review, see Mattson, 2010). A recent study genetically and pharmacologically dissected the role of presenilin in Schaffer collateral neurotransmitter release and found that presynaptic loss of presenilin impairs theta-burst-induced LTP through impairment of calcium release (Zhang et al., 2009). This corresponds with our observation that in addition to LTP, paired-pulse facilitation is altered in PS cDKO;WtTau mice, indicating a presynaptic defect. Although these alterations in Ca^{2+} release could also affect Mn^{2+}, our administration of saturating doses of $MnCl_2$ and the lack of axonal transport impairment in PS cDKO mice make it unlikely that such a phenomenon occurred in our study. Our inability to detect LTP deficits in PS cDKO mice could be explained by differences in experimental setup or variation in mouse strains and transgenes between our study and previous reports. Because of the trend toward a decrease in PS cDKO vs PS2 KO mice in our present study, however, it is possible that a statistically significant difference would be observed with an increased sample size or a modified stimulation protocol.

Together, our data support a combined role for presenilin and tau in AD pathogenesis. Substantial evidence supports the notion that familial AD mutations in presenilin genes primarily result in PS loss of function, particularly with regard to γ-secretase cleavage of Notch, cadherins, and APP (Chen et al., 2002; Moehlmann et al., 2002; Wiley et al., 2005). In PS mutants, the cleavage of APP generates β-amyloid species in a ratio that favors the pathogenic Aβ42 over Aβ40, but this could result from decreased production of Aβ40, not excess Aβ42 (for review, see De Strooper, 2007). Presenilin mutations can also impair γ-secretase-independent functions, leading to defects in Wnt signaling and Ca^{2+} homeostasis (for review, see Shen and Kelleher, 2007). This evidence supports the use of PS cDKO mice to study the roles of presenilin and tau in AD pathogenesis. We have established that loss of presenilin alone results in a phenotype that can be enhanced and even expanded by the presence of wild-type human tau, leading to impairment of axonal trafficking, neurotrophin signaling, learning and memory, and synaptic plasticity, all of which culminate in neurodegeneration. These findings provide evidence for the convergence of multiple pathways in the progression of AD and indicate that subtle differences in protein composition (i.e., human vs mouse tau) can dramatically affect cellular processes governing neuronal function and survival.

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PSYCHOGERIATRICS
The Official Journal of the Japanese Psychogeriatric Society

doi:10.1111/j.1479-8301.2010.00330.x PSYCHOGERIATRICS 2010; 10: 117–123

ORIGINAL ARTICLE

The production ratios of AICDε51 and Aβ42 by intramembrane proteolysis of βAPP do not always change in parallel

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Received 15 January 2010; accepted 29 January 2010.

Abstract

Background: During intramembrane proteolysis of β-amyloid protein precursor (βAPP) by presenilin (PS)/γ-secretase, ε-cleavages at the membrane-cytoplasmic border precede γ-cleavages at the middle of the transmembrane domain. Generation ratios of Aβ42, a critical molecule for Alzheimer's disease (AD) pathogenesis, and the major Aβ40 species might be associated with ε48 and ε49 cleavages, respectively. Medicines to down-regulate Aβ42 production have been investigated by many pharmaceutical companies. Therefore, the ε-cleavages, rather than the γ-cleavage, might be more effective upstream targets for decreasing the relative generation of Aβ42. Thus, one might evaluate compounds by analyzing the generation ratio of the βAPP intracellular domain (AICD) species (ε-cleavage-derived), instead of that of Aβ42.

Methods: Cell-free γ-secretase assays were carried out to observe *de novo* AICD production. Immunoprecipitation/MALDI-TOF MS analysis was carried out to detect the N-termini of AICD species. Aβ and AICD species were measured by ELISA and immunoblotting techniques.

Results: Effects on the ε-cleavage by AD-associated pathological mutations around the ε-cleavage sites (i.e., βAPP V642I, L648P and K649N) were analyzed. The V642I and L648P mutations caused an increase in the relative ratio of ε48 cleavage, as expected from previous reports. Cells expressing the K649N mutant, however, underwent a major ε-cleavage at the ε51 site. These results suggest that ε51, as well as ε48 cleavage, is associated with Aβ42 production. Only AICDε51, though, and not Aβ42 production, dramatically changed with modifications to the cell-free assay conditions. Interestingly, the increase in the relative ratio of the ε51 cleavage by the K649N mutation was not cancelled by these changes.

Conclusion: Our current data show that the generation ratio of AICDε51 and Aβ42 do not always change in parallel. Thus, to identify compounds that decrease the relative ratio of Aβ42 generation, measurement of the relative level of Aβ42-related AICD species (i.e., AICDε48 and AICDε51) might not be useful. Further studies to reveal how the ε-cleavage precision is decided are necessary before it will be possible to develop drugs targeting ε-cleavage as a means for decreasing Aβ42 production.

Key words: *AICDε51, Alzheimer's disease, presenilin/γ-secretase, βAPP, γ-cleavage, ε-cleavage.*

INTRODUCTION

The transmembrane domain of β-amyloid protein precursor (βAPP) is proteolysed by presenilin (PS)/γ-secretase.[1] Analysis of the resultant products has shown that the proteolysis proceeds by at least two distinct cleavages. The 'ε-cleavage' liberates its intracellular domain (i.e., βAPP intracellular domain (AICD)) into the cytoplasm, whereas the 'γ-cleavage' releases Alzheimer's disease (AD)-associated amyloid β-protein (Aβ).[2–6]

There are some variations in both the γ- and ε-cleavages of βAPP.[6–8] The major N-termini of AICD species consist of leucine-49, valine-50 and leucine-52 (Aβ-numbering), whereas the major C-termini of Aβ species are comprised of valine-40 and alanine-42. (Fig. 1a).[6] Among these, highly aggregatable Aβ42 is the major component of senile plaques in AD brains.[9]

Are there any relationships between the ε- and γ-cleavages? How do these cleavages occur? Ihara *et al.* have tried to address these questions and recently showed that ε-cleavage precedes γ-cleavage in *in vitro* γ-secretase assays.[10] βAPP-CTF stubs, βAPP membrane-tethered remnants after β-cleavage, first undergo ε-cleavage.[10] The ε-cleavage liberates AICD from the membrane and produces a membrane-bound 48/49 amino-acid-long Aβ species that undergoes further C-terminal truncation by PS/γ-secretase.[11] Stepwise cleavages remove every three amino-acid residues from the C-terminus of the long Aβ species, which finally secretes Aβ40/42.[12–14] For example, mutant PS causes increases in both ε48 and γ42 cleavages.[8] Thus, the γ-cleavage seems to occur in an ε-cleavage-dependent manner.[10] Furthermore, these results show that the production process for pathological Aβ42 is distinct from that of Aβ40.[15] That is, the major ε49 cleavage causes the production of Aβ40, whereas a minor ε48 cleavage causes the production of pathological Aβ42.[14]

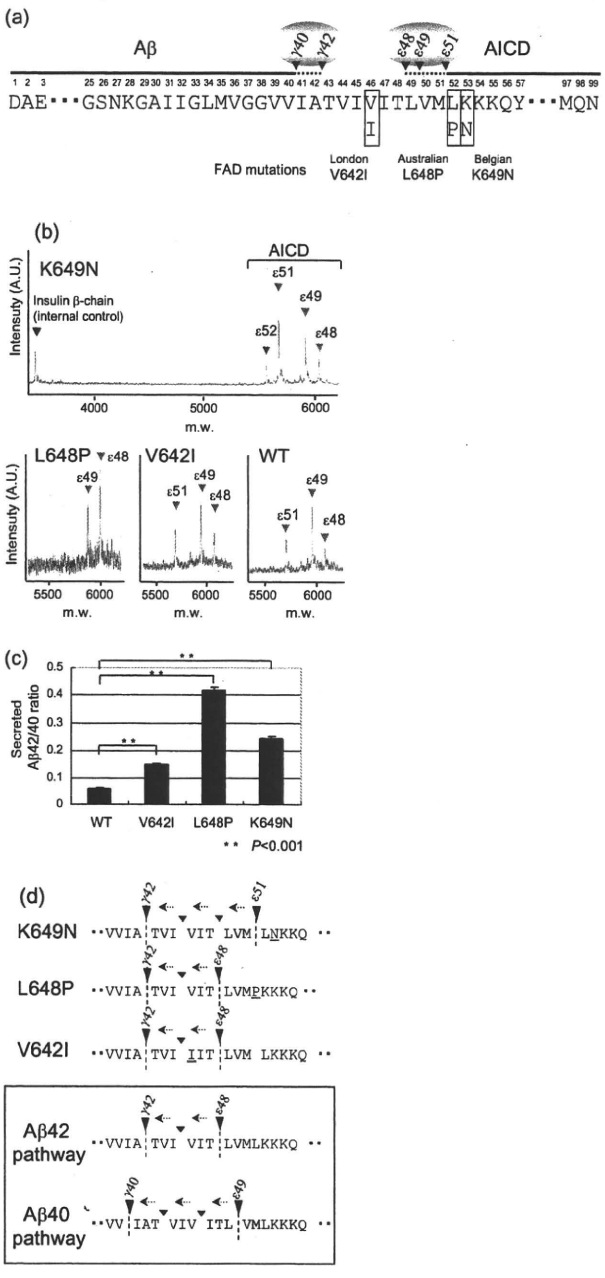

Figure 1 Effect of familial Alzheimer's disease (AD)-associated β-amyloid protein precursor (βAPP) mutations around the ε-cleavage site. (a) Schematic diagram of intramembrane cleavage sites of βAPP and the familial AD mutations used in the present study. The amino acid sequence around the juxta membrane region of human βAPP is described (amyloid β-protein (Aβ) numbering). Filled inverted triangles indicate the cleavage sites. Substituted amino acids of the familial AD mutations are shown in open rectangles. The site of each mutant is also shown using APP695 numbering. (b) Mass spectra of *de novo* βAPP intracellular domain (AICD) species in the cell-free assay. Crude membrane fractions obtained from wild-type (wt) βAPP and the indicated βAPP mutant cells were used. (c) Relative secreted Aβ42 to Aβ40 ratio in the conditioned media of wt βAPP and the indicated βAPP mutant cells. The asterisks indicate statistical significance (*$P < 0.05$, **$P < 0.001$, one-way analysis of variance (ANOVA) and Tukey–Kramer method). Error bars indicate standard error of the mean (SEM). (d) Hypothesis for explaining increased γ42 cleavage in each mutant βAPP (upper panels) and differential production of Aβ40 and Aβ42 (lower panels). FAD, familial Alzheimer's disease; m.w., molecular weight.

Modulation of γ-secretase function to specifically inhibit Aβ42 production is one of the promising strategies for developing drugs to modify the disease course of AD[16]. Given the possible correlation between the ε- and γ-cleavages, we think that targeting the upstream ε-cleavages will be a novel and more efficient method for developing Aβ42-lowering drugs. To test if precision of the ε-cleavage can be used as a novel target for drug development, we investigated the ε-cleavage pathway, particularly ε51 cleavage, which has previously not been well-characterized.[7]

MATERIALS AND METHODS

Cell culture and cDNA constructs

cDNAs of βAPP V642I, L648P and K649N mutants were generated by PCR-based mutagenesis using a Quickchange mutagensis kit (Stratagene, La Jolla, CA, USA) or KOD plus (Toyobo, Osaka, Japan) with wt βAPP695 cDNA as a template. K293 cells were transfected and cultured as previously described.[17]

Membrane preparation

The crude membrane fraction was prepared as previously described with a slight modification.[7,18] In the present study, the homogenization buffer contained 0.25 mol/L sucrose and 50 mmol/L HEPES (pH 7.4) containing a protease inhibitor cocktail (Roche Basel, Switzerland). To prepare the alkaline pretreated membrane, the membrane fraction was suspended in a 50 mmol/L bicarbonate buffer (pH 11.0) and incubated at 4°C for 1 h. The suspension was then centrifuged at 100 000 × *g* for 1 h followed by washing once with a 50 mmol/L Mes buffer (pH 6.0).

Cell-free γ-secretase assay

The cell-free γ-secretase assay was carried out as previously described with a modification.[7,18] The reaction buffer in the present study contained a 150 mmol/L citrate buffer (pH 6.0), 50 mmol/L MES (pH 6.0), 167 mmol/L NaCl and a protease inhibitor mixture comprised a 5x complete protease inhibitor cocktail (Roche), 0.5 mmol/L DIFP (WAKO, Osaka, Japan), 1 μg/ml TLCK (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/ml antipain (Peptide Institute, Osaka, Japan), 10 μg/ml leupeptin (Peptide Institute), 5 mmol/L 1,5 phenanthroline (Sigma-Aldrich), 10 μmol/L amastatin (Peptide Institute), 10 μmol/L bestatin (WAKO), 1 μmol thiorphan (Sigma-Aldrich), 10 μmol/L phosphoramidon (Peptide Institute) and 1 μmol/L pepstatin A (Peptide Institute). To prepare the pH 7.4 buffer, 50 mmol/L HEPES (pH 7.4) was used instead of the citrate and MES buffers.

Immunoprecipitation/MALDI MS analysis

Immunoprecipitation/MALDI MS (IP-MS) analysis followed by cell-free incubation was carried out as previously described.[7,18,19] The heights of the MS peaks and molecular weights were calibrated using angiotensin and bovine insulin β-chain as standards (Sigma-Aldrich).

ELISA analysis for Aβ

Aβ40 and Aβ42 levels in conditioned media were quantified by ELISA (WAKO).

Immunoblotting of Aβ

SDS-solubilized proteins were separated by SDS-PAGE using an 8 mol/L urea gel[17,18,19] and transferred to a nitrocellulose membrane. Immunoblotting of Aβ species using 82E1 (IBL) was carried out as previously described.[20]

RESULTS

The βAPP K649N Belgian mutant increased both the relative ratio of AICDε51 and Aβ42 production in a cell-free γ-secretase assay

To test if the ε51 cleavage precedes the γ42 cleavage, we analyzed the effects of three βAPP mutants (V642I,[21] L648P,[22] and K649N[23]) around the ε-site. The L648P and K649N mutants (βAPP695 numbering) are located downstream of the ε51 site, and the V642I mutant is located upstream of the ε48 site (Fig. 1a). Each of the three mutants is familial AD-associated and therefore increases the relative ratio of Aβ42 production. We raised K293 cells stably expressing each of the mutants, prepared the crude membrane fractions[24] and carried out the cell-free γ-secretase assays.[7,18]

As shown in Figure 1b, the K649N βAPP mutant caused a marked increase in the relative ratio of AICDε51 production (see also Table 1). However, the other two mutants caused completely different effects on the cleavage. The L648P mutant produced a barely detectable level of AICDε51, whereas in the V642I mutant cells, the ratio of AICDε51 production was comparable to that of wild-type (wt) expressing cells. It is of note that, instead of increased AICDε51 pro-

Table 1 Molecular species of β-amyloid protein precursor intracellular domain generated in the cell-free assay

AICD species		m/z Calculated (M + H)	Observed (M + H) Mean	SD
AICDε51 (52–99)	Wild-type	5677.79	5678.38	0.64
	V642I	5677.79	5678.30	0.70
	K649N	5663.74	5663.96	0.23
AICDε49 (50–99)	Wild-type	5907.9	5908.35	0.29
	V642I	5907.9	5908.49	0.21
	L648P	5891.87	5892.48	0.20
	K649N	5893.84	5894.10	0.27
AICDε48 (49–99)	Wild-type	6020.98	6021.36	0.40
	V642I	6020.98	6021.59	0.42
	L648P	6004.96	6005.59	0.33
	K649N	6006.93	6007.51	0.17
AICDε52 (53–99)	K649N	5550.65	5551.01	0.27

M + H, protonated molecular mass.

duction, these V642I and L648P mutants substitutively increased the relative ratio of AICDε48 production. Next we measured Aβ species secretion by the stable cells in conditioned media using ELISA (Fig. 1c). As expected, we observed a significant increase in the ratio of Aβ42 to total Aβ secretion in the conditioned medium of the mutant cells. This data shows that the K649N mutant increased the ratio of Aβ42 production through upregulation of the ε51 cleavage, whereas the V642I and L648P mutants increased Aβ42 production through the ε48 cleavage. Based on these results, we suggest that not only the ε48, but also the ε51 cleavage precedes Aβ42 production, possibly by sequential three amino-acid C-terminal truncation[14] (Fig. 1d).

Incubation in higher pH does not cancel the K649N βAPP mutant effects

We previously found that the precision of ε-cleavage changes depending on the buffer pH.[7,18] The relative ratio of AICDε51 production is the most sensitive to such changes. Therefore, we next determined whether the relative ratio of AICDε51 and/or Aβ42 production by the K649N mutant is affected by changing the buffer pH during the cell-free assay. As expected, incubation in the higher pH (pH 7.4 *vs* pH 6.0) buffer decreased the relative ratio of AICDε51 generation in both the K649N mutant and wt βAPP membrane fraction. However, the pH effect was not so strong as to cancel the AICDε51 upregulation effect by the K649N mutant (Fig. 2a). We further analyzed the pH effects on the increase in the relative ratio of Aβ42 production by the mutant (Fig. 2b). Surprisingly, the assay pH elevation did not cause any changes in the relative ratio of Aβ42 generation. Therefore, unlike the effects of the K649N mutant on the ε51- and γ42-cleavages, the elevation of the buffer pH causes a decrease in the relative ratio of AICDε51 production, but does not cause any changes in Aβ42 production. The data suggest that two distinct mechanisms might contribute to the determination of the relative ratio of AICDε51 production.

Alkali pretreatment of the crude membrane fraction cancels the effect of higher pH cell-free incubation on ε-cleavage

Because the ε51 cleavage occurs at the membrane-cytosol interface, we considered that membrane-bound substances might induce the pH-dependent effects on AICDε51 production. Many substances detach from the membrane on treatment with alkali solution.[25] To test this theory, we washed the wt βAPP membrane fraction in a pH 11 solution (see 'Materials and Methods'), then we carried out the cell-free assay at pH 6.0. The relative ratio of AICDε51 production markedly decreased (Fig. 3a), whereas that of the Aβ42 did not (Fig. 3b). The phenomena are reminiscent of the effects of raising the pH of the incubation buffer (see Fig. 2). Thus, we further considered that the decrease in the AICDε51 production resulting from the use of a higher incubation buffer pH might also be a result of detachment of substances from the membrane. When the membrane fraction was incubated in a pH 7.4 buffer after alkali treatment, we could no

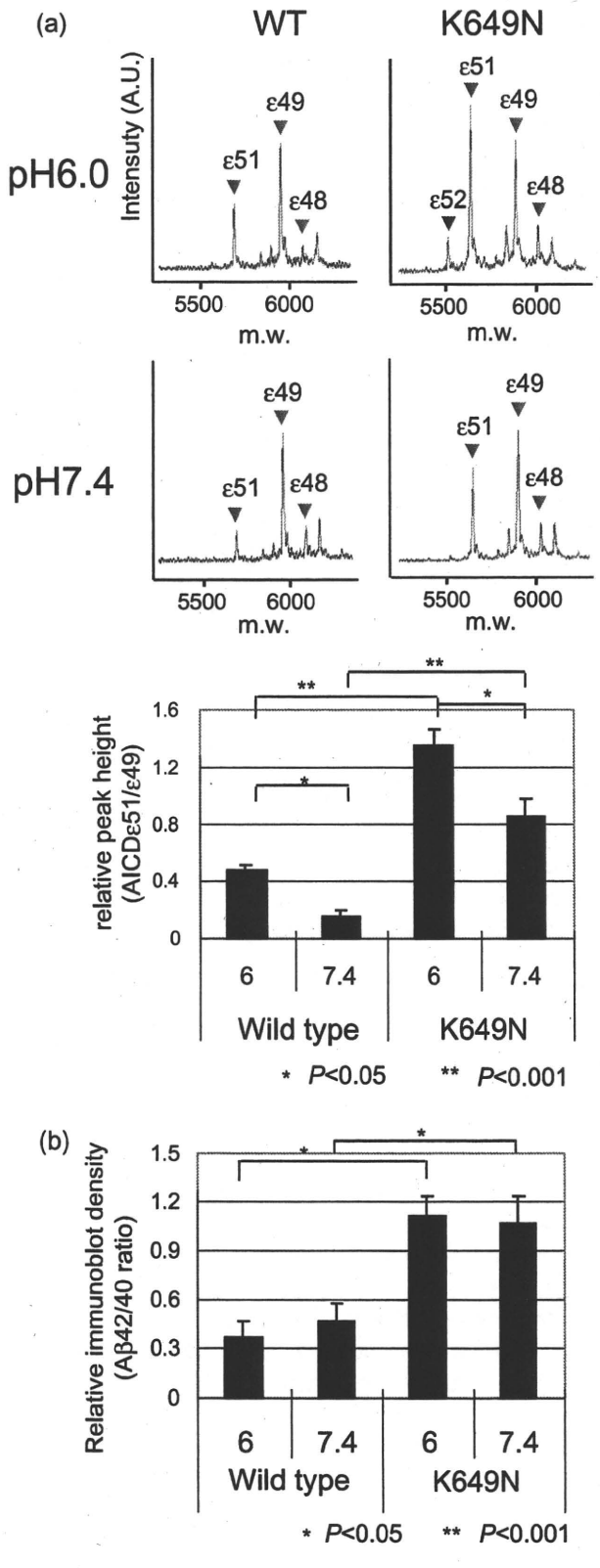

Figure 2 Effect of cell-free incubation pH levels on the precision of ε/γ-cleavages. (a) Mass spectra of β-amyloid protein precursor intracellular domain (AICD) generated in the cell-free assay carried out at the indicated pH (upper and middle panels). Peak heights of AICDε49 and ε51 were measured and the ratios of AICDε49 to ε51 were calculated (lower panel). The asterisks indicate statistical significance (*$P < 0.05$, **$P < 0.001$, one-way ANOVA and Tukey–Kramer method). Error bars indicate SEM. (b) Levels of amyloid β-protein (Aβ) generated at the indicated pH. Levels of Aβ40 and 42 were measured by western blotting and the Aβ42 to 40 ratios calculated. The asterisks indicate statistical significance. Error bars show SEM. m.w., molecular weight.

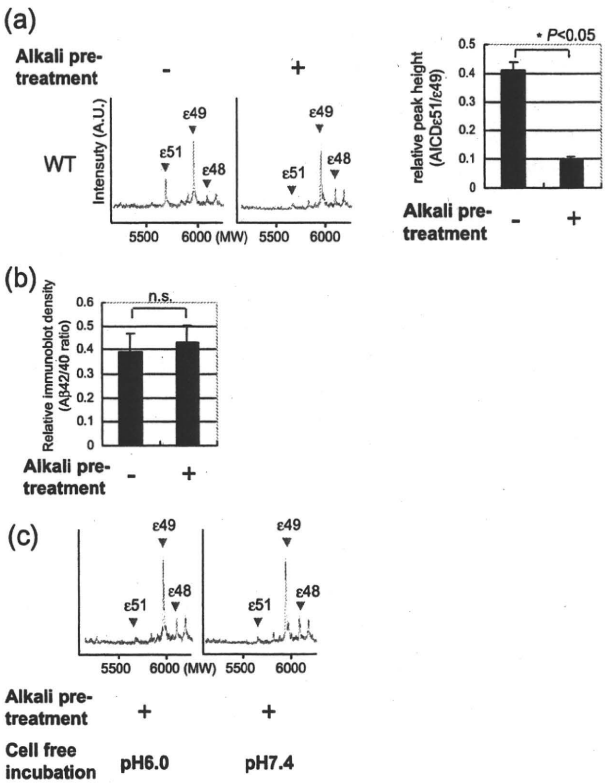

Figure 3 Effect of alkali pretreatment on the precision of ε/γ-cleavages of wild-type (wt) β-amyloid protein precursor. (a) Mass spectra of β-amyloid protein precursor intracellular domain (AICD) generated in the cell-free assay with and without alkali pretreatment. Peak heights of AICDε49 and ε51 were measured and the AICDε49 to ε51 ratios calculated. The asterisk indicates statistical significance (*$P < 0.05$, paired t-test). Error bars indicate SEM. (b) Levels of β-amyloid (Aβ) generated in the cell-free assay after alkali pretreatment. Levels of Aβ40 and 42 were measured by western blotting with and without alkali pretreatment and the Aβ42 to 40 ratios calculated. (c) Mass spectra of AICD generated in the cell-free assay at the indicated pH after alkali pretreatment. MW, molecular weight; WT, wild type.

longer observe the pH-dependent incubation buffer effects on the AICDε51 ratio (Fig. 3c). Collectively, although incubation at lower pH buffer increased in the AICDε51 ratio (Fig. 2a), the effects were cancelled by the alkali pretreatment (Fig. 3a). These results suggest that substances removed by the alkali treatment might induce the changes in the relative ratio of AICDε51 production.

Alkali pretreatment of the crude membrane fraction did not cancel the effects of the K649N mutant on the ε-cleavage

As shown in Figure 1, the K649N βAPP mutation causes upregulation of both the AICDε51 and Aβ42 ratio, whereas alkali pretreatment causes downregulation of only the AICDε51 ratio (Fig. 3). These data show that changes in the AICDε51 ratio caused by the mutation and by the treatment occur by two distinct processes. A further experiment was carried out to confirm whether the K649N mutation cause a change in the relative ratio of AICDε51 production through the effect of the alkali treatment (Fig. 4a). After treatment of the K649N mutant membrane fraction in the alkali solution, the cell-free assay was carried out at pH 6.0. As shown in Figure 4a, even after the alkali treatment, the K649N mutant membrane produced a relatively higher level of AICDε51 than that of the wt fraction (Fig. 3a). Furthermore, the elevated Aβ42 ratio was not changed by the pretreatment (Fig. 4b).

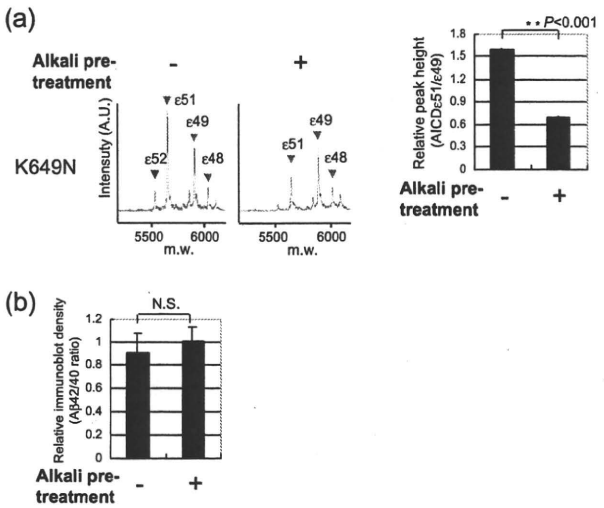

Figure 4 Effect of alkali pretreatment on the precision of ε/γ-cleavages of βAPP K649N Belgian mutant. (a) Mass spectra of β-amyloid protein precursor intracellular domain (AICD) generated in the cell-free assay with and without alkali pretreatment. (b) Levels of of β-amyloid generated in the cell-free assay following alkali pretreatment. N.S, not significant.

DISCUSSION

In the present study, we determined that there are at least two factors that change the precision of ε-cleavage: (i) a process induced by a pathological βAPP mutation; and (ii) another process induced by possibly unidentified substances removed from the membrane fraction by alkali pretreatment. In the case of βAPP mutations, the relative ratio of ε51 and ε48 production increases in parallel with the ratio of AD-associated Aβ42.

It has been reported that ε-cleavage precedes γ-cleavage and γ-cleavage seems to occur in an ε-cleavage-dependent manner.[10] Considering these reports and our own preliminary results, it seemed possible that measurement of the relative ratio of AICDε48/AICDε51 production might help develop Aβ42-lowering anti-AD drugs. Further study showed, however, that the relative level of AICDε51 production is drastically affected by the removal of unidentified substances from the membrane as a result of alkali pretreatment. Interestingly, the alkali pretreatment did not cause any changes in the relative ratio of Aβ42 generation. These results show that changes in the precision of ε-cleavage do not always cause parallel alterations in the precision of γ-cleavage, even though ε-cleavage occurs upstream of the γ-cleavage. Therefore, although measuring the levels of AICD species is a potentially attractive new target for developing Aβ42 lowering compounds, challenges must still be overcome before screening methods for such compounds can be established. For example, the paradoxical mechanism discussed previously must first be understood before an assay in which the ε-cleavage precision accurately reflects the γ-cleavage precision can be developed.

How does alkali pretreatment result in a decreased ratio of AICDε51 production? One might consider the presence of unknown substances that (i) transiently associate with the PS/γ-secretase and affect its intramembrane cleavage precision, or (ii) truncate a couple of N-terminal amino-acid residues of AICD produced by the ε-cleavage. The second possibility is reminiscent of angiotensin-converting enzyme activity to truncate the C-terminus of Aβ42.[26] Of course, the

possibility that alkali pretreatment might change the character of PS/γ-secretase itself also cannot be excluded.

CONCLUSION

Our current data suggest that the precision of ε-cleavage does not always change in parallel with the precision of γ-cleavage, even though ε-cleavage occurs upstream of the γ-cleavage. Thus, to measure the levels of AICD species might be an attractive new target for developing Aβ42 lowering compounds, there still remain some challenges.

ACKNOWLEDGMENTS

M.O and coworkers are funded by the National Institute of Biomedical Innovation (05–26), the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan. The authors declare no competing financial interests.

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Original Research Article

Dementia
and Geriatric
Cognitive Disorders

Dement Geriatr Cogn Disord 2010;30:302–308
DOI: 10.1159/000320482

Accepted: August 16, 2010
Published online: September 25, 2010

KIBRA Genetic Polymorphism Influences Episodic Memory in Alzheimer's Disease, but Does Not Show Association with Disease in a Japanese Cohort

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Key Words
Alzheimer's disease · Episodic memory · Genetics · Neuropsychological assessment · *KIBRA* gene · Rivermead Behavioral Memory Test

Abstract
Background/Aims: A single-nucleotide polymorphism (SNP) in the *KIBRA* gene, rs17070145, was reported to be significantly associated with episodic memory in cognitively normal cohorts. This observation has expanded genetic studies on *KIBRA* to Alzheimer's disease (AD). Importantly, the association between *KIBRA* and episodic memory in AD has never been addressed. In this study, we investigated whether the *KIBRA* rs17070145 SNP influences AD episodic memory and the disease in a Japanese cohort. ***Methods:*** Blood samples from 346 AD patients and 375 normal cognitive controls were collected and genotyped for rs17070145. Episodic memory was measured in 32 AD patients, diagnosed for the first time, by use of the Rivermead Behavioral Memory Test (RBMT). ***Results:*** We found that *KIBRA* C allele carriers scored significantly lower than *KIBRA* non-C carriers on both RBMT total profile score ($p = 0.042$, effect size = 0.84) and RBMT total screening score ($p < 0.001$, effect size = 1.42). The *KIBRA* gene did not show association with AD in our Japanese cohort. ***Conclusion:*** Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD, but show no influence on AD in our Japanese cohort. We propose that *KIBRA* might have an effect similar to cognitive reserve.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by a progressive deterioration of cognitive abilities and memory loss. For the famil-

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ial occurrence of the disease (early-onset), the existence of familial AD-responsible genes has been demonstrated, with mutations in 3 genes, *APP*, *PSEN1* and *PSEN2*, consistently reported. However, the genetic component that underlies sporadic AD (late-onset), which accounts for over 95% of all AD cases, is still poorly understood.

KIBRA (also known as WW and C2 domain-containing protein 1) is a protein mainly expressed in the brain and kidney [1], whose functions are still being characterized, but that, importantly, has been shown to be involved in the control of synaptic plasticity in the brain [2]. Recently, it was reported that KIBRA regulated the Salvador/Hippo/Warts network which restricted tissue size [3]. In 2005, a *KIBRA* gene single-nucleotide polymorphism (SNP), rs17070145, was reported to be significantly associated with episodic memory in 3 independent cognitively normal cohorts from Switzerland and the USA [4]. This result was later confirmed in a German sample of healthy individuals [5], a Japanese sample of healthy individuals [6] and in a cohort in which nearly 50% of individuals had a diagnosis of mild cognitive impairment [7].

These observations led to studies of the *KIBRA* rs17070145 SNP in AD, whose core feature is dysfunction of episodic memory [8]. Recently, Corneveaux et al. [9] reported an association of the *KIBRA* CC genotype (*KIBRA* CC carriers) with increased risk for late-onset AD (n = 702). Conversely, the *KIBRA* T allele (*KIBRA* CT and *KIBRA* TT carriers) was shown to be associated with an increased risk for AD in a Spanish cohort [10]. Despite the available information, *KIBRA* has not yet been established as an AD risk gene, and, importantly, no studies have ever addressed the association between *KIBRA* and episodic memory in AD.

Therefore, in this study, we investigated whether the *KIBRA* SNP rs17070145 influences AD episodic memory and AD in a Japanese cohort.

Methods

Subjects

We collected blood samples from 346 consecutive AD patients who visited Osaka University Hospital between July 27, 2001, and June 10, 2010, and from 375 cognitively normal controls, who were population-based elderly subjects (Suita City, Japan) tested by a questionnaire including the date, orientation and history. Blood samples were collected after written informed consent had been obtained from subjects and/or representatives. This study was approved by the genome ethical committee of the Osaka University Graduate School of Medicine. AD patients met the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria for probable AD [11].

We also have a research-oriented clinic for patients with cognitive impairment in the Department of Neuropsychiatry of the Osaka University Medical Hospital. It is also a clinic for early identification of dementia. In this clinic, all patients were examined comprehensively by specialists of geriatric psychiatry, and they underwent standard neuropsychological examinations including the Mini Mental State Examination (MMSE), routine laboratory tests, electroencephalography, cranial magnetic resonance imaging and radionuclear neuroimaging studies. Blood drawing for the genome study was not routine in this clinic. Eighty first-time diagnosed AD patients agreed to it, and 32 out of them agreed to an additional visit for the memory examination by use of the Rivermead Behavioral Memory Test (RBMT) between September 30, 2002, and May 23, 2007 (RBMT-AD specialized clinic subjects). RBMT-AD specialized clinic subjects were excluded from the study if they (1) had the complication of other neurological diseases, (2) had any evidence of focal brain lesions on magnetic resonance images or of cerebral arterial occlusive lesions on magnetic resonance angiography, or (3) did not have a caregiving family member familiar with their everyday life.

Rivermead Behavioral Memory Test

The RBMT, developed by Wilson et al. [12, 13], is a standardized, validated and reliable test for everyday memory, including personal events, name of persons, newspaper articles, places visited, routes followed, schedules and appointments. It is difficult to assess everyday memory with traditional memory tests [14], but the RBMT differs from conventional tests in that each of its 12 items is an analog of an everyday task, rather than a test based on experimental material, such as paired associates or list of words. The Japanese version of the RBMT was developed by Watamori et al. [15], and its reliability and validity have been previously confirmed [16–19]. Concretely, the authors reported that the RBMT can distinguish AD from both mild cognitive impairment and normal control, and strongly correlates with objective memory tests, such as the Everyday Memory Checklist caregiver rating and Clinical Dementia Rating (CDR) memory domain.

Although the RBMT has 4 parallel forms (A, B, C and D) for repeated uses, only the RBMT-A form was administered to subjects in this study. The subtests of the RBMT are (1) remembering a first name and a surname with a facial portrait, (2) remembering to ask for a personal item belonging to the subject, (3) remembering to ask about an appointment, (4) picture recognition, (5) remembering a short story (immediate), (6) remembering a short story (delayed), (7) face recognition, (8) remembering a new route (immediate), (9) remembering a new route (delayed), (10) remembering to deliver a message (immediate and delayed), (11) orientation for time, place and persons, and (12) date. In 8 of the subtests, i.e. points 1–4, 6, 7, 9 and 10 (delayed), the subjects were instructed to remember the information that they were about to be given. The subtests were then conducted 5–30 min after the information had been given. Subtests 2, 3 and 10 are tests of prospective memory. In subtest 2, the subjects were asked to hand in a personal item at the start of the session and instructed to ask for it at the conclusion of the session. The item was then placed out of sight. In subtest 3, subjects were instructed at the beginning of the test session to remember to ask for their next appointment when they heard a buzzer 20 min later. In subtest 10, they had to remember to de-

Table 1. Characterization of the *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT (n = 12)	TT (n = 20)	p
Mean age ± SD, years	68.5 ± 10.0	72.2 ± 8.1	0.267
Mean age of first abnormal memory loss episode reported by caregivers ± SD, years	63.8 ± 2.6	69.3 ± 2.0[1]	0.104
Male/female, n	5/7	8/12	1.000
APOE ε4+/−, n	10/2	12/8	0.248
CDR score 0.5/1/2, n	1/9/2	3/14/2	1.000
Mean MMSE score ± SD	17.8 ± 2.7	20.4 ± 4.6	0.093
Mean ADAS score ± SD	20.0 ± 5.8	17.6 ± 7.1[1]	0.335
Years of education			
Median	12	10	0.151
IQR	10.25–15	9–14[1]	

p values assessed by t test (continuous variables) and Fisher's exact test (categorical variables). IQR = Interquartile range (Q1–Q3).

[1] One datum was missed.

liver a message in the course of retracing a route around the room. For each subtest of the RBMT, a raw score was given. Then, two kinds of score were produced, a simple pass/fail or screening score ranging in each case from 0 to 1, and a standardized profile score ranging in each case from 0 to 2. A total screening score ranging from 0 to 12 and a total profile score ranging from 0 to 24 were used as indices of overall everyday memory status of the subjects.

Genotyping

Genotyping of *KIBRA* rs17070145 polymorphism was performed by the Taq-Man SNP assay and ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., USA) as previously described [20–23]. The apolipoprotein E *(APOE)* genotype was determined by the PCR-RFLP method [20–23].

Statistical Analysis

Baseline characteristics are presented as means ± standard deviation, medians or interquartile ranges for continuous variables, and frequencies for categorical variables. Comparisons for continuous variables and categorical variables were performed with the t test and χ^2 test or Fisher's exact test, respectively. The analysis of covariance model was used to investigate the effect of treatment on the RBMT scores with the following covariate: presence of the *KIBRA* SNP C allele (*KIBRA* CT and *KIBRA* CC), *APOE* ε4, age, the age of first abnormal memory loss episode reported by caregivers, gender, CDR stage, MMSE score, Alzheimer's Disease Assessment Scale for Japanese cognitive subscale (ADAS-Jcog) and/or years of education. The best set of covariates was selected by using Akaike's information criterion [24]. All tests were two-sided, and the statistical significance level was set at 5%. Statistical analysis was performed with SAS software version 9.02 (SAS Institute, Cary, N.C., USA), and all p values and confidence intervals (CI) presented are the original and were not corrected for multiple testing. Meta-analysis of *KIBRA* CC AD odds ratio and 95% CI was performed by the Der-Simonian-Laird method.

Results

From the RBMT-AD specialized clinic subjects, we found 1 patient with *KIBRA* CC, 11 patients with *KIBRA* CT and 20 patients with *KIBRA* TT (*KIBRA* non-C carriers). *KIBRA* CC and CT groups (*KIBRA* C carriers) were combined because there was only 1 *KIBRA* CC patient and that patient displayed memory performance similar to that of the *KIBRA* CT group (total profile score was 2, total screening score was 0). A lower frequency of the *KIBRA* C allele was observed, which was in accordance with the National Center for Biotechnology Information database of genetic variation (dbSNP) for the Asian population. Most of the patients were in an early stage of dementia (table 1). No significant differences in age, gender, *APOE* ε4, CDR, MMSE score, ADAS score and years of education were found between *KIBRA* C and *KIBRA* non-C carriers.

When analyzing the RBMT scores of the two groups, we found that C carriers scored significantly lower than non-C carriers on both the profile score ($p = 0.042$, effect size = 0.84) and screening score ($p < 0.001$, effect size = 1.42; table 2), evidencing an association of *KIBRA* rs17070145 polymorphism with episodic memory impairment in our Japanese AD cohort. We then assigned RBMT total scores as dependent variables and *KIBRA* C, age, age of first abnormal memory loss episode reported by caregivers, gender, *APOE* ε4, CDR stage, MMSE score, ADAS-Jcog score and/or years of education as independent variables and performed multiple linear regression analysis. For all the different combinations, we selected the appropriate models to which Akaike's information criteria were the smallest [24]. Model 1 was appropriate for total profile score and model 2 for total screening score. *KIBRA* C was found to be significantly associated with both total profile and screening scores after adjustment with the models shown in table 2.

We also analyzed 346 AD patients and 375 cognitively normal controls. As expected, we found significant differences in gender and *APOE* ε4 allele frequencies (table 3). *KIBRA* rs17070145 genotype and allele distribution in control and AD groups are shown in table 4. The genotype frequencies were in accordance with the Hardy-

Table 2. RBMT scores (total profile score and total screening score) between *KIBRA* C carriers and non-C carriers of the RBMT-AD specialized clinic subjects

	CC/CT	TT	p	Effect size
Total profile score (not adjusted)	2.17, 0.60–3.17	4.26, 3.01–5.51	0.042	0.84
Total profile score (model 1)	1.88, 0.42–3.34	4.22, 3.16–5.29	0.012	1.07
Total profile score (model 2)	1.79, 0.31–3.27	4.28, 3.20–5.35	0.010	1.13
Total screening score (not adjusted)	0.10, 0.00–0.36	0.93, 0.58–1.39	<0.001	1.42
Total screening score (model 1)	0.07, 0.00–0.35	0.91, 0.56–1.37	<0.001	1.54
Total screening score (model 2)	0.05, 0.00–0.31	0.93, 0.58–1.40	<0.001	1.66

Scores are expressed as mean estimates, followed by 95% CI. p values assessed by ANCOVA; model 1: adjusted for *APOE* ε4, years of education and ADAS score (this model is appropriate for total profile score); model 2: adjusted for *APOE* ε4, years of education, ADAS score and age (this model is appropriate for total screening score).

Table 3. Characterization of cognitively normal controls (NC) and AD patients

	NC (n = 375)	AD (n = 346)	p
Mean age ± SD, years	75.5 ± 4.9	75.2 ± 8.6	0.600
Male/female, n	170/205	110/236	<0.001
APOE ε4+/–, n	60/315	172/174	<0.001

p values assessed by t test (continuous variable) and Fisher's exact test (categorical variables).

Table 4. rs17070145 genotype and allele distribution in cognitively normal controls (NC) and AD patients

	CC	CT	TT	p^a	p^b	p^c
NC	13 (3.5)	128 (34.1)	234 (62.4)	0.673	0.414	0.694
AD	16 (4.6)	104 (30.1)	226 (65.3)	0.669		

Results are numbers, with percentages in parentheses.
[a] p for Hardy-Weinberg equilibrium tests (Pearson χ^2 test).
[b] p for genotype distribution (Fisher's exact test).
[c] p for allele distribution (Fisher's exact test).

Weinberg equilibrium. The *KIBRA* SNP did not show any association with AD in our Japanese cohort (table 4), even after adjustment for age, gender and *APOE* ε4 (data not shown).

Figure 1 shows KIBRA CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts. Our cohort's KIBRA CC AD odds ratio was 1.35 (95% CI = 0.64–2.85). Meta-analysis of them was not significant (OR = 1.10, 95% CI = 0.92–1.30).

Discussion

Despite numerous reports evidencing association of *KIBRA* with episodic memory, the relevance of *KIBRA* to AD still remains elusive. In our study, we addressed for the first time whether *KIBRA* genetic variation is associated with episodic memory impairment in AD. Our results evidence a strong association between the *KIBRA* gene and episodic memory impairment in AD and suggest a role for *KIBRA* similar to cognitive reserve, with no impact on diagnosis of AD.

There are several memory test batteries available, such as the Auditory-Verbal Learning Test (AVLT) [25], the Revised Wechsler Memory Scale Logical Memory Test [26], the Rey-Osterrieth complex figure [27] and the Takeda Three Colors Combination Test [28]. Association of *KIBRA* rs17070145 with episodic memory was shown for the first time by AVLT in 3 independent cognitively normal cohorts [3], and it has been recently confirmed in a Scottish cohort study (n = 2,091) [29]. In addition, the latter reported no association of the *KIBRA* SNP with the Revised Wechsler Memory Scale Logical Memory Test that rewards relational coding (Lothian Barth cohort, n = 542) [29], suggesting that *KIBRA* is not specific for complex episodic memory such as the Revised Wechsler Memory Scale Logical Memory Test but for simple episodic memory such as the AVLT instead. In our study, we

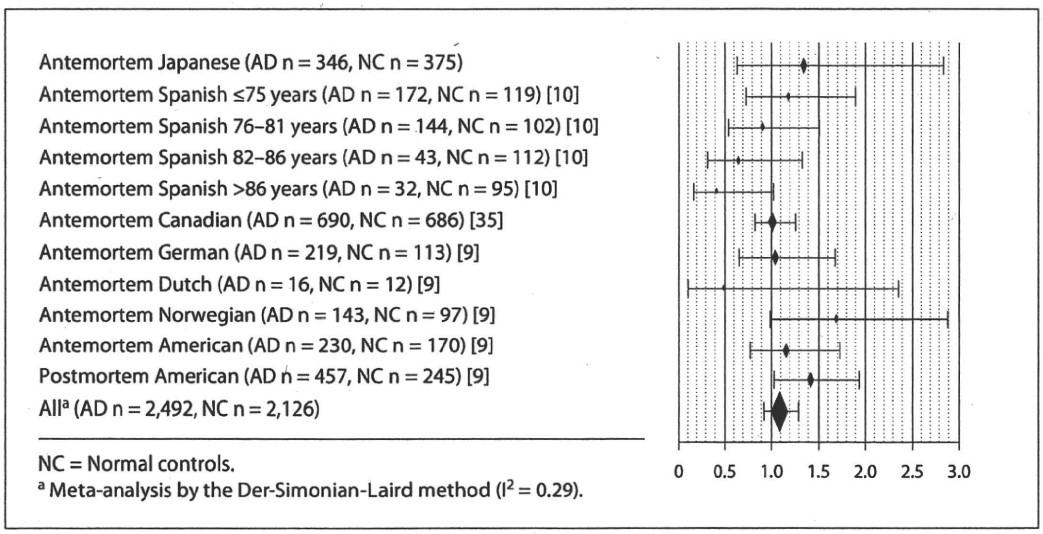

Fig. 1. *KIBRA* CC AD odds ratio and 95% CI in our Japanese cohort and previously reported cohorts.

used the RBMT, which assesses multidimensional aspects of everyday memory such as orientation, prose recall, visual recognition, prospective memory and so on. It also includes both complex episodic memory and simple episodic memory. Our results show that the *KIBRA* rs17070145 polymorphism is strongly associated with episodic memory impairment in our specialized clinic Japanese AD cohort (table 2). MMSE and ADAS-cog showed no differences between *KIBRA* C and non-C carriers (table 1). These results suggest that the *KIBRA* gene specifically seems to affect memory functions but not global cognitive status.

This association remained significant after adjustment for covariant components, which indicates that the *KIBRA* SNP might be an independent risk factor for episodic memory impairment.

Whereas an association of *KIBRA* with episodic memory has been repeatedly reported [4, 5, 7, 29], the impact of *KIBRA* on AD is still controversial (fig. 1). In a Spanish cohort, *KIBRA* CC AD odds ratio decreased continuously with age. *KIBRA* CC AD patients perhaps had earlier onsets and died soon. Hence, we tested the association between *KIBRA* and age of onset and course of the disease in RBMT-AD specialized clinic subjects. We defined the age of first abnormal memory loss episode reported by caregivers as onset age of AD. Although we found no significant differences between *KIBRA* C carriers and non-C carriers, the age of first abnormal memory loss episode reported by caregivers tended to be later in *KIBRA* non-C carriers (table 1). It is possible that the presence of the *KIBRA* T allele delays diagnosis of some AD clinical symptomatology. This effect could be similar to the well-reported effect of cognitive reserve, reflected in years of education. Highly educated individuals have better cognitive performance and, thus, tend to be judged as cognitively normal, albeit AD neuropathology is already present [30–32]. On the other hand, it appears that AD symptomatology progresses faster in people with higher education once AD is diagnosed [33]. Incidentally, in our cohort, duration from first memory loss episode to AD diagnosis was significantly shorter in the *KIBRA* TT group (2.6 ± 1.7 vs. 4.7 ± 1.2 years, $p = 0.001$). We propose that *KIBRA* might have an effect similar to cognitive reserve, particularly in simple word recall.

The impact of *APOE*, an established AD risk gene that accelerates AD brain pathology, on episodic memory was also examined. A recent study reported no differences, suggesting that *APOE* ε4 does not influence episodic memory (AVLT delayed recall) in cognitively normal individuals under 60 years of age [34]. In accordance, our results evidenced no significant differences in both RBMT total profile score (*APOE* ε4–: 3.40 ± 2.12 vs. *APOE* ε4+: 3.64 ± 3.16; $p = 0.831$) and RBMT total screening score (*APOE* ε4–: 0.80 ± 0.63 vs. *APOE* ε4+: 0.86 ± 1.17; $p = 0.873$) between *APOE* ε4 carriers and non-*APOE*-ε4 carriers. This lack of correlation between *APOE* and the episodic memory is intriguing and is in contrast with our findings for *KIBRA*, which seems to

have a less certain effect on AD but a more significant impact on episodic memory in young [4] and elderly subjects [4, 5, 7, 29] and even in mild AD patients, as our study shows (table 2). It is possible that *KIBRA* does not have a direct impact on AD neuropathology but could have an effect on the clinical diagnosis of AD, in a manner similar to cognitive reserve.

Compared to many reports based on Caucasian samples, our cohort evidenced lower frequencies of *KIBRA* CC. Thus, comparison of our results with those based on Caucasian samples must be carried out with caution. As our research-oriented clinic is specialized in the early identification of dementia, we should take selection bias into consideration. Further studies with larger samples, including cognitive functional and pathological data, will be carried out in the future in order to clarify the importance of the *KIBRA* SNP for episodic memory and AD pathology.

Acknowledgements

This work was supported by a Kakenhi (Grant-in-Aid for Scientific Research) on priority areas applied genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Health and Labor Sciences Research Grants for a research on dementia (Dementia-General-003) from the Ministry of Health, Labor and Welfare of Japan.

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Neuroscience Letters 484 (2010) 43–46

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Neuroscience Letters

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Post-treatment of a BiP inducer prevents cell death after middle cerebral artery occlusion in mice

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ARTICLE INFO

Article history:
Received 25 May 2010
Received in revised form 3 August 2010
Accepted 6 August 2010

Keywords:
Apoptosis
ER stress
Immunoglobulin heavy chain binding protein (BiP)
Ischemia
Mouse

ABSTRACT

We previously reported the effect of a selective inducer of BiP (a BiP inducer X; BIX) after permanent middle cerebral artery occlusion (MCAO) in mice. However, in acute stroke, almost all drugs have been used clinically after the onset of events. We evaluated the effect of post-treatment of BIX after permanent MCAO in mice, and examined its neuroprotective properties in *in vivo* mechanism. BIX (intracerebroventricular injection at 20 μg) administered either at 5 min or 3 h after occlusion reduced both infarct volume and brain swelling, but at 6 h after occlusion there was no reduction. BIX protected against the decrease in a dose-dependent manner. Furthermore, BIX reduced the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells induced by the ischemia in ischemic penumbra. These findings indicate that post-treatment with BIX after ischemia has neuroprotective effects against acute ischemic neuronal damage in mice even when given up to 3 h after MCAO. BIX may therefore be a potential drug for stroke.

Stroke is the third most common cause of death after heart attack and cancer, and it has profound negative social and economic effects. The only preventive treatment for stroke is anti-platelet therapy for patients with transient ischemic attack or stroke, which produces a modest but clinically worthwhile benefit [3]. In acute stroke, only a small fraction of patients benefit from intravenous administration of recombinant tissue plasminogen activator (t-PA), which is the only drug with proven effectiveness in reducing the size of infarct in humans [1,17].

BiP is one of the molecular chaperones localized to the ER membrane, and is a highly conserved member of the 70-kDa heat shock protein family [10,11]. It has been reported that the expression of BiP, an endoplasmic reticulum (ER) molecular chaperone, was upregulated by ischemia in focal and global transient ischemia models [7,16,19]. Furthermore, previous reports showed that the induction of BiP prevents neuronal death induced by ER stress [8,14,15,21]. By contrast, inhibition of GRP78 (78 kDa glucose-regulated protein) mRNA induction increases cell death in response to calcium release from the ER, oxidative stress, hypoxia, and T-cell-mediated cytotoxicity [4,13,18]. Therefore, BiP activators will be effective agents against cerebral ischemia.

ER stress, which is caused by an accumulation of unfolded proteins in the ER lumen, is associated with stroke and with neurodegenerative diseases such as Parkinson's and Alzheimer's. An earlier study showed that pretreatment with BIX (intracerebroventricular injection at 5 or 20 μg) protects cells from ER stress [9]. BIX is an inducer of BiP mRNA found by using a BiP reporter assay system [9]. Our previous report showed that BIX selectively induces BiP in SK-N-SH cells and pretreatment with BIX (intracerebroventricular injection at 5 or 20 μg) reduces the area of infarction and the neuronal cell death due to focal cerebral ischemia in mice [9].

In the present study, we examined the neuroprotective effects of post-treatment with BIX on infarction, brain swelling, neurological deficits, and apoptosis in a murine permanent focal cerebral ischemia model.

The experimental designs and all procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University, and approved by the Animal Experimental Committee of Gifu Pharmaceutical University. All *in vivo* experiments were per-

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doi:10.1016/j.neulet.2010.08.015

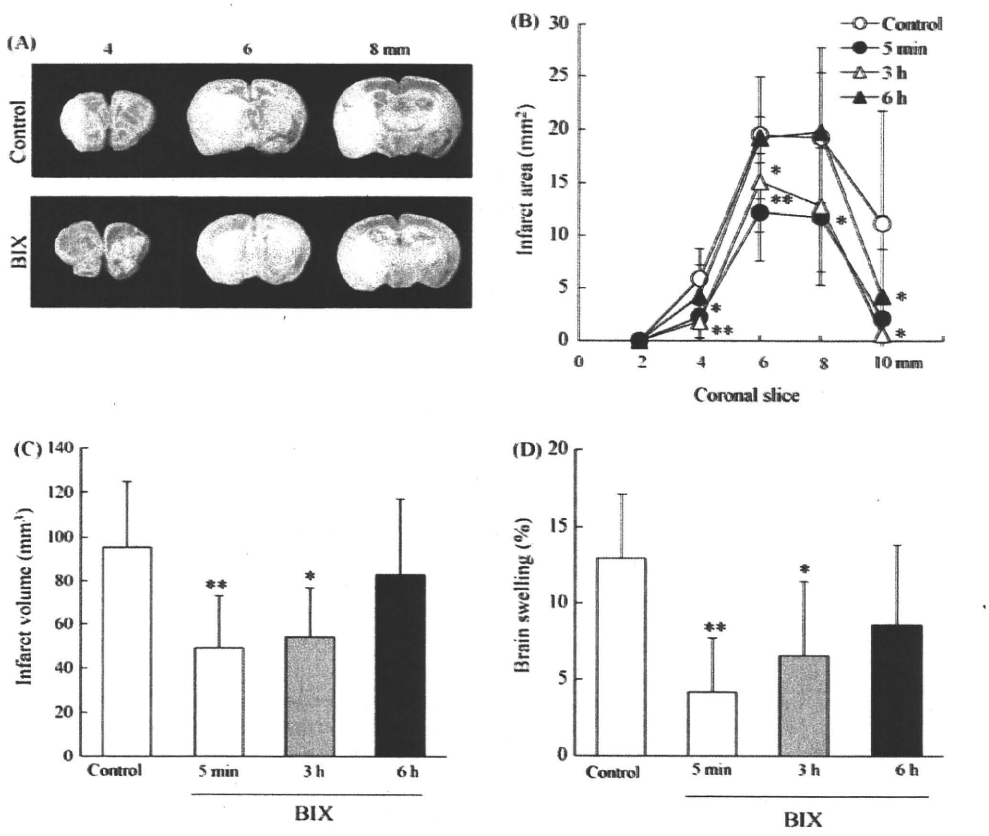

Fig. 1. Effects of BIX administered after ischemia on a therapeutic window after MCAO in mice. (A) TTC staining of coronal brain sections (2 mm thick) at 24 h after permanent MCAO in representative mice. Upper panels, vehicle-injected (control) mice. Lower panels, BIX (intracerebroventricular injection at 20 μg)-treated mice. (B) Brain infarct area measured at 24 h after MCA occlusion. Brains were removed and the forebrains sliced into five coronal 2 mm sections. $^{*}P<0.05$, $^{**}P<0.01$ vs. control ($n=7$–11). (C and D) Effects of BIX on infarct volume and brain swelling (measured at 24 h after MCAO). Values are expressed as the mean ± S.D. $^{*}P<0.05$, $^{**}P<0.01$ vs. control ($n=7$–11).

formed using male adult ddY mice (body weight 26–32 g; Japan SLC Ltd., Shizuoka, Japan). The animals were housed at 24 ± 2 °C under a 12 h light/dark cycle (lights on from 07:00 to 19:00). Each animal was used for one experiment only.

Anesthesia was induced using 2.0–3.0% isoflurane, and maintained using 1.0–1.5% isoflurane (both in 70% N_2O/30% O_2) by means of an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at 37.0–37.5 °C with the aid of a heating pad and heating lamp. After a midline skin incision, the left external carotid artery was exposed, and its branches were occluded **[5,6]**. An 8-0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with a mixture of silicone resin (Xantopren; Bayer Dental, Osaka, Japan) was introduced into the left internal carotid artery through the common carotid artery so as to occluded the origin of the middle cerebral artery. Then, the left common carotid artery was occluded. After the surgery, the mice were kept in the preoperative condition (room temperature; 24 ± 2 °C) until sampling.

BIX was dissolved in 10% DMSO, and fresh solution was made daily. Two microliters of vehicle (10% DMSO in saline) or BIX 1, 5 or 20 μg was administered intracerebroventricularly at 5 min, 3 h, and 6 h after ischemia. Used animals were divided into each group so as not to make significant differences in average body weight.

To analyze infarct volume, mice were euthanized using sodium pentobarbital at 24 h after MCAO, and forebrains were sectioned coronally into five slices (2 mm thick). These were placed in 2% TTC at 37 °C for 30 min. The infarcted areas and volumes were recorded as images using a digital camera (Coolpix 4500; Nikon, Tokyo, Japan), then quantified using Image J, and calculated as in our previous report **[5]**. Brain swelling was calculated according to the following formula: (infarct volume + ipsilateral undamaged volume − contralateral volume) × 100/contralateral volume (%) **[6]**. To minimize potential bias in infarct volume assessment, the investigator who analyzed the cerebral infarction was blinded.

Mice were tested for neurological deficits at 24 h after MCAO. Scoring was done as described in our previous study **[6]**, using the following scale: (0) no observable neurological deficits (normal); (1) failure to extend the right forepaw (mild); (2) circling to the contralateral side (moderate); and (3) loss of walking or righting reflex (severe). The investigator who rated the mice was blinded as to the group to which each mouse belonged.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals Inc., Mannheim, Germany). Ischemic areas of cortical brain sections 0.4–1.0 mm anterior to bregma (through the anterior commissure) were excised and used. The brains were removed, fixed overnight in 4% paraformaldehyde, and immersed for 1 day in 25% sucrose with phosphate-buffered saline (PBS). The brains were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek). Cerebral sections 20 μm thick were cut on a cryostat at −25 °C, and stored at −80 °C until staining. After washing twice with PBS, sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37 °C for 1 h. The sections were washed three times in PBS for 1 min at room temperature. Sections were subsequently incubated with an anti-fluorescein antibody-peroxidase conjugate at room temperature in a humidified chamber for 30 min, and then developed using DAB tetrahydrochloride peroxidase substrate. To quantify the number of DNA-fragmented cells present after MCAO, the numbers of

TUNEL-positive cells such as necrotic and apoptotic cells) in the caudate-putamen (as the ischemic core) and cortex (as the ischemic penumbra, two areas) were counted in a high-power field (×200) on a section through the anterior commissure by a blinded investigator. Each count was expressed as number/mm^2 (n = 7).

Data are presented as the mean ± S.D. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's test and Mann–Whitney U-test (using STAT VIEW version 5.0: SAS Institute, Cary, NC). $P < 0.05$ was considered to indicate statistical significance.

Using TTC staining, we examined whether BIX would reduce infarct volume. Twenty-four hours after MCAO, the mice had developed infarcts affecting the cortex and striatum (**Fig. 1**A). When administered at 5 min or 3 h after MCAO, BIX significantly reduced the infarct area, infarct volume, and brain swelling, but had no such effect when administered at 6 h (**Fig. 1**B and D). Our previous results indicate that the induction of BiP by BIX was transient, peaking at 4 h after treatment, but the levels of BiP protein continued to increase until 12 h **[9]**. Although some drugs for cerebral infarction are permitted for clinical use, most drugs aim to improve the CBF. These drugs must be administered in the early phase of infarction; otherwise they cause adverse effects. For example, t-PA, a most remarkable therapeutic agent for cerebral infarction, must be administered within 3 h after the onset of the infarction, but sometimes causes serious complications such as cerebral hemorrhage **[20]**. In our previous report, we checked the physiological parameters between control and BIX-treated in permanent MCAO model, and there were no significant differences **[9]**. It would appear that BIX could save neurons from cell death even if it was given as late as 3 h after the onset of ischemia.

At 24 h after MCAO, an ischemic zone was consistency identified in the cortex (penumbra area) and subcortex (core area) of the left cerebral hemisphere. By measuring infarction, we noted that BIX significantly reduced both the infarct area and the volume in a dose-dependent manner (**Fig. 2**A and B). Moreover, BIX (intracerebroventricular injection at 20 μg) improved neurological deficits (**Fig. 2**C). When BIX was administered at 5 min after MCAO, it exhibited dose-dependent neuroprotective effects and, with a dose of 20 μg, reduced both the infarct volume and the neurological deficits significantly. BIX induced BiP mRNA in a concentration-dependent manner; its effects were significant at 1–50 μM **[9]**. Furthermore, BIX does not induce other ER stress-associated signals (such as XBP-1 splicing or CHOP), and does not evoke ER stress. In cultured human neuroblastoma SK-N-SH cells, BIX at 5 μM inhibited tunicamycin (Tm)-induced cell death **[9]**. Even a high dosage of BIX at 50 μM did not induce BiP mRNA mediated by non-activating transcription factor 6 pathways **[9]**. These results imply that the mechanism of BiP induction utilized by BIX may be different from those used by ER stressors, such as thapsigargin and Tm. It has been reported that the activation of transducers of ER stress is caused by dissociation of BiP from luminal domains of PKR (protein kinase regulated by RNA)-like ER-associated kinase and inositol-requiring kinase 1 **[2]**. It may be assumed that the artificial induction of BiP induced by BIX disturbs the activation of transducers of ER stress, because abundant BiP remains bound to these transducers preventing their activation.

The morphological features of TUNEL-stained cells (indicative of the ischemic damage and apoptotic cell death induced by 24 h MCAO) are shown in **Fig. 3**B. Cells exhibiting shrunken cell bodies and condensed nuclei were distributed in both the ischemic core and penumbra of the territory affected by MCAO, with the TUNEL-positive cells being among the population displaying such features. Lei et al. **[12]** reported that numerous cells in the penumbra were TUNEL-positive at 24 h after MCA occlusion, while only a small number of cells in the core were TUNEL-positive at that time. In this study, TUNEL-positive cells (necrotic and apoptotic cells) were

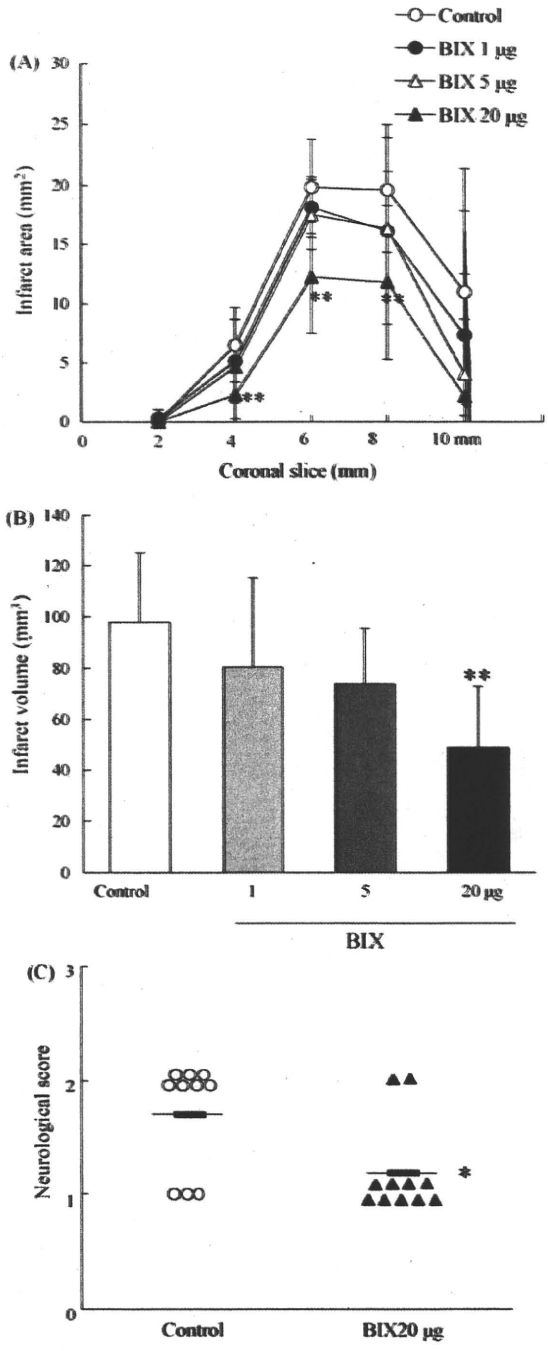

Fig. 2. Effects of BIX administered at 5 min after ischemia on infarction and neuronal damage at 24 h after MCAO in mice. (A) Brain infarct area measured at 24 h after MCA occlusion. Brains were removed and the forebrains sliced into five coronal 2 mm sections. *$P < 0.05$, vs. control (n = 7–11). (B) Effects of BIX on infarct volume (measured at 24 h after MCAO). BIX protected against the decrease in a dose-dependent manner. Values are expressed as the mean ± S.E. **$P < 0.01$ vs. control (n = 7–11). (C) Effects of BIX on neurological score (assessed at 24 h after MCAO). Values are expressed as the mean ± S.D. *$P < 0.05$ vs. control (n = 10–11).

predominantly located in the ischemic core region rather than in the ischemic penumbra, and BIX significantly reduced the number of TUNEL-positive cells in the ischemic penumbra (**Fig. 3**C). We next distinguished apoptotic cells from necrotic cells, and each type was counted. Only densely labeled cells showing cell shrinkage, chromatin condensation, and fragmented nuclei indicating apoptosis were considered to be apoptotic cells, whereas cells with light dif-

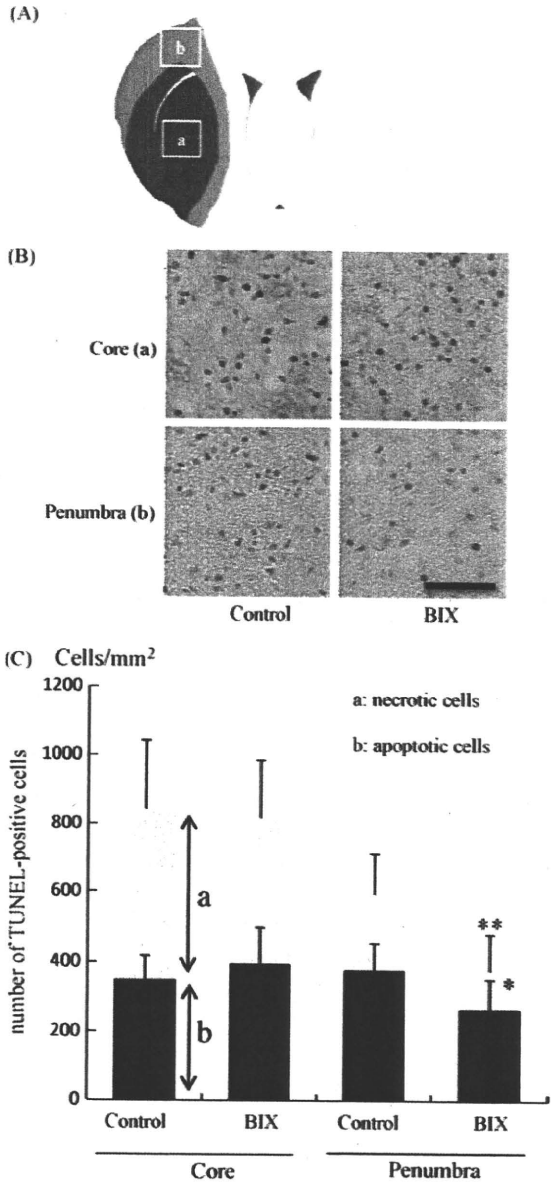

Fig. 3. Effects of BIX on TUNEL staining at 24 h after MCAO in mice. (A) Schematic drawing showing brain regions at 0.4–1.0 mm anterior to bregma (through the anterior commissure): measurement areas (a and b) in **Fig. 1**A. (B) Representative photomicrographs of TUNEL-positive cells in ischemic core and penumbra. Scale bar = 50 μm. (C) Quantitative analysis of TUNEL-positive cells (necrotic and apoptotic cells). TUNEL-positive cells were significantly reduced in ischemic penumbra at 24 h after MCAO. (a and b): the number of necrotic cells and apoptotic cells, respectively, among all positive cells. Values are expressed as the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control ($n = 7$).

fuse labeling were taken as necrotic. BIX reduced the number of necrotic and apoptotic cells significantly in the ischemic penumbra area at 24 h after MCAO (**Fig. 3**C).

In conclusion, we suggest that post-treatment of BIX, even if it was administered at 3 h after ischemia, provides significant protection against focal ischemia and has a wide therapeutic window. Hence, drugs which selectively induce BiP may exert a neuroprotective effect and may be the seeds of new treatment of stroke.

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