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A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains

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Abstract P25, a calpain cleavage product of the cyclin-dependent kinase 5 (Cdk5) activator p35, causes prolonged activation of Cdk5. Although p25 has been shown to accumulate in brains of patients with Alzheimer's disease (AD), it is not known whether p25 accumulation in AD is brain region-specific. We analyzed the amounts of p25 and p35 in human autopsy samples from multiple brain regions including frontal cortex, inferior parietal cortex and hippocampus using immunoblotting assays. Our results show that the p25–p35 indices are higher in AD than in the control groups in all three brain regions. The most significant difference in p25–p35 indices between AD and control groups is in the frontal cortex. No significant difference in calpain activity between AD and control groups is observed, indicating that postmortem calpain activation cannot account for the elevation of p25/p35 ratios in AD brains. Our results support the notion that p25 accumulation deregulates Cdk5 activity in AD brains, and the deregulated Cdk5 activity may contribute to the pathogenesis of AD.

Key words: Cyclin-dependent kinase 5; Cyclin-dependent kinase 5 activator; Proteolysis; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is the leading cause of senile dementia. Recent evidence suggests that cyclin-dependent kinase 5 (Cdk5) plays a role in the pathogenesis of AD. The activity of Cdk5 has been shown to be higher in the prefrontal cortex of AD brains [1]. Immunohistochemical analyses indicate the presence of Cdk5 in the early stage of neurofibrillary tangles [2], one of the two pathological hallmarks in AD. Neurofibrillary tangles are comprised primarily of hyperphosphorylated microtubule-associated protein tau. Indeed, Cdk5 is one of the major protein kinases that phosphorylate tau on multiple sites [3].

Cdk5 is a ubiquitous serine/threonine protein kinase [4]. Enzymatic activity of Cdk5 requires association with one of its regulatory activators, p35 or p39. P35 and p39 are 60% identical in sequence. Both proteins are predominantly expressed in the nervous system [5,6]. P35 is subjected to two alternative proteolytic pathways. First, p35 is normally degraded by the ubiquitin-dependent proteasome pathway. This results in a short half-life of p35 which allows for stringent regulation of Cdk5 catalytic activity [7]. Second, the calcium-dependent cysteine protease calpain, which is activated under neurotoxic conditions, cleaves p35 between Phe99 and Ala100 to liberate a 25 kDa fragment termed 'p25' [8–10]. The half-life of p25 is 5–10-fold longer than that of p35 [7]. Generation of p25, therefore, causes prolonged activation of Cdk5 [7]. Furthermore, the localization of p25 is diffuse in cells, whereas p35 is mostly restricted to the cell membrane, as judged by immunocytochemical analysis [8]. Several lines of evidence suggest that p25 is neurotoxic. In primary cortical neurons, overexpression of p25 results in apoptosis [7]. Transgenic mice expressing human p25 show cytoskeletal disruptions and hyperphosphorylation of tau in certain areas of the brain, providing evidence for a role of p25/Cdk5 in the pathologic phosphorylation of tau [11]. In addition, application of calpain or Cdk inhibitors markedly reduces p25 neurotoxicity induced by the β-amyloid peptides [8,12], suggesting that p25 is a downstream effector of the amyloid β peptides. Interestingly, we found that the ratio of p25 to p35 is elevated in postmortem brains of AD patients. This led us to propose that increase in p25 level contributes to the pathogenesis of AD [7].

Contrary to our findings, Yoo and Lubec reported that p25 was decreased in AD brain samples [13]. Taniguchi and colleagues also argued that p25 was not significantly increased in AD brains [14]. To address the relationship of p25 and AD more specifically, we performed a large survey on the levels of p25 and p35 in human brains. In addition, we also analyzed p25 and p35 levels in three different brain regions including the frontal cortex, inferior parietal cortex and hippocampus. Postmortem intervals of all tissues analyzed were less than 7 h. The activity of calpain in the postmortem brain samples was also examined by detecting the breakdown products of cytoskeletal spectrin. Statistical analyses of these experiments suggest that the p25/p35 ratio in AD brains is significantly higher than that in age-matched control brains.

2. Materials and methods

2.1. Materials and buffers

RIPA buffer contained 500 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 2 mM EGTA, 2 mM EDTA and 50 mM Tris base, pH 7.5. The 1% SDS buffer contained 1% SDS in RIPA buffer. All solutions contained 1 mM dithiothreitol, 25 mM β-glycerolphosphate, 5 mM NaF, 0.5 mM Na_3VO_4, 0.1 mM phenylmethylsulfonyl fluoride,

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1 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 μM calpeptin. PBST solution was the phosphate-buffered saline containing 0.2% Tween 20. Chemiluminescence reagents used in immunoblotting assay were purchased from NEN Life Science Products (Boston, MA, USA). Polyvinylidene difluoride (PVDF) membrane (Immobilon-P) was purchased from Millipore (Bedford, MA, USA). Chemicals are reagent-grade. Polyclonal antibody p35 was raised against full-length p35, recognizing p25 and p35. A monoclonal antibody against non-erythroid spectrin was obtained from Chemicon (Temecula, CA, USA). Film used in the chemiluminescent detection was Kodak X-Omat Blue XB-1 (Eastman Kodak, New York, NY, USA).

2.2. Preparation of brain tissue samples

Human brain samples from the frontal cortex and inferior parietal cortex were provided by Dr. Yong Shen at the Sun Health Research Institute (Sun City, AZ, USA). Samples from the superior frontal gyrus, a region in the frontal cortex, and hippocampus were provided by Dr. Barbara Sahagan at Pfizer (Groton, CT, USA). The superior frontal gyrus samples were considered the frontal cortex in this report. Samples were shipped on dry ice and immediately stored at −80°C upon arrival. Descriptions of the brain samples analyzed in this report are listed in Table 1. The samples from the frontal cortex, superior frontal gyrus, and inferior parietal cortex were homogenized in RIPA buffer using a pre-chilled Dounce homogenizer. Hippocampal tissue was prepared in a 1% SDS buffer. Cell lysates of each sample were clarified by centrifugation at 4000×*g* at 4°C for 30 min.

2.3. Immunoblotting assay, imaging and statistical analysis

A 100 μg aliquot of proteins from each lysate was loaded and resolved on 10% SDS–polyacrylamide gels using a standard Tris–glycine SDS-PAGE protocol. Proteins were electrically transferred into PVDF membranes. Immunoblotting was performed according to the protocol provided by NEN Life Science Products. The polyclonal p35 antibody (2 μg/ml) was prepared in PBST containing 5% non-fat dried milk. The p35 antibody raised against p35 recognized both p35 and p25, used to label p35 and p25. The spectrin antibody was used at a concentration of 0.01 μg/ml. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Amersham Biosciences, CA, USA) was used at 1:5000 dilution in the p25 and p35 experiment and HRP-conjugated anti-mouse antibody (Amersham Biosciences) was diluted at 1:5000 in the spectrin experiment. The labeled proteins (p25, p35, and spectrin) were visualized by chemiluminescent method and film exposure. The images were digitalized and imported into a Windows 2000 computer using a calibrated ImageScanner scanner (Amersham Biosciences). The digital images were obtained at a 16-bit gray mode to provide 65 636 gray levels. The volumes of image spots were quantified using the ImageMaster 3 software (Amersham Biosciences). The unit of volumes given by the image measurement is an arbitrary unit. The total units per spot were representations of the p25 or p35 levels in the analyzed samples. The p25/p35 ratio was established and the logarithm of each p25/p35 ratio was calculated and referred to as p25–p35 index. Statistical calculations, including two-tailed Student's *t*-test, were performed using Microsoft Excel (Redmond, WA, USA).

3. Results and discussion

Tissues from a total of 28 AD brains and 25 age-matched control brains were analyzed and shown in Table 1. All of the brain samples came from different individuals. To minimize the postmortem effect of p25 production, we only analyzed brain samples with postmortem intervals less than 7 h. The average of the postmortem intervals in all sample groups was approximately 2.5 h (Table 1).

The p35 antibody raised against full-length p35 recognizes both p25 and p35 [7]. The levels of p25 and p35 in each sample were determined by immunoblotting using the p35 antibody and analyzed using ImageMaster 3 software (see Section 2). Using the p25/p35 value for comparison may seem more straightforward but the conclusion reached is misleading because a completely different result can be obtained when the p35/p25 ratio is used instead of p25/p35. To eliminate the discrepancy caused by using p25/p35 or p35/p25, we used the logarithm of p25/p35, termed p25–p35 index, to study p25 and p35. The use of logarithmic transformation can resolve the non-additivity and heterogeneity of variance [15] and has been applied in biological study [16]. A 99% confidence limit was set. Samples located within the 99% confidence intervals were analyzed here. The logarithm of p25/p35 for each case (25 controls and 28 ADs) was plotted on a scatter plot (Fig. 1A). The mean, S.D. and results from Student's *t*-test are listed in Table 2. In Fig. 1A, the p25–p35 indices in AD are, in general, higher than in the control. The mean of the p25/p35 ratios in AD (0.705, shown in Table 2) is higher than that in the control (0.243, shown in Table 2). In Student's *t*-test, the *P* value is 0.0000359 when we compare p25–p35 indices between the two groups (Table 2), suggesting that the p25–p35 index in AD is significantly higher than that in the control.

To study p25 and p35 more specifically, we analyzed p25 and p35 levels by brain region, including frontal cortex, inferior parietal cortex and hippocampus (Fig. 1B–D). The p25–p35 indices in the AD groups tend to have higher values compared to the corresponding controls. The means of p25–p35 indices in the three AD groups are higher than those in the corresponding controls. These results indicate that p25–p35 indices in AD are elevated.

Statistical assays were further utilized to evaluate the p25–p35 index in AD versus control brains, shown in Table 2. In control groups, the means of p25–p35 indices in the frontal

Table 1
Basic information of analyzed brain samples

Brain region	Gender (male/female)	P.M.I.[a] (mean ± S.D.)	Age of death (mean ± S.D.)
Frontal cortex			
Control	8/2	3.06 ± 0.90	80.3 ± 10.3
AD	9/6	2.59 ± 0.86	83.6 ± 5.4
Inferior parietal cortex			
Control	3/6	2.49 ± 0.74	80.0 ± 9.6
AD	7/0	3.32 ± 1.85	84.3 ± 5.1
Hippocampus			
Control	4/2	2.93 ± 1.16	76.7 ± 14.4
AD	3/3	2.07 ± 0.51	83.7 ± 3.6
Total			
Control	15/10	2.83 ± 0.92	79.2 ± 10.3
AD	19/9	2.66 ± 1.18	83.8 ± 5.0

[a]P.M.I.: postmortem interval.

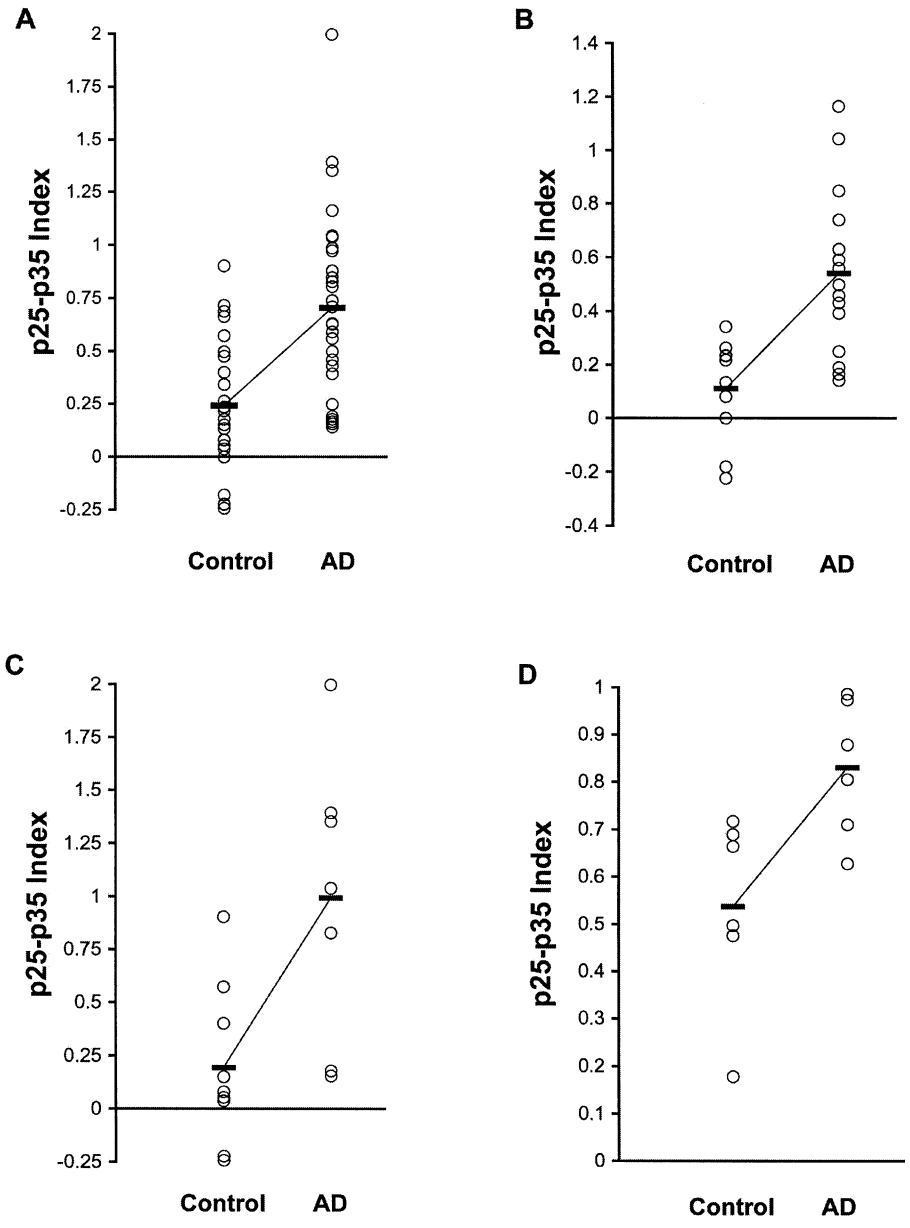

Fig. 1. Comparison of p25–p35 indices of human control and AD brains. The result of log (p25/p35) for each brain sample is represented by a circle. Bars represent the mean value of the group. A: Total brain samples. B: Frontal cortex. C: Inferior parietal cortex. D: Hippocampus.

cortex, inferior parietal cortex, and hippocampus are 0.111, 0.194, and 0.537, respectively. These numbers showed that p25–p35 indices were different in different brain regions. In the AD groups, the mean p25–p35 indices in the three corresponding regions are 0.541, 0.992, and 0.831 (Table 2). To determine the change in p25/p35 ratios, we took the exponential of [log $(p25/p35)_{AD}$ − log $(p25/p35)_{control}$]. The results showed that the means of the p25/p35 ratios in the AD groups were 2.0–6.3-fold higher than those in the control groups, depending on the particular brain region analyzed. The highest p25/p35 case (p25/p35 = 99.5, log (p25/35) = 2.00) appeared in the AD inferior parietal cortex (Fig. 2C). The lowest p25/p35 (p25/p35 = 0.574, log (p25/p35) = −0.241) was present in the control inferior parietal cortex (Fig. 2C). Student's *t*-tests on the frontal cortex, inferior parietal cortex and hippocampus demonstrate that the p25–p35 indices in the AD and control are significantly different ($P < 0.05$) (Table 2). Based on these results, we conclude that, in general, p25/p35 in the AD brain is indeed increased. Owing to the paucity of the tissue material that can be obtained and the limitation of the semi-quantitative immunoblot assay method more detailed studies in specific sub-cortex regions could not be made. However, the p25–p35 index procedure described here appears to be an acceptably reliable approach for comparative purposes.

We previously showed an increased conversion of p35 to p25 in Brodmann's areas 11 and 21 in AD brains which are part of the frontal cortex [7]. We also showed that the p25/p35 ratios were as high as 20-fold in AD brains in the previous report [7]. In this study, we report that the p25/p35 ratios in AD are higher not only in frontal cortex but also in inferior parietal cortex and hippocampus. The p25/p35 ratios in AD can reach 10–100-fold. Our results are contradictory to the

result reported by Yoo and Lubec where they find a decreased p25 level in the frontal cortex of AD brains [13]. There are several possible explanations for the discrepancy. One possibility is the difference in postmortem delays of samples. Samples with long postmortem delays (>12 h) can have complicated enzymatic activities, including protease activities. The protease calpain that degrades p35 to generate p25 can be activated during the postmortem period, thereby complicating the analysis of the tissue samples. Another complication can be caused by the variability within the control cases. In rodent models, ischemia can trigger the generation of p25 [8]. Moreover, cycles of freeze–thawing also affected the p25/p35 ratios, as reported in a previous study [14]. Therefore, careful sample handling is required in the analysis of p25 and p35.

Recent studies showed that the protease calpain was responsible for conversion of p35 to p25 [8–10]. Calpain has been shown to be active during the postmortem period or in hypoxia/ischemia conditions [17–19]. To determine if the increased p25 in AD brains was generated during the postmortem period, we examined postmortem calpain activity in the AD and control frontal cortex samples by measuring the cleavage of α-spectrin [18,19]. Large breakdown products of spectrin are molecular markers used to represent postmortem calpain activity [18]. Upon calpain cleavage, the full-length spectrin (280 kDa) is broken down into 150 kDa and 140 kDa fragments, respectively [8,18]. Four samples for each group (control and AD frontal cortices) were selected and analyzed. The p25–p35 index in the selected AD samples was significantly higher than that in the control ($P<0.05$). The breakdown levels of either p150 or p140 in the control and AD groups are similar (Fig. 2). The result of a statistical analysis on the breakdown levels of spectrin (Student's t-test, $P>0.05$) indicates that there is no significant difference between the control and AD groups (Fig. 2). Taniguchi et al. speculated that p25 in AD brains is perhaps generated postmortem [14]. However, in this report, the breakdown levels of spectrin in the AD frontal cortex are similar to those in the control groups, suggesting that AD brains do not harbor higher postmortem calpain activity than control brains. This is consistent with a previous biochemical analysis on calpain activity in AD brains [20]. Thus, the observed increase in p25/p35 ratio in AD can hardly be explained by de novo generation of p25 postmortem. Rather, the elevated p25/p35 ratio in AD is presumably the result of accumulation of p25 in particular afflicted neurons over the course of the disease.

Table 2
Statistical summary of p25–p35 indices in brain samples

Brain region	Log of p25/p35 (mean ± S.D.)	P values of Student's t-test
Frontal cortex		
Control, $n=10$	0.111 ± 0.191	0.00267
AD, $n=15$	0.541 ± 0.309	
Inferior parietal cortex		
Control, $n=9$	0.194 ± 0.373	0.0199
AD, $n=7$	0.992 ± 0.6670	
Hippocampus		
Control, $n=6$	0.537 ± 0.203	0.0177
AD, $n=6$	0.831 ± 0.144	
Total		
Control, $n=25$	0.243 ± 0.314	0.0000359
AD, $n=28$	0.705 ± 0.472	

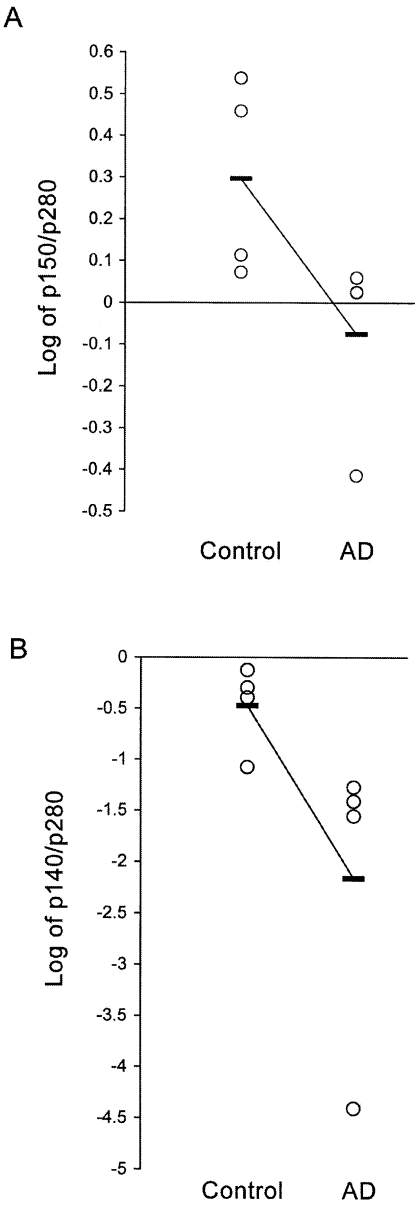

Fig. 2. Comparison of spectrin breakdown products in the human control and AD frontal cortex. The values of log (p150/p280) and log (p140/p280) of each sample are shown in A and B, respectively. The bars represent the mean values of the groups. There are four samples analyzed in each group. P values of Student's t-test in A and B are 0.6172 and 0.1337, respectively.

Real direct proof of p25 accumulation, based on definite chemical analysis, is not available.

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