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Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains

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Abstract Tau in Alzheimer neurofibrillary tangles has been shown to be hyperphosphorylated and CDK5, GSK3, MAP kinase and SAP kinases are the candidate kinases for the phosphorylation of tau. Recently, it was reported that the conversion of p35, the activator of CDK5, to p25 was upregulated in Alzheimer's disease (AD) brains, and that p35 is cleaved to yield p25 by calpain. Here we show that p35 is rapidly cleaved to p25 in rat and human brains within a short postmortem delay and that the conversion of p35 to p25 is partially dependent on calpain activity. Immunoblot analysis of brains prepared from patients with AD or age-matched control individuals with a short postmortem delay revealed no specific increase in the levels of p25 in AD brains, whereas the levels of active form of calpain were increased in AD brains compared to the those in controls. These observations suggest that the conversion of p35 to p25 is a postmortem degradation event and may not be upregulated in AD brains.

Key words: Calpain; p25; p35; CDK5; Alzheimer's disease; Tau

1. Introduction

Abundant neurofibrillary lesions and senile plaques are the neuropathological hallmarks of Alzheimer's disease (AD). Neurofibrillary lesions are composed of filamentous structures, paired helical filaments (PHFs) and the related straight filaments, and hyperphosphorylated microtubule-associated protein tau (PHF-tau) is the major component of the PHFs [1–3]. Aggregation of hyperphosphorylated tau is also central to a number of other dementia disorders [4,5]. Extensive protein chemical and immunochemical studies have identified about 20 hyperphosphorylated amino acid residues in PHF-tau [6–8], and approximately half of these sites are serine/threonine-proline sequences. Based on in vitro experiments, mitogen-activated protein (MAP) kinase [9], neuronal cdc2-like kinase (NCLK) or CDK5 [10,11], glycogen synthase kinase-3 (GSK3) [12,13], and stress-activated protein kinases (SAPKs) [14,15] are known as candidate protein kinases for the hyperphosphorylation of tau. It has recently been shown that p25, a truncated form of CDK5 activator p35, is upregulated and accumulated in neurons in AD brains [16] and that the expression of the p25-CDK5 complex induces cytoskeletal disruption and neurodegeneration in cultured neurons [16]. It has also been shown that p35 can be cleaved to p25 by calpain in vitro and in vivo [17,18]. Although calpain has been shown to be activated in AD brains [19], correlation between the p35 processing and calpain activation is unknown.

We investigated here cleavage of p35 to p25 and activation of calpain in AD and control brains, and in rat brains with various postmortem incubation. We show that calpain is surely activated in AD brains. We also show that p35 is degraded to produce p25 in rat brains within a few hours of postmortem incubation and that the conversion of p35 to p25 is mediated by calpain. Notably, we observed no difference in the relative levels of brain p25 between AD and age-matched controls.

2. Materials and methods

2.1. Antibodies

A polyclonal antibody to p25·p35 (C19) and a polyclonal antibody to CDK5 (C8) were purchased from Santa Cruz Biotechnology Inc. A polyclonal antibody to μ-calpain, which specifically recognizes the active form of human μ-calpain (76-kDa post-autolytic μ-calpain), and a polyclonal antibody to spectrin are described previously [20,21]. A monoclonal antibody AT8 is a previously described phosphorylation-dependent anti-tau antibody [22]. Antibodies were diluted at the following ratios: p25·p35 (C19) (1:1000), CDK5 (C8) (1:1000), μ-calpain (1:500), spectrin (1:500), AT8 (1:1000) for immunoblotting of brain extracts.

2.2. Preparation of brain extracts from the brains of patients with AD and age-matched control individuals

Frozen brain tissues with a short postmortem delay (range: 1–6.5 h) from patients with AD (eight cases, Table 1) and age-matched controls (nine cases, Table 1) were homogenized in four volumes of extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM EGTA, 1 mM DTT, plus protease inhibitors and phosphatase inhibitors; see [16]), and centrifuged at $13\,000\times g$ for 30 min. The resulting supernatants were used as brain

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extracts. We paid special attention to keep the w/v ratio of wet brain tissue/extraction buffer exactly at 1:4.

Sarkosyl-insoluble fractions were prepared from the first pellet of brain homogenates by extracting with 10 volumes of 50 mM Tris buffer (pH 7.5) containing 5 mM EDTA, 0.8 M NaCl, 10% sucrose and 1% Sarkosyl, followed by incubation at 37°C for 1 h. Following centrifugation, the pellets were resuspended in SDS sample buffer.

2.3. Preparation of rat brain extracts

10-week-old Wistar rats (Japan-SLC) were lethally anesthetized and the brains were excised at 0, 1, 3, 6, 14 and 24 h after anesthetization. Rats were kept at room temperature prior to brain excision. The brains were homogenized as described in Section 2.2.

To inhibit calpain activation in postmortem rat brains, rats were anesthetized and perfused transcadially with PBS, PBS plus 10 mM EGTA or PBS plus 60 μM calpain inhibitor I, and the brains were subsequently excised at 3 or 6 h after perfusion.

2.4. SDS–PAGE and immunoblotting

SDS–PAGE was performed using 12.5% polyacrylamide gels for immunoblotting of p25·p35 and CDK5, 10% gels for tau protein or 7.5% gels for μ-calpain and spectrin. For immunoblotting, about 100 μg proteins were loaded in each lane and transferred to Immobilon P. The blots were incubated with primary antibodies, followed by peroxidase-conjugated anti-mouse or rabbit IgG antibodies (Dako) as described [17]. The reactions were detected using the ECL Western blotting systems (Amersham-Pharmacia Biotech, Tokyo, Japan). Relative intensities of immunolabelled bands were quantified using Luminescent image analyzer LAS-1000plus (Fuji Film Co.). The intensities of the immunoreactive bands were always within the linear ranges.

3. Results

3.1. p25 level is not increased in AD brains

To investigate whether the p35 processing and calpain activation are correlated in AD brains, we collected control and AD brain tissues with relatively short postmortem intervals (< 6.5 h) which had been preserved in good condition without any freeze–thaw processes (Table 1). We then extracted proteins with the extraction buffer containing EGTA and protease inhibitors. The frozen brains used in this study are listed in Table 1. As shown in Fig. 1A, hyperphosphorylated PHF-tau was detected in Sarkosyl-insoluble fractions of all AD brains with a phosphorylation-dependent tau antibody AT8, whereas no immunoreactivities were seen in control brains, showing that all AD patients had tau pathology. Immunoblot analysis of brain extracts with a p25·p35 antibody showed no significant difference in the ratios of p25 to p35 between control and AD brains (Figs. 1B and 2A), although total levels of both p25 and p35 were decreased by about 50% in AD brains compared with those in normal controls. The levels of CDK5 immunoreactivities were also reduced by approximately 50% in AD brains compared with those in control brains (Fig. 1C).

Because calpain has been implicated in the conversion of p35 to p25 [17,18], we investigated whether calpain is activated in AD brains using an antibody which specifically recognizes the active form of μ-calpain [20]. As shown in Fig. 1D, the μ-calpain antibody specifically labelled the active form of calpain which migrated at 76 kDa in human brain extracts, and the immunoreactivities detected in AD brains were about 7-fold stronger than those in control brains (Figs. 1D and 2B). Fig. 2 shows quantification of the p25/p35 ratio and the band intensities of the active form of μ-calpain in AD and control brains.

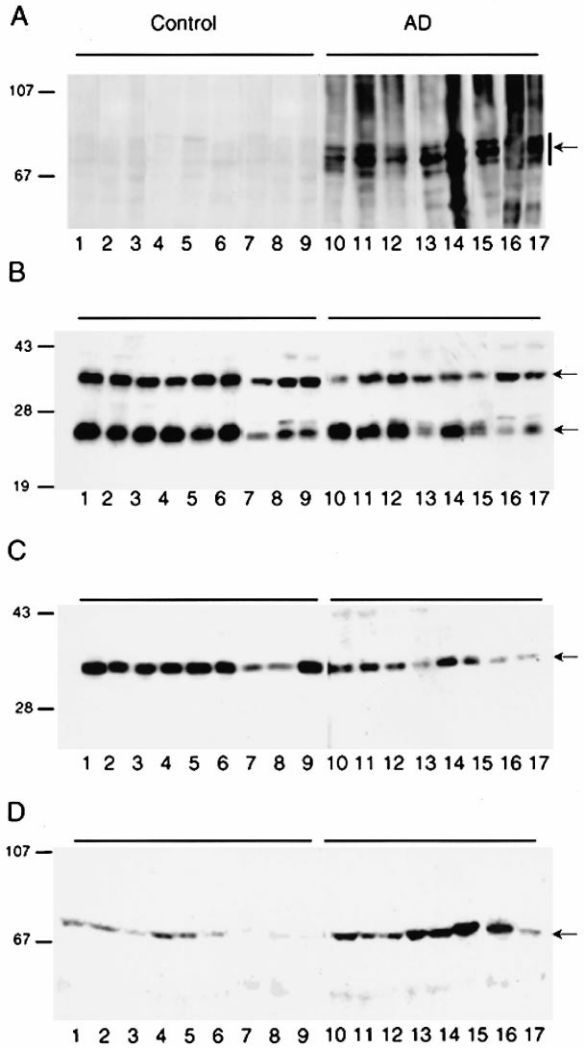

Fig. 1. Immunoblots of Sarkosyl-insoluble pellets with anti-tau antibody AT8 (A), and brain extracts with anti-p25·p35 antibody (B), anti-CDK5 antibody (C) or anti-μ-calpain antibody (D) from control and AD brains. lanes 1–9: brain extracts from control brains; lanes 10–17: brain extracts from AD brains.

Table 1
Age at death, sex, and postmortem interval (PMI) of control and AD brains used in this study

Case no	Age	Sex	PMI (h)
Control			
1	74	M	3.00
2	83	F	3.83
3	75	M	2.33
4	78	F	3.50
5	83	M	2.50
6	67	F	2.50
7	78	F	1.00
8	80	M	1.00
9	71	M	1.50
AD			
10	83	F	6.50
11	83	M	2.50
12	86	F	3.00
13	87	F	2.50
14	71	F	2.17
15	79	M	3.67
16	86	F	3.00
17	83	F	2.67

3.2. Degradation of p35 in situ

Immunoblot analysis of human brain tissues with various postmortem delay revealed a marked variation in the levels of p25 or p35. This prompted us to hypothesize that this variation might be due to postmortem changes (e.g. postmortem delay and/or difference in agonal conditions). To investigate the effect of postmortem delay on p35 protein degradation, we examined the amount of p25 in rat brains with different postmortem intervals by leaving the sacrificed rats at room temperature for 1, 3, 6, 14 and 24 h. As shown in Fig. 3A, p35 was rapidly degraded during incubation at room temperature after death. At 0 h, i.e. when the brain was immediately excised and homogenized within a few minutes after death, a strong p35 band and a very weak p25 band were detected. During postmortem incubation of 3–14 h, the levels of p35 band gradually decreased, whereas those of p25 in turn increased. The intensity of p25 band reached maximum at 14 h and then slightly decreased at 24 h. Fig. 3B shows quantification of the band intensities of p25 during postmortem incubation.

To further characterize p35 degradation in situ in postmortem rat brain, we analyzed calpain activation in brains by detecting spectrin degradation with an anti-spectrin antibody that specifically detects the cleavage site by calpain [21]. Spectrin (full-length, 230 kDa) has been shown to be cleaved by calpain to generate a 150-kDa fragment, which has been regarded as an indicator of calpain activation. A very weak 150-kDa fragment was detected in rat brain extract at 0 h of delay. Then the 150-kDa band gradually increased at 1, 3, 6 h after death, suggesting activation of calpain during postmortem incubation (Fig. 3C,D). The levels of the 150-kDa spectrin fragment during postmortem incubations were closely correlated with those of p25 (Fig. 3B,D).

To obtain further evidence for the calpain-dependent degradation of p35 in postmortem rat brains, we perfused rat brains with a calcium-chelating buffer or a buffer containing a calpain inhibitor peptide, and incubated for 3–6 h prior to preparation of brain samples. Interestingly, degradation of p35 was significantly inhibited by this treatment (Fig. 4A,B). These results strongly suggest that p35 is converted to p25 within several hours of postmortem interval and that calpain is at least partially involved in this process.

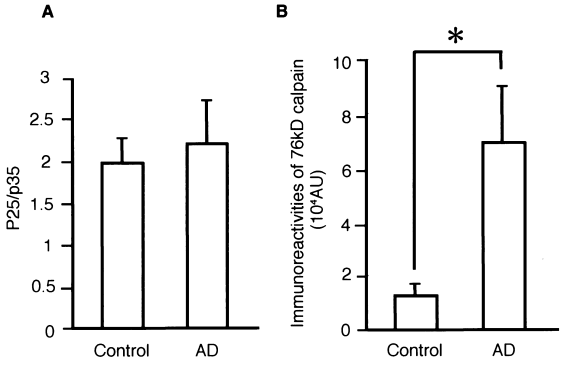

Fig. 2. A: p25/p35 ratios in control and AD brains. B: Levels of the active form of 76-kDa μ-calpain in control and AD brains. Relative intensities of immunolabelled bands were quantified using a Luminescent image analyzer. The intensities of the immunoreactive bands were always within the linear ranges. $^{*}P < 0.05$.

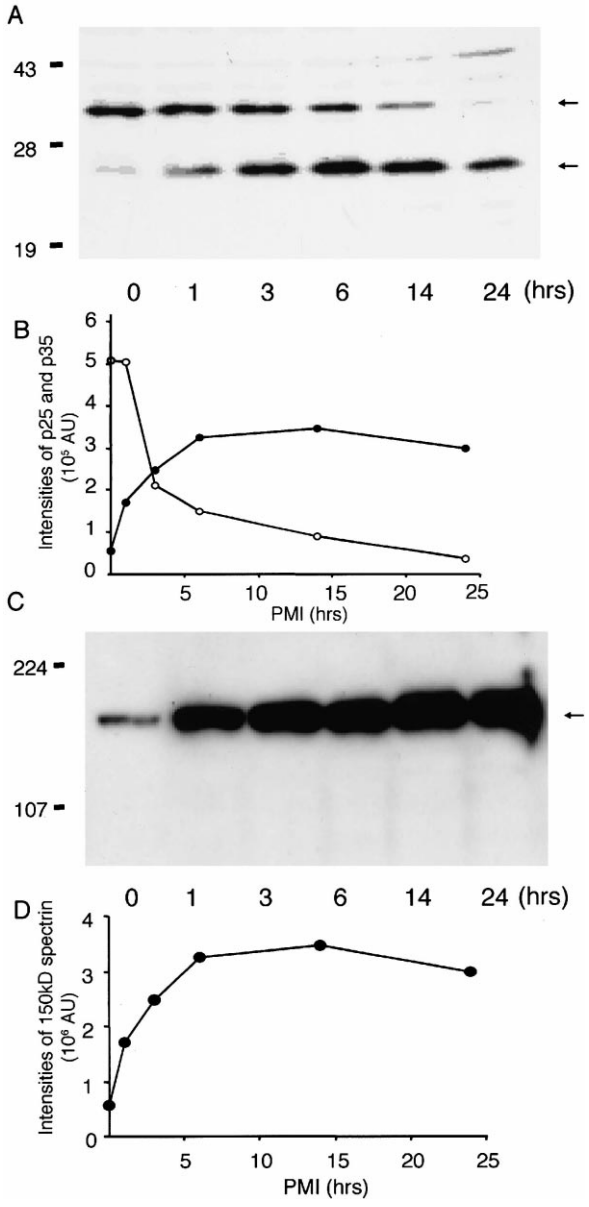

Fig. 3. A: Immunoblots of brain extracts from rats with different postmortem intervals with anti-p25·p35 antibody (C19). B: Time course of degradation of p35 (○) and production of p25 (●) in postmortem rat brains. C: Immunoblots of brain extracts from rats with different postmortem intervals with anti-spectrin antibody. D: Time course of production of the 150-kDa spectrin fragment in postmortem rat brains. Intensities of immunolabelled bands were quantified using a Luminescent image analyzer.

4. Discussion

In this study, we have shown that the levels of p25 in AD brains with relatively short postmortem intervals were not increased compared with those in age-matched controls. We also have shown that the total levels of p25·p35 and CDK5 were decreased by about 50% in AD brains in which hyperphosphorylated PHF-tau was accumulated, and μ-calpain was activated. We investigated the degradation of p35 to p25 during postmortem incubation in rat brains and showed that the conversion of p35 to p25 was rapid and partially mediated by calpain.

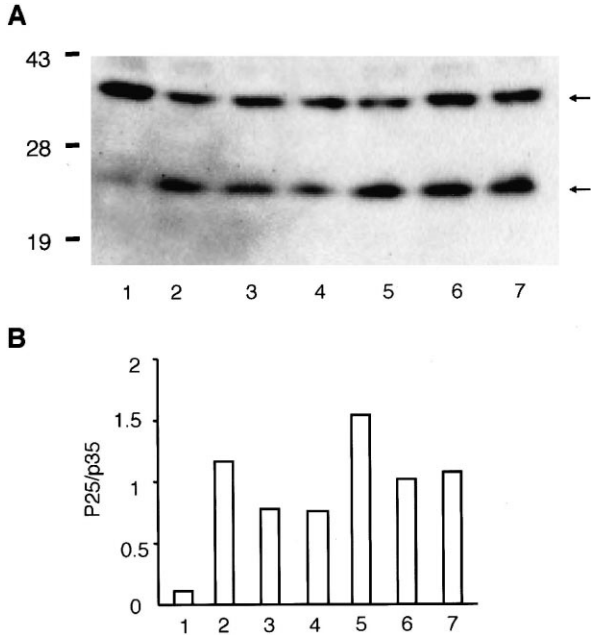

Fig. 4. Immunoblots of rat brain extracts with anti-p25·p35 antibody (A) and relative intensities of p25 and p35 (B) in rat brains perfused with PBS, PBS plus 10 mM EGTA or PBS plus 6 μM calpain inhibitor I. Lane 1, rat brain extract with 0-h postmortem interval, lanes 2, 3 and 4, with 3-h postmortem interval, lanes 5, 6 and 7, with 6-h interval; lanes 1, 2 and 5, extracts from brains perfused with PBS, lanes 3 and 6, extracts from brains perfused with calpain inhibitor I, lanes 4 and 7, rats perfused with EGTA.

Our present results are not consistent with those by Patrick et al. [16], in which the level of p25 was shown to be 20–40-fold upregulated in AD brains, whereas the p35 levels remained unchanged, suggesting that the p25 protein is accumulated in the tangle-bearing neurons of AD brains [16]. We are not certain about the reasons why they detected increased levels of p25 in AD brains. We prepared brain extracts exactly according to their method with the same buffer as they used. The difference in the specificities of the antibodies used to detect p25·p35 proteins might have affected the results to some extent.

However, it also might be due to difference in postmortem delays of brain tissues. As we have shown here, postmortem intervals significantly affected the levels of p25 and p35. By immunoblot analysis of rat brains with various controlled postmortem delays, we have shown that p35 degraded to p25 during 1–6 h of postmortem incubation and that the levels of p25 were drastically increased within a few hours of postmortem intervals. The freeze–thaw process also increased the levels of p25 or p35 in tissues, probably because conversion of p35 to p25 is stimulated by a treatment that damages cells and activates calpain (data not shown). Several previous reports also have shown that p35, but not p25, predominates in the crude brain extracts [23–25], whereas the p35–CDK5 complex has never been purified from mammalian brains [26,27], probably because p35 degrades to p25 by calpain during purification.

Several studies have shown that the CDK5–p35 complex is involved in the migration or positioning of neural cells through phosphorylation of cytoskeletal proteins during development [28,29], and tau and neurofilaments (NF-H and NF-M) [26,30] have been identified as substrates for this complex. The CDK5–p25 complex has also been purified from microtubule-enriched fractions of bovine and porcine brain extracts [26,27]. In neurons of AD brains, tau becomes hyperphosphorylated and microtubules are disrupted and replaced by the abnormal tau filaments, i.e. PHFs. The levels of CDK5 and p25·p35 may be decreased in degenerating neurons of AD brains as a result of these cytoskeletal changes.

Using a specific antibody to active form of μ-calpain, we have shown that the activation of μ-calpain was elevated in AD brains. This confirmed the previous observations by Saito et al. [19] in which the ratio of active form of μ-calpain to the precursor form was elevated in AD but not in Huntington disease brains. Although calpain is shown to be able to cleave p35 to p25 in vitro and in vivo, we could not see any difference in the p25/p35 ratios between control and AD brains. These contrast to those seen in postmortem rat brains where the p35 degradation is closely correlated with μ-calpain activation. The reason for this discrepancy is unclear at present; however, one possibility would be that p25 detected in autopsied control and AD brains is largely derived by postmortem degradation. Further studies in autopsied and biopsied brain samples as well as in model animals will help to clarify whether p25 levels are upregulated in AD brains due to calpain activation.

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