol/L $CaCl_2$). Then the supernatant was examined by Western blot with tau Ab-2. (b) Relative level of total tau shown in panel (a) was quantified by densitometry (■: Con; □: 1 mmol/L $CaCl_2$). The results were expressed as $(\bar{x} \pm s)$ ($n = 9$; $*P < 0.01$ vs. control at the same time point). *1* ~ *5*: incubated for 0, 5, 10, 15, 30 min respectively.

2.2 Inhibition of calcium-induced tau degradation by calpain inhibitors

Calpain is a calcium-activated protease, and it degrades tau protein in PC12 cell. Therefore, we examined whether the cleavage of tau protein in the extracts was catalyzed by calpain. As shown in Figure 3, addition of calcium to the crude extracts induced the

cleavage of tau. This proteolysis was nearly completely suppressed by 552 μmol/L calpeptin (Figure 3b-*4*), indicating that this cleavage is mediated by calpain.

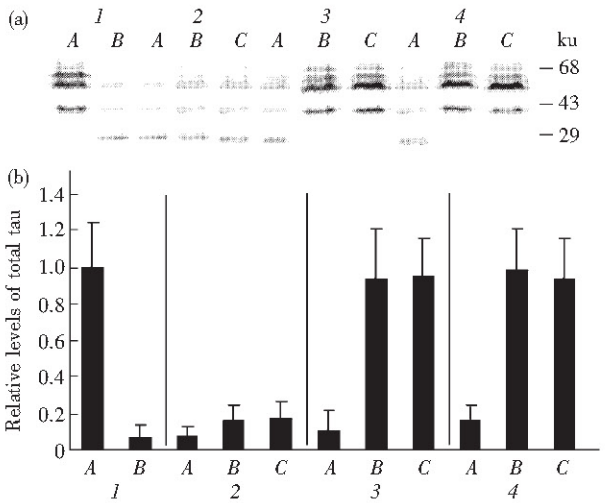

Fig. 3　Concentration dependent inhibition of tau degradation by calpastatin, calpain inhibitor Ⅳ and calpeptin on the level of total tau

(a) The supernatant containing 1 mmol/L $CaCl_2$ was incubated with calpastatin, calpain inhibitor Ⅳ or calpeptin at the indicated concentrations for 15 min. Then the supernatant was examined by Western blot with tau Ab-2. (b) Relative level of total tau shown in panel (a) was quantified by densitometry. The results were expressed as ($\bar{x} \pm s$). *1A*: 0 mmol/L Ca^{2+} + 0 μmol/L calpastatin + 0 μmol/L calpain inhibitor Ⅳ + 0 μmol/L calpeptin; *1B*: 1 mmol/L Ca^{2+}; *2A* ~ *2C*: 1 mmol/L Ca^{2+} + calpastatin (0.01, 0.05, 0.25 μmol/L respectively); *3A* ~ *3C*: 1 mmol/L Ca^{2+} + calpain inhibitor Ⅳ (20, 100, 500 μmol/L respectively); *4A* ~ *4C*: 1 mmol/L Ca^{2+} + calpeptin (276, 552, 1 104 μmol/L respectively).

2.3　Both μ-calpain and m-calpain activated by calcium resulted in tau degradation

There are two ubiquitously distributed calpains namely μ- and m-calpain, activated either by micromolar or millimolar of free Ca^{2+}, respectively. To study the nature of different subtypes of calpain on tau degradation, the brain cortex extracts were treated with various concentrations of μ-calpain inhibitor (calpastatin) or m-calpain inhibitor (calpain inhibitor Ⅳ) at 37℃ for 15 min in the presence of 1 mmol/L $CaCl_2$. It was found that the Ca^{2+}-induced tau degradation was decreased in the samples treated with inhibitors. This data suggested that both μ-calpain and m-calpain in brain cortex extracts were activated by Ca^{2+} and both of them degraded tau (Figure 3 b-*1*, b-*2*, b-*3*). When the extracts containing 1 mmol/L $CaCl_2$ were treated with 0.05 μmol/L of calpastatin (completely block μ-calpain) or with 100 μmol/L calpain inhibitor Ⅳ (completely block m-calpain) or both (block both subtypes), the inhibition rate of Ca^{2+}-induced degradation of tau was 8.6%, 92.5% and 97.8% of the total level, respectively (Figure 4). It is implied that the capacity of m-calpain-induced tau degradation is roughly 10 times greater than that of μ-calpain.

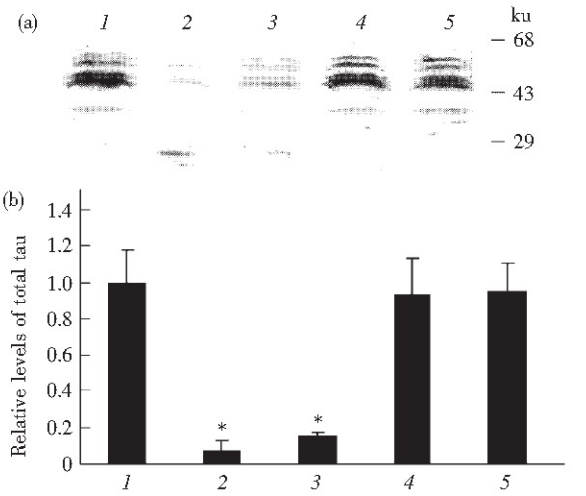

Fig. 4　Effect of m- and μ-calpain on the degradation of tau

(a) The supernatant with 1 mmol/L $CaCl_2$ was incubated with 0.05 μmol/L calpastatin, 100 μmol/L calpain inhibitor Ⅳ, 0.05 μmol/L calpastatin + 100 μmol/L calpain inhibitor Ⅳ or 552 μmol/L calpeptin for 15 min. Then the supernatant was examined by Western blot with tau Ab-2. (b) Relative level of total tau shown in panel (a) was quantified by densitometry. The results were expressed as ($\bar{x} \pm s$) ($*P < 0.01$ vs control). *1*: 0 mmol/L Ca^{2+} + 0 μmol/L calpastatin + 0 μmol/L calpain inhibitor Ⅳ; *2*: 1 mmol/L Ca^{2+}; *3*: 1 mmol/L Ca^{2+} + 0.05 μmol/L calpastatin; *4*: 1 mmol/L Ca^{2+} + 100 μmol/L calpain inhibitor Ⅳ; *5*: 1 mmol/L Ca^{2+} + 0.05 μmol/L calpastatin + 100 μmol/L calpain inhibitor Ⅳ.

3　Discussion

A major pathological abnormality in AD brain is the intraneuronal accumulation of abnormally hyperphosphorylated tau. The hyperphosphorylation of tau is mainly the result of an "inactivation" of protein phosphatases including calcium-dependent calcineurin (PP2B). In addition to the phosphorylation mechanism, tau *in vivo* undergoes dynamic turnover by proteolytic degradation preferentially by calpain[11]. We have demonstrated in the present study that both μ- and m-calpain in rat brain cortex extracts are activated by different concentration of Ca^{2+}. Although both subtypes can degrade tau, m-calpain is much more effective than μ-type. The findings together with Johnson's suggest that calpain may modulate the turnover of tau and contribute to the neurodegenerative process, such as seen in AD brain. As the Ca^{2+}-induced tau degradation was entirely blocked by total calpain inhibitor, we speculated that it is calpain, a calcium-activated protease, but not the other protease degraded tau in this system. Additionally, the antibody used in the present study recognizes total tau, therefore, we are unable to define the origin of the degraded fragments, including the most dominantly

seen peptide with molecular mass around 29 ku.

Calpain modulates the phosphorylation and proteolysis of tau, two initiating factors in the formation of paired helical filaments (PHF)[12, 13]. Regarding to the regulation in tau phosphorylation, some researchers believe that calpain is capable of converting p35 to p25, and thus plays a pathological role in activating cdk5 and phosphorylating tau in AD brain[14, 15]. However, it is also reported that the cleavage of p35 to p25 by calpain does not induce tau hyperphosphorylation[16]. As a calcium-activated protease, calpain plays a more important role in regulating the turnover of cytoskeletal proteins. The supporting data for this view are the findings that PHF-1 positive aggregates are co-localized with the active form of calpain in patients of frontotemporal dementia (FTD)[17]; that μ-calpain is activated in AD brain[18]. It is suggested that both μ-calpain and m-calpain might be involved in AD pathogenesis.

Why does not activated calpain degrade tau efficiently? Like many other cytoskeletal proteins, the dynamic turnover of tau through a signal-mediated phosphorylation/dephosphorylation/proteolysis scheme is an integral part of the cellular homeostasis. Both dephosphorylation and degradation of tau are necessary steps in its turnover, and both events involve calcium-dependent processes, therefore, the apparent hyperphosphorylation and accumulation of tau would point to a calcium deficit, which would inactivate calcineurin and calpain. Based on this fact, we speculate that instead of calcium overload seen in advanced AD, it must have a calcium deficit in the early phase of AD. Decreased calcium signal will lead to inactivation of calpain and accumulation of undegraded tau, which aggravates tau hyperphosphorylation and tangle formation. To prove this hypothesis, further studies are required to elucidate the effect of calpain on degradation of hyperphosphorylated tau *in vivo*.

In summary, the data obtained in this study suggest that among different subtypes of calpains, m-type is pivotal in proteolysis of tau, and may be indicative in the treatment in early phase of AD.

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Calpain 对细胞骨架蛋白 tau 降解作用的研究*

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摘要 Calpain 是钙依赖性中性蛋白酶，根据其对钙敏感性的不同，可分为 m-和 μ-calpain 两型. 分别用不同浓度 $CaCl_2$ 溶液孵育 Wistar 大鼠脑皮质匀浆液，并用蛋白质印迹和定量图像分析技术检测不同亚型 calpain 对 tau 蛋白的降解作用. 研究发现：在 37℃用 1 mmol/L Ca^{2+} 孵育底物 15 min，可见 tau 蛋白明显降解，并在分子质量为 29 ku 处出现 tau 蛋白降解片段；当 Ca^{2+} 浓度为 5 mmol/L 时，tau 蛋白几乎全部被降解；这种 tau 蛋白降解可被 calpain 特异性抑制剂完全逆转. 进一步的研究发现，分别用 μ-calpain 抑制剂（0.05 μmol/L calpastatin），m-calpain 抑制剂（100 μmol/L calpain inhibitor Ⅳ）或总 calpain 抑制剂（552 μmol/L calpeptin）与 1 mmol/L Ca^{2+} 共同孵育 Wistar 大鼠脑皮质匀浆液，Ca^{2+} 激活的 tau 蛋白降解分别被抑制 8.6%，92.5% 和 97.8%. 结果表明一定浓度的 Ca^{2+} 可同时激活 μ-calpain 和 m-calpain，这两种亚型 calpain 均参与降解 tau 蛋白，但 m-calpain 的作用比 μ-calpain 更强.

关键词 钙依赖性中性蛋白酶，tau 蛋白，钙离子，阿尔茨海默病

学科分类号 R3

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