Conversion of Aβ43 to Aβ40 by the successive action of angiotensin-converting enzyme 2 and angiotensin-converting enzyme

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Running title: ACE2 and ACE convert Aβ43 to Aβ40

Abstract

The longer and neurotoxic species of amyloid β-protein (Aβ), Aβ42 and Aβ43, contribute to Aβ accumulation in Alzheimer's disease (AD) pathogenesis and are considered to be the primary cause of the disease. In contrast, the predominant secreted form of Aβ, Aβ40, inhibits amyloid deposition and may have neuroprotective effects. We have reported that angiotensin converting-enzyme (ACE) converts Aβ42 to Aβ40 and that Aβ43 is the earliest-depositing Aβ species in the amyloid precursor protein (APP) transgenic mouse brain. Here we found that Aβ43 can be converted to Aβ42 and to Aβ40 in mouse brain lysate. We further identified the brain Aβ43-to-Aβ42-converting enzyme as ACE2. The purified human ACE2 converted Aβ43 to Aβ42 and this activity was inhibited by a specific ACE2 inhibitor, DX600. Notably, the combination of ACE2 and ACE could convert Aβ43 to Aβ40. Our results indicate that the neurotoxic longer forms of Aβ can be converted to the shorter less toxic or neuroprotective forms of Aβ by ACE2 and ACE. Moreover, we also found that ACE2 activity showed a tendency to decrease in the serum of AD patients compared with normal controls, suggesting an association between lower ACE2 activity and AD. Thus, maintaining brain ACE2 and ACE activities may be important for preventing brain amyloid neurotoxicity and deposition in Alzheimer's disease.

Keywords

Alzheimer's disease; amyloid β-protein; angiotensin-converting enzyme; angiotensin-converting enzyme 2.

Introduction

Alzheimer's disease (AD) is a progressive, ultimately fatal degenerative disease of the brain and is the most common cause of dementia in the elderly. A defining neuropathological hallmark is the presence of senile plaques due to the aberrant accumulation of amyloid β-protein (Aβ), which is composed mainly of 40-43 amino acid species. Aβ is generated from the cleavage of amyloid precursor protein (APP) through successive proteolytic processing by the aspartic proteinases, β- and γ-secretase. β-secretase removes the large APP ectodomain and leaves a membrane-bound C-terminal fragment (β-CTF), which is then subsequently hydrolyzed by γ-secretase (Haass and Selkoe 2007; Masters and Selkoe 2012).

Aβ42 and Aβ40 are the main components of senile plaques, with Aβ42 being more neurotoxic as a result of its higher hydrophobicity, which leads to faster oligomerization and aggregation (Blennow et al. 2006; Brouillette et al. 2012). In contrast, some studies suggest that Aβ40 inhibits amyloid deposition in vitro and in vivo and has a neuroprotective effect (Kim et al. 2007; Zou et al. 2003). Recent studies have revealed that another longer Aβ species, Aβ43, emerges as important in the pathogenesis of AD. Aβ43 deposition is found more frequently than Aβ40 in both sporadic Alzheimer's disease (SAD) brain and familial Alzheimer's disease (FAD) brain (Iizuka et al. 1995;

Welander et al. 2009). Moreover, Aβ43 shows a higher propensity to aggregate and is more neurotoxic than Aβ42 (Saito et al. 2011) and we have demonstrated that Aβ43 is the earliest depositing Aβ species in APP transgenic mouse (Zou et al. 2013). Another study shows that even small elevations in the ratio of Aβ42 (43) to total Aβ in secreted Aβ due to presenilin (PS, a component of the γ-secretase complex), or APP, mutations may trigger the onset of FAD (Okochi et al. 2013). Thus, converting the longer and more toxic Aβ43 to the neuroprotective Aβ40 may be a novel therapeutic strategy for AD treatment.

Our previous studies have described that angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase, which participates in and plays an important role in the renin-angiotensin system (RAS), cleaves two amino acids from the C-terminus of Aβ42 and converts it to Aβ40 (Zou et al. 2009; Zou et al. 2007). However, current knowledge on the conversion of Aβ is quite limited as compared with the extensive information available concerning the generation or degradation of Aβ. Therefore, we further investigated whether any enzymes are conducive to producing Aβ40 from Aβ43. Interestingly, we found that angiotensin-converting enzyme 2 (ACE2), which is also distributed in brain (Xia and Lazartigues 2008), could convert Aβ43 to Aβ42 and that the combination of ACE2 and ACE converted Aβ43 to Aβ40.

ACE2 is a zinc metalloprotease comprising 805 amino acids and the ACE2 gene maps to a hypertension-related trait on the X chromosome (Tipnis et al. 2000). The ectodomain of human ACE2 is shed by ADAM17 from the membrane to the extracellular space (Lai et al. 2011; Lambert et al. 2005). As a homologue of ACE, ACE2 is also membrane bound, but it is a monocarboxypeptidase that generates angiotensin 1-9 (Ang1-9) and angiotensin 1-7 (Ang1-7) from the decapeptide angiotensin I (Ang I) and the octapeptide angiotensin II (Ang II), respectively (Crackower et al. 2002; Donoghue et al. 2000; Tipnis et al. 2000). ACE 2 has direct effects on cardiac function, and is expressed predominantly in vascular endothelial cells (Danilczyk and Penninger 2006; Katovich et al. 2005). In addition, in spite of a great deal of information that has described ACE2 as a new component of RAS in the brain, which participates in the regulation of cardiovascular and metabolic functions (Xia et al. 2009), there have not been any clear studies addressing the impact of this enzyme on conversion or degradation of Aβ. Our present results showed that ACE2 is involved in Aβ43 conversion, suggesting that brain ACE2 may play an important role in the conversion of a longer and more toxic Aβ species to a shorter and neuroprotective Aβ species.

Materials and Methods

Mice and tissue preparation

C57BL/6J mice were fed with standard mouse chow and water at the Iwate Medical University Animal Center. All mice were bred on a 12-hour light/dark schedule with adlibitum access to food and water. Five-month-old mice were killed by inhalation of CO_2. The brain and other tissues were homogenized in an equal volume (w/v) of lysis buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5% NP-40) and centrifuged at 10,000 rpm, 4 °C for 10 min. The supernatants were then collected to examine enzymatic activity. To examine the protein expression of ACE2, a proteinase inhibitor cocktail (Roche, Mannheim, Germany) containing EDTA was added into the lysis buffer. All animal procedures were approved by the Iwate Medical University Committee for Animal Use.

Purification of recombinant secreted ACE2 protein

ACE2 cDNA minus the transmembrane and C-terminal domains is designated as soluble ACE2 (sACE2). The cDNA encoding sACE2 was conjugated to a C-terminal FLAG tag and cloned into a pCI-neo expressing plasmid. The plasmids were then transfected into HEK293 cells and the cells constantly expressing sACE2 were used for

purification. Using anti-FLAG M2 Gel (Sigma, Saint Louis, MO, USA), sACE2 was purified by affinity purification from the culture media of HEK293 cells. The purified sACE2 protein was dialyzed in 10 mM HEPES, 50 mM NaCl, 1 μM $ZnCl_2$, pH 7.5 and then concentrated with Amicon Ultracel 50 k (Millipore, Carrigtwohill, Ireland). The purity of the sACE2 protein was examined by SDS-PAGE and Coomassie staining.

ACE2 activity assay

ACE2 activity was measured by a SensoLyte 390 ACE2 activity assay kit (AnaSpec, San Jose, CA, USA), using Mc-Ala/Dnp fluorescence resonance energy transfer peptide. The fluorescence of Mc-Ala was monitored at excitation/emission 330 nm/390 nm. All reactions were carried out in duplicate in 96-well, clear, flat-bottom polystyrene microplates (Greiner bio-one, Frickenhausen, Germany) at a final volume of 100 μl. Specificity was confirmed by use of the ACE2 inhibitor DX600 (AnaSpec). To determine the ACE2 activity, human serum was collected as described previously (Zou et al. 2013). The age-matched normal controls consisted of 10 males and 8 females with no known neurological disorder whose average age was 75.1 ± 1.8 (means ± SEM). The 28 patients with AD included 11 males and 17 females had a mean age of 75.4 ± 1.2 (means ± SEM). The clinical diagnosis of AD was based on NINCDS-ADRDA

Alzheimer's Criteria. The mean Mini-Mental State Examination (MMSE) score (means ± SEM) of AD patients was 18.6 ± 1.0. This study was examined and approved by the Ethics Committee of Iwate Medical University hospital.

Measurement of angiotensin II in human serum

Angiotensin II levels in human serum were measured by enzyme-linked immunosorbent assay (ELISA) (Sigma). Biotinylated angiotensin II peptide was mixed with human serum and the mixture was added into an anti-angiotensin-II-antibody-coated plate. The bound (uncompeted) biotinylated angiotensin II peptide in the samples interacted with streptavidin-horseradish peroxidase (SA-HRP), which catalyzed a color development reaction. The plates were then washed with the washing buffer before incubating with 3, 3', 5, 5'- tetramethylbenzidine (TMB) solution under light-protected conditions at room temperature for 15 min. The absorbance value was measured at 450 nm. All samples were measured in duplicate.

Immunohistochemistry

The brain sections from 8 months old mice were prepared as previously described (Zou et al. 2007). Immunostaining was performed using an anti-ACE2 polyclonal antibody

(Biochain, Newark, CA, USA, 1:50 dilution) and an Alexa Fluor 568-tagged secondary antibody (Invitrogen, Carlsbad, CA, USA, 1:200 dilution).

Western blot

To examine ACE2 protein expression, the protein was subjected to SDS-PAGE using a 5-20% gradient gel and blotted on a nitrocellulose membrane. An anti-human ACE2 goat polyclonal antibody (R&D, Minneapolis, MN, USA) was used for the detection of recombinant human sACE2. An anti-mouse ACE2 rabbit polyclonal antibody (Biochain) was used for the detection of ACE2 expression in mouse brain and other tissues. For Aβ Western blot, synthetic human Aβ40, Aβ42 and Aβ43 were purchased from Peptide Institute (Osaka, Japan) and dissolved in 0.1% NH_4OH. Purified human kidney ACE was purchased from (CHEMICON, Temecula, CA, USA). To enhance the reactivity, the membranes were boiled in PBS for 3 min prior to blocking (Zou et al. 2007). The primary antibodies were anti-human amyloid β (35-40) mouse monoclonal IgG, anti-human amyloid β (1-42) rabbit polyclonal IgG and anti-human amyloid β (1-43) rabbit polyclonal IgG (IBL, Takasaki, Japan). Protease inhibitors, pepstatin A, leupeptin, E-64, Z-LLL-al and phosphoramidon were purchased from the Peptide Institute (Osaka, Japan). Chloroquine, EDTA, captopril, amastatin, and bestatin were

from Sigma. TPCK, aprotinin, and chymostatin were from Roche Diagnostics (Mannheim, Germany). Detection was undertaken with chemiluminescent reagents (Thermo Scientific).

Thioflavin T binding assay

Aβ43 was dissolved in 0.5 ml PBS at a concentration of 5 μM. To determine whether ACE2 inhibits Aβ43 aggregation, Aβ43 was also mixed with ACE2 or with ACE2 and DX600. The mixture was incubated at 37 °C for 24 hours and then mixed with 5 μM thioflavin T in 50 mM glycine-NaOH, pH 8.5. The fluorescence intensities were measured at an excitation wavelength of 450 nm and an emission wavelength of 492 nm using a multiplate reader (Infinite F500, TECAN, Kanagawa, Japan). Each sample was measured in triplicate.

Statistical analysis

All data are presented as means ± standard error of the mean (SEM). Student's *t*-test and Mann-Whitney-U test were used for statistical analysis. Statistical significance was set at $*P < 0.05$.

Results

Mouse brain lysate converts Aβ43 to Aβ42, and this activity is inhibited by an ACE2 inhibitor.

In the present study, we used three specific antibodies to examine the synthetic Aβ species in different concentrations. There was no cross-reaction among them, which was confirmed by Western blot analysis (Fig. 1A). The generations of converted Aβ42 and converted Aβ40 were detected in the mixture of Aβ43 and brain homogenate by Western blot, suggesting that Aβ43 is converted to Aβ42 and to Aβ40 by converting enzymes in mouse brain lysate (Fig. 1B). To further characterize which enzyme converts Aβ43 to Aβ42, we examined the inhibitory effect of various protease inhibitors by Western blot. Among the protease inhibitors tested, only EDTA caused a clear and complete inhibition on both Aβ42 and Aβ40 conversions (Fig. 1C and Table 1). Therefore, one or more metalloproteases contribute to the conversion of Aβ43 to Aβ42 and to Aβ40 in mouse brain.

ACE, as a dipeptidyl carboxypeptidase, cleaves a dipeptide from the C terminus of a substrate, hence converts Aβ42 to Aβ40 and degrades Aβ peptides under physiological conditions, thereby contributing to the prevention of Aβ deposition in the brain (Zou et al. 2007). To further assess which enzyme converts Aβ43 to the shorter forms of Aβ, we

focused on the ACE homologue, ACE2. ACE2 is a zinc metalloprotease and, as a carboxypeptidase, cleaves a single C-terminal amino acid from a peptide, and hence may convert Aβ43 to Aβ42. To confirm whether ACE2 converts synthetic Aβ43 to generate Aβ42, we incubated the mixture of Aβ43 and mouse brain homogenate with or without specific ACE2 inhibitor, DX600. The Aβ43-to-Aβ42-converting activity was completely inhibited by EDTA and DX600, suggesting that ACE2 is a major Aβ43-to-Aβ42-converting enzyme in brain (Fig. 1D). Using an ACE2 activity assay kit, ACE2 activity was clearly detected in mouse brain lysate, which could be inhibited partially by DX600 (Fig. 1E). This suggests that other enzymes are also associated with ACE2-like activity.

Next, we examined whether ACE2 protein is expressed in mouse brain. Previous report demonstrated a moderate ACE2 expression level in human brain (Hamming et al. 2004). Similar with the results from human brain, we also found that ACE2 was moderately expressed in mouse cerebrum, though its expression level was lower than that in kidney and pancreas (Fig. 2A). In addition, immunostaining revealed that ACE2 was expressed in the granular cells of the dentate gyrus in mouse hippocampus and in the endothelium of blood vessels (Fig. 2B and C). These results suggest that ACE2 may play a potential physiological role in modulating Aβ43 levels in the brain and in the

blood.

Recombinant ACE2 converts Aβ43 to Aβ42

To further confirm ACE2 Aβ43-to-Aβ42-converting activity, we examined the conversion of Aβ43 to Aβ42 by purified ACE2. The purified recombinant ACE2 (sACE2cFlag) protein from HEK293 cell medium migrated with a molecular mass of 120 kDa, and its purity was greater than 90% as shown by Coomassie-staining and Western blot (Fig. 3A and 3B). The purified recombinant ACE2 protein activity was confirmed and inhibited completely by 2 μM DX600 (Fig. 3C). Purified ACE2 protein was mixed with synthetic Aβ43, and the mixture was incubated for 0, 2, 4, 8 and 16 hours. Aβ42 converted from Aβ43 by recombinant ACE2 protein was detected from 4 hours of incubation, and increased at 8 and 16 hours (Fig. 3D, middle panel). However, incubation of mixtures for 16 hours did not generate Aβ40 (Fig. 3D, top panel). To further investigate ACE2 Aβ43-to-Aβ42-converting activity, we examined whether the ACE2 mediated generation of converted Aβ42 was inhibited by DX600 at 16 and 24 hours. ACE2 Aβ43-to-Aβ42-converting activity was affected by 1 μM DX600, and was completely inhibited by 10 μM (Fig. 3E). Thus, the present findings indicate that ACE2 converts Aβ43 to Aβ42, but not to Aβ40, and that the ACE2 inhibitor inhibits the ACE2

Aβ43-to-Aβ42-converting activity.

Combination of ACE2 and ACE converts Aβ43 to Aβ40.

Because ACE can convert Aβ42 to Aβ40, we explored a new assumption whether Aβ43 converts to Aβ40. We mixed purified recombinant ACE2 and Aβ43, and then incubated with ACE at 37 °C from 1 hour to 16 hours. Converted Aβ40 was markedly increased after 4 hours of incubation, demonstrating that ACE2 and ACE convert Aβ43 to Aβ40 (Fig. 4A, top panel). Aβ42 was also converted from Aβ43 consistent with the previous results (Fig. 4A, middle panel). In addition, the level of synthetic Aβ43 was decreased, indicating degradation of Aβ43 (Fig. 4A, bottom panel). Therefore, Aβ43 was converted stepwise to Aβ40, by the combination of ACE2 and ACE. Next, we examined whether ACE2 degraded Aβ43 and inhibited Aβ43 aggregation by the thioflavin-T assay, and whether DX600 inhibited ACE2-mediated Aβ43 degradation. ACE2 significantly inhibited Aβ43 aggregation and DX600 reversed this inhibitory effect (Fig. 4B). This result also suggests that the ACE2 inhibitor inhibits the ACE2 Aβ43-to-Aβ42-converting activity and Aβ43-degrading activity.

ACE2 activity shows a tendency to decrease in AD patients.

We previously reported that the serum Aβ43 in the AD group was higher than that of the control group (Zou et al. 2013). To explore whether ACE2 activity is related to Aβ43 levels, we examined ACE2 expression in human serum by Western blot and measured ACE2 activity in the serum of AD patients and an age-matched normal control group. ACE2 protein was detected at 120 kDa in human serum (Fig. 5A) and its activity showed a tendency to decrease in the serum of AD patients compared with that of the control group (control, 836.3 ± 77.5 a.u.; AD, 693.9 ± 50.0 a.u., $P = 0.079$). We further examined the serum angiotensin II, which is also degraded by ACE2. The angiotensin II level of AD patients was similar to that of control group, suggesting that angiotensin II is prone to be maintained at a constant level in the serum (Fig. 5C).

Discussion

Substantial evidence indicates that Aβ42 oligomers are the causal factor in AD pathology (Haass and Selkoe 2007). In addition, the most recent studies of the aggregation and degradation of amyloid peptides has led to a consensus view that Aβ43 is another key factor in AD (Saito et al. 2011; Zou et al. 2013). A degree of cognitive impairment in patients with AD is not related to the insoluble form of the peptide in brain, but is associated with oligomeric forms of Aβ species (Shankar et al. 2007). Aβ43 showed a higher propensity to aggregate and was more neurotoxic than Aβ42 (Saito et al. 2011). In this regard, we found that some enzymes in mouse brain, converted Aβ43 to Aβ42 and to Aβ40. Converted Aβ40 disappeared faster than Aβ42 in the brain lysate, which is probably due to Aβ42 being prone to aggregate and to form amyloid fibrils rapidly, thereby preventing further degradation (Fig. 1B). We used a highly sensitive method for Aβ42 Western blot (boiling the membrane) because the amount of generated Aβ42 is extremely low. On the contrast, the concentration of synthesized Aβ43 was high and Aβ43 was detected using much less sample volume. Thus, the slight degradation of Aβ43 may be enough to generate detectable Aβ42 and the band intensity of Aβ42 and Aβ43 was not comparable (Fig.1B and 1D). Using various protease inhibitors, we found that ACE2, as a metallocarboxypeptidase and a homologue of ACE, by cleaving a single

amino acid, converted Aβ43 to Aβ42, but not to Aβ40 (Fig. 3D), likely resulting in reduction of Aβ43 neurotoxicity. A conserved glutamic acid residue, 24 amino acids following a particular HEXXH zinc-binding domain, aligns with the critical glutamate necessary for the catalytic activity of ACE2. ACE2 differs in substrate specificity from ACE in that it functions exclusively as a carboxypeptidase. The main differences between ACE and ACE2 occur in the ligand-binding pockets, particularly at the S2' subsite and in the binding of the peptide carboxy-terminus. The third zinc ligand in ACE, provided by a glutamate residue, is contained within the Glu-$(Xaa)_3$-Asp motif. The aspartate residue within this motif serves to precisely position the first histidine zinc ligand. However, this aspartate residue is replaced by a glutamate (Glu406) in ACE2 (Guy et al. 2003), which might be an explanation for why ACE2 converts Aβ43 to Aβ42, while ACE converts Aβ43 to Aβ41. We have demonstrated that the removal of N-linked glycosylation of ACE abolished its angiotensin-converting and Aβ42-to-Aβ40-converting activities, whereas its Aβ-degrading activity was not affected (Zou et al. 2009). Thus, the glycosylation of ACE2 and ACE may be another functional modifier of their substrate selectivity.

By Western blot, we found that ACE2 was expressed in mouse brain (Fig.2A). We then determined that ACE2 was distributed predominantly in blood vessels and in the

granular cells in the dentate gyrus of hippocampus (Fig. 2B and 2C). Mouse ACE2 is substantially less potent than human ACE2 in transformation of Ang1-10 to Ang1-9 (Poglitsch et al. 2012). We speculate that human ACE2 might have stronger cleavage activity than that of mouse ACE2 to convert Aβ43 to Aβ42. We used the purified recombinant human ACE2, and confirmed that Aβ43-to-Aβ42-converting activity was completely inhibited by EDTA and DX600 (Fig. 3E and 4B). The ACE2 inhibitor, DX600, with a Ki of 2.8 nM, is specific for ACE2, and does not inhibit ACE activity (Huang et al. 2003). However, the DX600 did partially inhibit the ACE2 activity in mouse brain lysate (Fig. 1E), suggesting that other enzymes may also have ACE2-like activity. In our present studies, it is difficult to compare the ACE2 activity or the Aβ43-to-Aβ42-converting activity between the purified recombinant ACE2 and the brain lysate. The brain lysate also have many other Aβ-degrading enzymes, which may degrade the generated Aβ42 immediately. In addition, the unit of fluorescence intensity is arbitrary unit, which is randomly optimized for each measurement, thus, the fluorescence intensity is not suitable to be compared between different experiments. 1 μg ACE2 generated Aβ42 after 4 hours incubation, suggesting that 1 μg recombinant ACE2 may have less Aβ43-to-Aβ42-converting activity than 250 μg brain lysate (Fig. 1B and 3D). However, it is difficult to conclude that because the amount of Aβ42

degradation was unknown.

We further assume the existence of successive enzyme reactions, which convert Aβ43 to Aβ42, and then to Aβ40. Interestingly, a combination of ACE2 and ACE converted Aβ43 to Aβ40 (Fig. 4A). We hypothesized that there are two possible pathways: in one, Aβ43 firstly is converted to Aβ42 by ACE2, and then converted to Aβ40 by ACE; in the other one, Aβ43 starts to be converted to Aβ41 by ACE, and then to Aβ40. We speculate that Aβ41 may be converted to Aβ40 by ACE2, which needs to be confirmed by a suitable anti-Aβ41 antibody when one becomes available in the future.

We have previously reported that in AD patient serum, the Aβ43 level was higher compared with the age-matched control group. Here, we found that ACE2 was expressed in human serum and ACE2 activity showed a tendency to decrease in the serum of AD patients compared with that of the control group (Fig. 5B). Whether serum ACE2 activity is associated with higher Aβ43 level needs to be investigated in future using larger sample size. We also measured the serum angiotensin II level in AD patients because ACE2 also degrades it. Serum angiotensin II in AD patients was similar to the normal control group (Fig. 5B), suggesting that the serum angiotensin II level might be maintained at a constant level by regulation of angiotensinogen secretion or other enzymes in the brain RAS. In brain, the existence of associated enzymes which

are involved in converting Aβ43 to Aβ42 and/or to Aβ40 has yet to be determined. One interesting highlight of our description is that ACE2 converts Aβ43 to Aβ42, and even converts to Aβ40 successively by ACE. However, whether the ratio of Aβ43/Aβ42 or Aβ43/Aβ40 is influenced by the combination of ACE2 and ACE needs to be confirmed. The conversion of a longer Aβ species to a shorter species emerges as a therapeutic strategy for AD treatment. The γ-secretase modulators are such candidates, which can shift the γ-cleavage from producing longer Aβ species to shorter species. However, targeting γ-secretase always has the associated difficulties of preventing changing the processing of Notch and other important γ-secretase substrates (Selkoe 2011). Instead of modulating γ-secretase, ACE2 and ACE could convert the longer species Aβ43 to a shorter species Aβ40 without changing the processing of Notch and other γ-secretase substrates.

Taken together, an extensive amount of research indicates that peripheral ACE2 exerts a pivotal role in blood pressure regulation as well as in cardiovascular, renal and pulmonary function or other peripheral tissues (Hamming et al. 2004; Oudit et al. 2010; Zhong et al. 2010). Remarkably, for the first time, we provide strong evidence demonstrating the impact of ACE2 on these neurotoxic amyloid peptides in brain, to assess the benefits of central ACE2 in AD. Moreover, we hypothesized a pathway that

involves both ACE2 and ACE as factors in the brain RAS, not only in the regulation of blood pressure but also being associated with the conversion of Aβ43 to Aβ40, which might reduce the β-amyloid accumulation. Therefore, maintaining ACE2 and ACE activities in the brain could act as a protective and defensive mechanism in the initial stages of AD to limit its pathological development.

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Figure legends

Figure 1. Mouse brain lysate converts Aβ43 to Aβ42 and this activity is inhibited by an ACE2 inhibitor. A, Western blot analysis of Aβ40, Aβ42 and Aβ43 testing the specificity of antibodies at different loading concentrations. Even at the lowest concentrations, the peptides were clearly detectable. B, Synthetic Aβ43 (70 μM) was mixed with mouse (C57BL/6, 5-month-old) brain homogenates (250 μg) and incubated at 37 °C in an increasing time course to detect the generation of converted Aβ42 and Aβ40. C, Western blot of converted Aβ40 and Aβ42 from synthetic Aβ43 with or without various protease inhibitors. VC, vehicle control. D, Western blot analysis of converted Aβ42 after a 4 hour incubation when treated with 10 μM EDTA and 10 μM or 100 μM ACE2 inhibitor (DX600). Aβ43-to-Aβ42-converting activity was inhibited confirming ACE2 action. E, DX600 inhibited the ACE2 activity in mouse brain homogenate.

Figure 2. Expression of ACE2 in mouse tissues. A, Western blot of ACE2 in mouse tissues revealed a moderate expression of ACE2 in mouse cerebrum. B and C, Confocal microscopy images demonstrated that ACE2 was expressed in the endothelium of blood vessels and in the granular cells of dentate gyrus in hippocampus (white arrows). Scale bar was 100 μm in B and 20 μm in C.

Figure 3. Recombinant ACE2 converts Aβ43 to Aβ42. A, The purified recombinant ACE2 protein was subjected to SDS-PAGE in a 5-20% gel and stained with Coomassie Blue. B, Western blot of 10 μl cell culture medium and 1 μg of purified ACE2 probed with a polyclonal anti-ACE2 antibody. C, Recombinant ACE2 activity was measured by a SensoLyte 390 ACE2 activity assay kit. The fluorescence intensity was monitored at an excitation/emission of 330 nm/390 nm. D, 1 μg of recombinant ACE2 protein was mixed with synthetic Aβ43 (40 μM), and was incubated for 0, 2, 4, 8 and 16 hours. Western blots of the mixtures were probed with anti-Aβ40, anti-Aβ42 and anti-Aβ43 antibodies. E, DX600 dose dependently inhibited ACE2-mediated generation of Aβ42 from Aβ43.

Figure 4. Combination of ACE2 and ACE converts Aβ43 to Aβ40. A, Western blot analysis of the generation of converted Aβ40 and Aβ42, followed by degradation of Aβ43 by a combination of ACE2 and ACE. Aβ43 was mixed with recombinant ACE2 protein and human kidney ACE protein and incubated in an increasing time course. B, Thioflavin-T assay of Aβ43 aggregation after 24 hour incubation with or without ACE2. The fluorescence was measured at an excitation/emission of 450 nm/492 nm. DX600 blocked ACE2-mediated Aβ43 degradation. All data are presented as mean ± standard

error (SEM) of four samples. ***$P < 0.001$, post hoc Bonferroni-Dunn test.

Figure 5. ACE2 was expressed in human serum and its activity showed a tendency to decrease in AD patients. A, Western blot of ACE2 in 3 μl human serum samples from normal controls and AD patients. B, Serum ACE2 activity of AD patient group exhibited a tendency to decrease. C, Angiotenion II levels in the serum of AD patients did not differ from that of normal controls. Clear circles represent each sample of the control group, while the black circles represent AD patients. Data are expressed as means ± SEM. Mann-Whitney-U test was used for statistical analysis.

Table1.

Effect of various protease inhibitors on Aβ40 and Aβ42 levels formed from Aβ43 by mouse brain homogenate.

Protease Inhibitor	Concentration	Target Peptidases	Inhibition of Aβ40 Level	Inhibition of Aβ42 Level
E-64	200 μM	cysteine	-	-
leupeptin	200 μM	serine	-	-
phosphoramidon	200 μM	neprilysin-like	-	-
chloroquine	200 μM	lysosomal	-	-
aprotinin	200 μM	serine	-	-
bestatin	200 μM	aminopeptidase	+	-
amastatin	200 μM	aminopeptidase	+	-
EDTA	200 μM	metallopeptidase	+++	++
puromycin	200 μM	aminopeptidase	+	-
captopril	200 μM	ACE	-	+
pepstatin A	200 μM	aspartate	+	+
Z-LLL-al	200 μM	cysteine	-	-
chymostatin	200 μM	chymotrypsin	+	+
TPCK	200 μM	serine	-	-

Table1. Suppression of Aβ conversion by specific peptidase inhibitors. Mouse brain homogenates were treated with or without protease inhibitors and then were incubated with Aβ43 for 4 hours. + indicates potent inhibition, while – represents little or no inhibition. +, > 30% inhibition; ++, > 50% inhibition; +++, > 90% inhibition; -, < 10% inhibition. EDTA, ethylenediaminetetraacetic acid; Z-LLL-al, benzyloxycarbonyl-Leu-Leu-leucinal; TPCK, tosyl phenylalanyl chloromethyl ketone. Aβ40 and Aβ42 levels were analyzed by Image J software.

Figure 1, Liu et al.

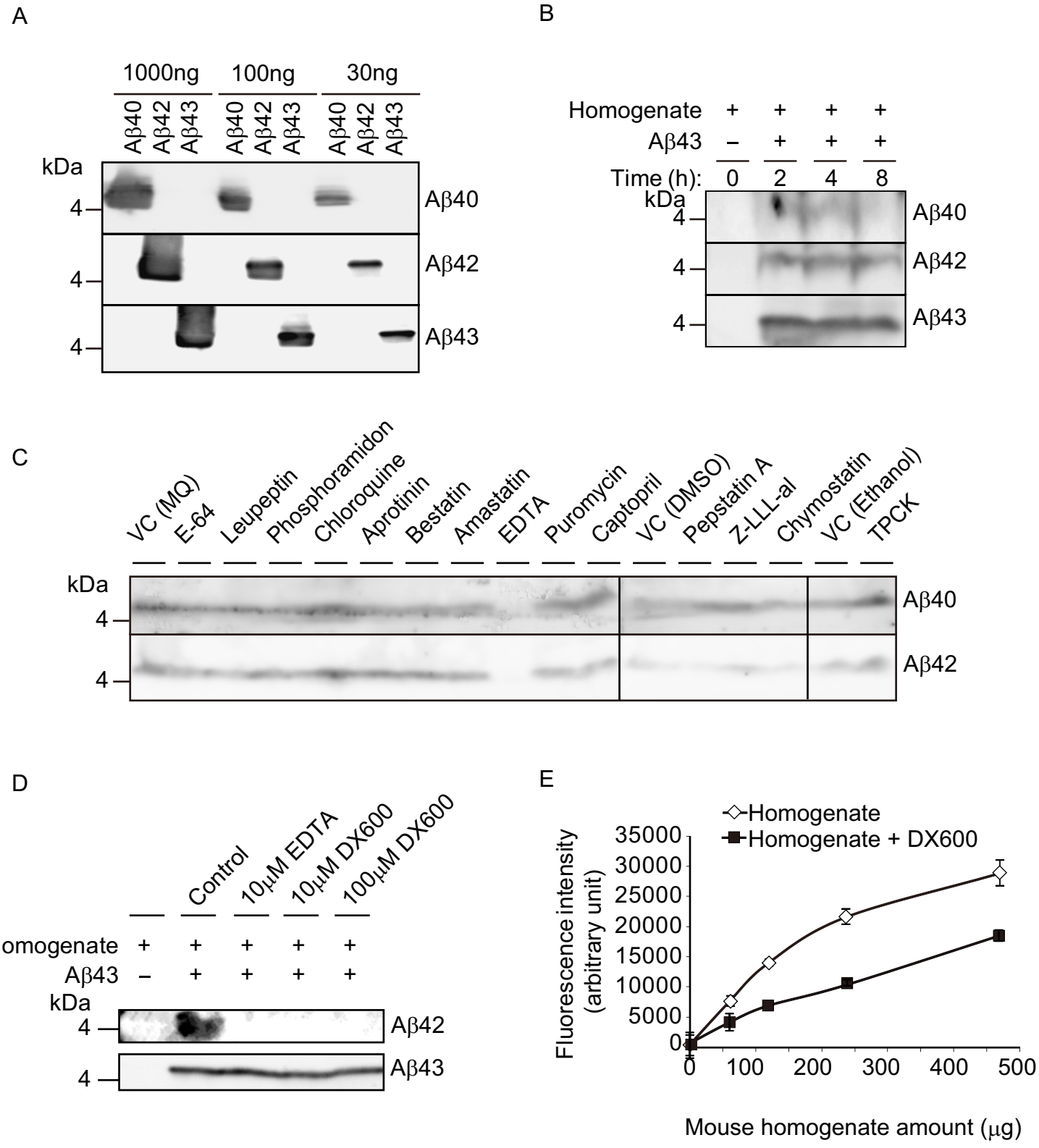

Figure 2, Liu et al.

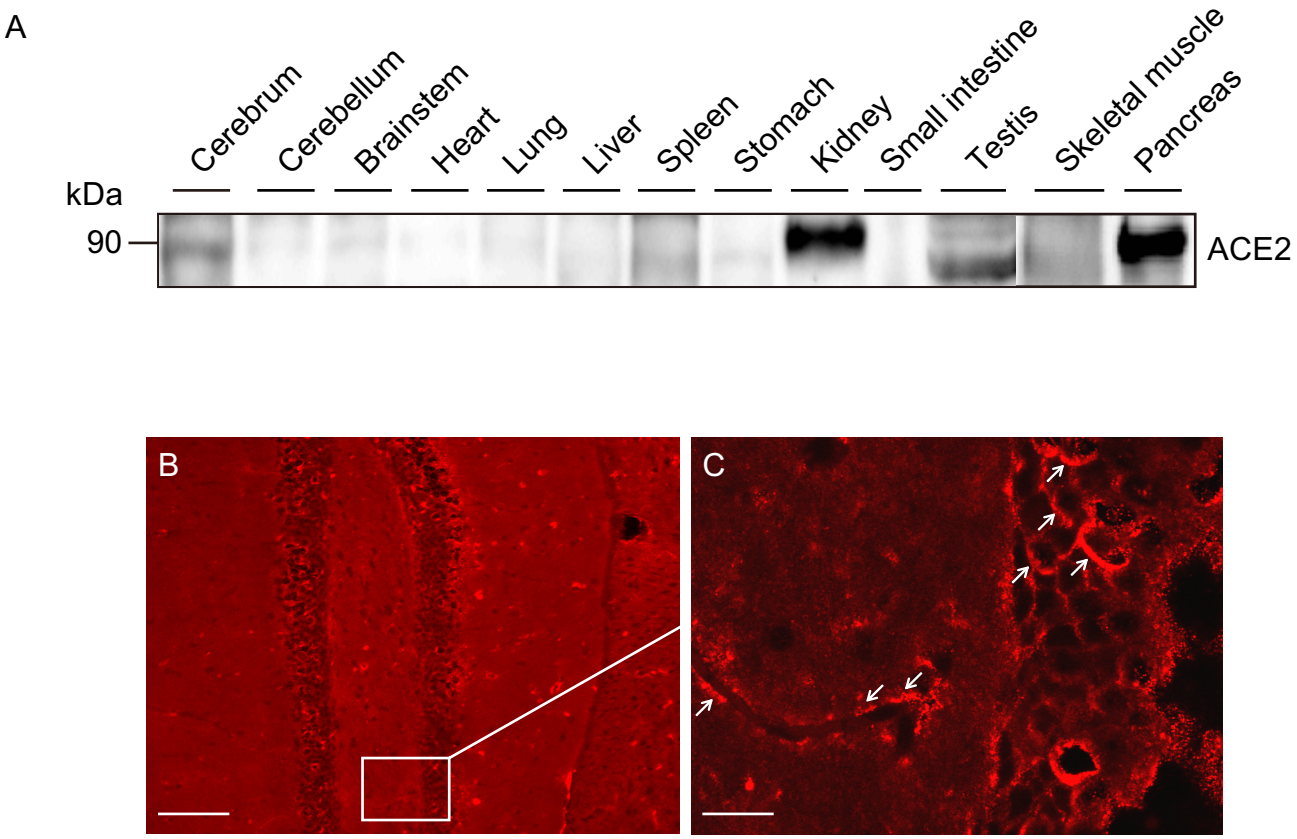

Figure 3, Liu et al.

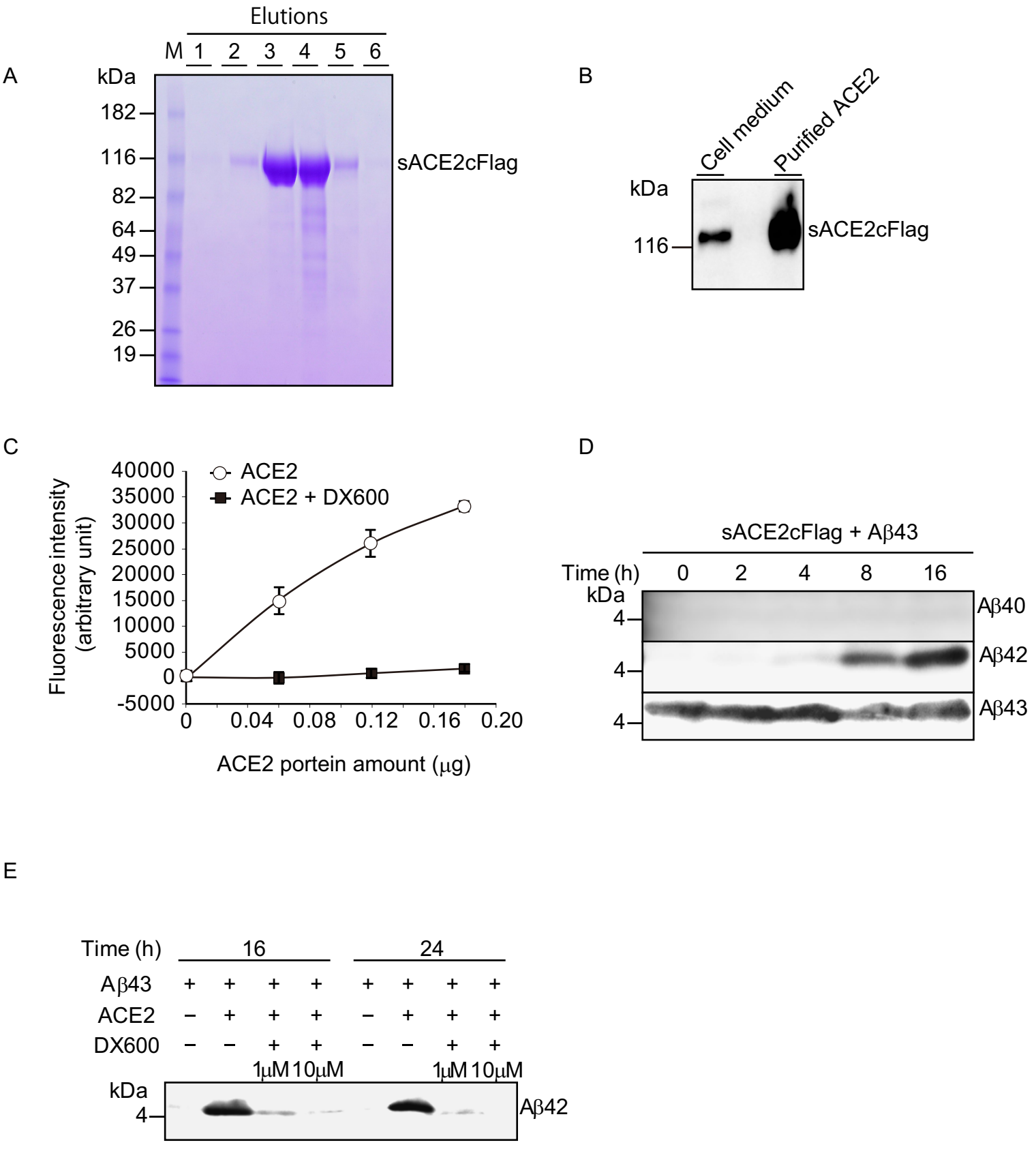

Figure 4, Liu et al.

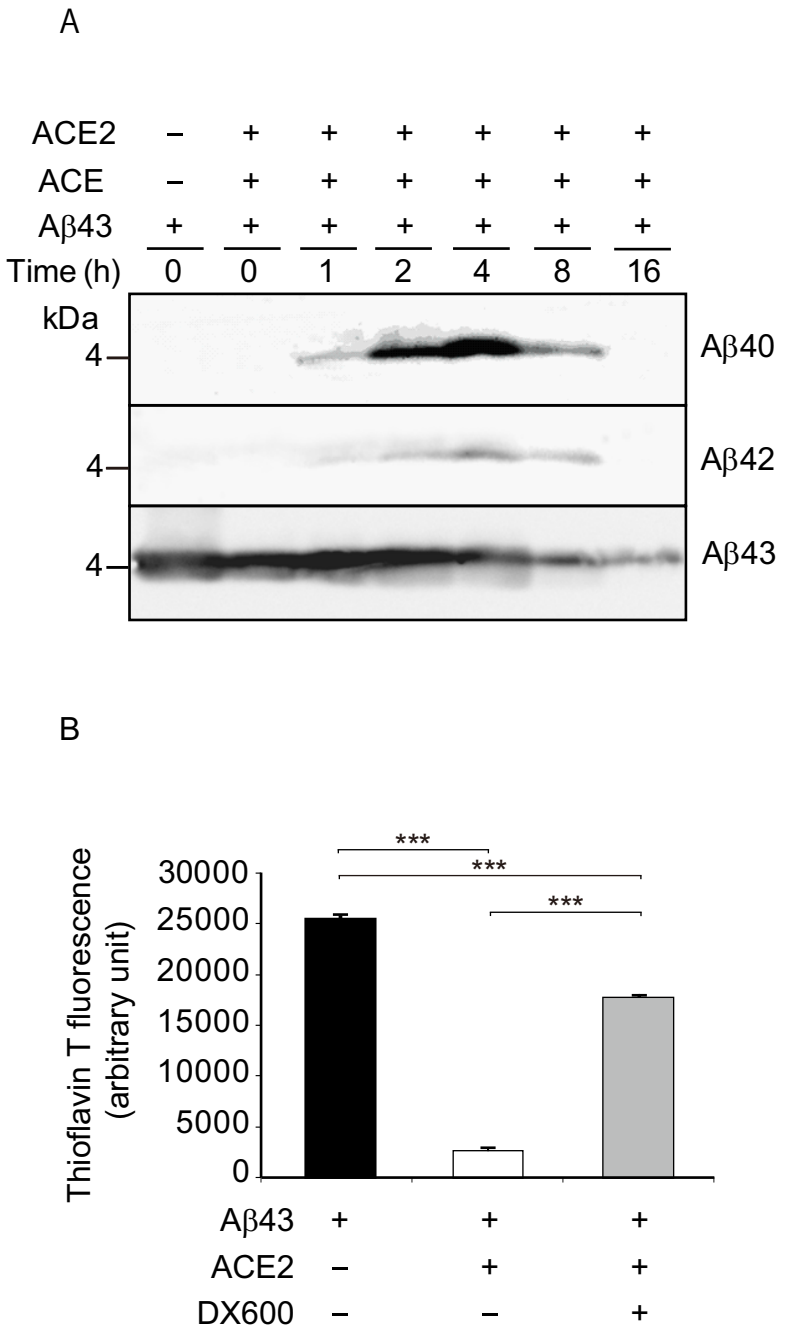

Figure 5, Liu et al.