

# Angiotensin-Converting Enzyme Converts Amyloid $\beta$ -Protein 1–42 ( $A\beta_{1-42}$ ) to $A\beta_{1-40}$ , and Its Inhibition Enhances Brain $A\beta$ Deposition

Kun Zou,<sup>1,3</sup> Haruyasu Yamaguchi,<sup>4</sup> Hiroyasu Akatsu,<sup>5</sup> Takaaki Sakamoto,<sup>1</sup> Mihee Ko,<sup>1</sup> Kazushige Mizoguchi,<sup>2</sup> Jian-Sheng Gong,<sup>1</sup> Wenxin Yu,<sup>1</sup> Takayuki Yamamoto,<sup>5</sup> Kenji Kosaka,<sup>5</sup> Katsuhiko Yanagisawa,<sup>1</sup> and Makoto Michikawa<sup>1</sup>

Departments of <sup>1</sup>Alzheimer's Disease Research and <sup>2</sup>Geriatric Medicine, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Obu, Aichi 474-8522, Japan, <sup>3</sup>Japan Society for the Promotion of Science, Tokyo 102-8471, Japan, <sup>4</sup>Gunma University School of Health Sciences, Maebashi 371-8514, Japan, and <sup>5</sup>Choju Medical Institute, Fukushima Hospital, Toyohashi 441-8124, Japan

The abnormal deposition of the amyloid  $\beta$ -protein ( $A\beta$ ) in the brain appears crucial to the pathogenesis of Alzheimer's disease (AD). Recent studies have suggested that highly amyloidogenic  $A\beta_{1-42}$  is a cause of neuronal damage leading to AD pathogenesis and that monomeric  $A\beta_{1-40}$  has less neurotoxicity than  $A\beta_{1-42}$ . We found that mouse and human brain homogenates exhibit an enzyme activity converting  $A\beta_{1-42}$  to  $A\beta_{1-40}$  and that the major part of this converting activity is mediated by the angiotensin-converting enzyme (ACE). Purified human ACE converts  $A\beta_{1-42}$  to  $A\beta_{1-40}$  as well as decreases  $A\beta_{1-42}/A\beta_{1-40}$  ratio and degrades  $A\beta_{1-42}$  and  $A\beta_{1-40}$ . Importantly, the treatment of Tg2576 mice with an ACE inhibitor, captopril, promotes predominant  $A\beta_{1-42}$  deposition in the brain, suggesting that ACE regulates  $A\beta_{1-42}/A\beta_{1-40}$  ratio *in vivo* by converting secreted  $A\beta_{1-42}$  to  $A\beta_{1-40}$  and degrading  $A\beta$ s. The upregulation of ACE activity can be a novel therapeutic strategy for AD.

**Key words:** angiotensin-converting enzyme; ACE; Alzheimer's disease; amyloid  $\beta$ -protein;  $A\beta$ ;  $A\beta$  deposition;  $A\beta$  degradation; APP transgenic mouse

## Introduction

The progressive accumulation and deposition of the amyloid  $\beta$ -protein ( $A\beta$ ) in the brain are early pathogenically important features of Alzheimer's disease (AD) (Selkoe, 2004). It has been assumed that insoluble  $A\beta$  amyloid is a culprit for inducing the pathological processes of AD, leading to neuronal dysfunction; however, recent studies have shown that not the insoluble form of  $A\beta$  but the soluble form of  $A\beta$  oligomers is pathogenic (Kirkitadze et al., 2002; Walsh et al., 2002).  $A\beta_{1-42}$  is deposited early and selectively in senile plaques, and this deposition is an invariant feature of all forms of AD (Iwatsubo et al., 1994). A high  $A\beta_{42}/A\beta_{40}$  ratio is a major determinant for AD development in familial AD with presenilin mutations (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996; Citron et al., 1997).

$A\beta$ s ( $A\beta_{1-40}$  and  $A\beta_{1-42}$ ) are constantly secreted by many

types of cell and are normally found in body fluids, including CSF. Recently, we have shown that monomeric  $A\beta_{1-40}$  has neuroprotective effects against metal-induced oxidative damage and  $A\beta_{1-42}$ -induced neuronal death, whereas  $A\beta_{1-42}$  is highly amyloidogenic and thus forms oligomers rapidly at very low concentrations, exerting strong neurotoxicity (Zou et al., 2002, 2003).  $A\beta_{1-40}$ , but not  $A\beta_{1-42}$ , rescues neurons from  $\beta$ - or  $\gamma$ -secretase inhibitor-induced cell death (Plant et al., 2003). Moreover, the inhibition of the effects of some nonsteroidal anti-inflammatory drugs, which reduce the risk of AD (in 't Veld et al., 2001), directly blocks  $A\beta_{1-42}$  generation by changing presenilin conformation and shifting  $\gamma$ -secretase function toward the production of a shorter soluble  $A\beta$  (Weggen et al., 2001; Lleo et al., 2004).

A recent study demonstrated that  $A\beta_{42}$  is essential for parenchymal and vascular amyloid deposition in mice and that mice expressing a high  $A\beta_{1-40}$  level do not develop overt amyloid pathology (McGowan et al., 2005). Both  $A\beta_{1-42}$  and  $A\beta_{1-40}$  levels were elevated coordinately in late-onset sporadic AD brains (Selkoe, 2004) and non-AD human brains (Morishima-Kawashima et al., 2000); however, insoluble  $A\beta_{42}$  level increases exponentially and steeply in an age-dependent manner, accompanied by much smaller increases in  $A\beta_{40}$  level (Morishima-Kawashima et al., 2000). Thus, although it is still debatable whether  $A\beta_{1-40}$  is nontoxic or neuroprotective, these findings suggest that decreasing neurotoxic  $A\beta_{1-42}$  level could be a strategy for developing AD treatments.

We considered that there is a carboxyl peptidase that converts

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Correspondence should be addressed to Dr. Makoto Michikawa, Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. E-mail: michi@nils.go.jp.

K. Zou's present address: Department of Neuroscience, Faculty of Pharmaceutical Sciences, Iwate Medical University, Yahaba 028-3694, Japan.

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secreted neurotoxic  $A\beta_{1-42}$  to  $A\beta_{1-40}$ , thus decreasing  $A\beta_{42}/A\beta_{40}$  ratio. This notion has led us to experimentally identify an  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting enzyme in the mouse and human brains. Interestingly, we found that the angiotensin-converting enzyme (ACE) is a major  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting enzyme in the brain. This suggests that the modulation of ACE activity can be a novel therapeutic strategy for AD.

## Materials and Methods

**Captopril treatment of Tg2576 mice and tissue preparation.** Male human amyloid precursor protein Swedish mutation (hAPP<sup>sw</sup>) transgenic Tg2576 mice were purchased from Taconic Farms (Germantown, NY). Mice at 6 months of age were fed with captopril-supplemented diet (0.25 mg/g) or control diet *ad libitum* for 7 or 11 months. There were 6–15 animals in each group. Animals were housed singly in individual cages. There were no significant differences in the amount of feed consumed or in the weight of the mice within or between treatment groups. The average captopril intake per animal was 30 mg/kg of body weight/d. Mice were killed by inhalation of  $CO_2$ , and 0.5 ml of blood was collected from the right atrium for the determination of serum ACE activity. Mice were then transcardially perfused with cold PBS. The left hemisphere of the brain was fixed in 4% buffered paraformaldehyde solution at 4°C for 48 h and incubated in 30% sucrose at 4°C for 48 h for histological processing. The right hemisphere of the brain was separated into the cerebral cortex, hippocampus, thalamus, and brainstem, and these samples were rapidly frozen in liquid nitrogen and stored at  $-80^\circ C$  until analysis.

**Human postmortem brain tissue.** Frontal cortex tissue samples from autopsied control subjects ( $n = 15$ ; male, 9, female, 6; neuropathological diagnosis, physiological aging) and AD subjects ( $n = 15$ ; male, 9, female, 6; clinical and neuropathological diagnosis, AD) were obtained from Fukushima Hospital (Toyohashi, Japan). Tissues were frozen immediately in liquid nitrogen at autopsy and then stored at  $-80^\circ C$  until use. The average postmortem delay was 7.7 h and was not significantly different between the two groups. The average age of the subjects of the control group was  $84.7 \pm 1.9$ , and that of the subjects of the AD group was  $85.5 \pm 1.9$  (data represent means  $\pm$  SEM;  $p = 0.75$ , ANOVA, Bonferroni/Dunn test). Experiments using human brains were performed after obtaining the informed consent of the patients' guardians for diagnosis and biochemical, molecular biological, and genomic research. This study was examined and approved by the Ethics Committee of Fukushima Hospital on October 6, 2005, and assigned the application number 180.

**ACE activity assay.** Cortical tissue (50–100 mg) was homogenized in a fourfold (w/v) volume of ACE homogenization buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 25  $\mu M$   $ZnCl_2$ , and 0.5% Triton X-100) and centrifuged at 4°C at  $10,000 \times g$  for 15 min. ACE activity in the supernatant against the synthetic substrate *N*-hippuryl-L-histidyl-L-leucine was determined using an ACE colorimetric kit (Buhlmann Laboratories, Schönenbuch, Switzerland). The reaction time was 6 h. ACE activity was then normalized by the initial weight of the brain tissue. For mouse serum ACE activity assay, serum was diluted at 1:2. All samples were measured in triplicate.

**Western blot analysis for determining conversion of  $A\beta_{1-42}$  to  $A\beta_{1-40}$ .** Somatic ACE-deficient mice (002680) were obtained from The Jackson Laboratory (Bar Harbor, ME).  $A\beta_{1-42}$  (Peptide Institute, Osaka, Japan) was freshly dissolved in 0.1%  $NH_3 \cdot H_2O$  at 200  $\mu M$  for each experiment. The wild-type mouse brain cortex, including the hippocampus, was homogenized in a twofold volume (w/v) of a buffer containing 10 mM Tris.HCl, pH 7.5, and 0.15 M NaCl, and the homogenate was centrifuged at  $500 \times g$  for 10 min. The supernatant was mixed with synthetic  $A\beta_{1-42}$  to a final concentration of 30  $\mu M$  and incubated at 37°C for 8 h. Ten microliters of the mixture was subjected to SDS-PAGE and blotted on a nitrocellulose membrane. To enhance the reactivity to an anti- $A\beta_{1-40}$  antibody, the membrane was boiled in PBS for 3 min after blotting, probed with an anti- $A\beta_{1-40}$  monoclonal antibody (1A10) (IBL, Takasaki, Japan), and visualized with SuperSignal (Pierce, Rockford, IL). Because of the high level of exogenous  $A\beta_{1-42}$ , the membrane was not boiled before the reaction with a polyclonal anti- $A\beta_{1-42}$  antibody.

**Thioflavin-T binding assay for aggregated  $A\beta$ .** Determination of the

aggregated state of  $A\beta$  was performed on the basis of a previously established method (Levine, 1995, 1999). The incubated  $A\beta$  peptides were centrifuged at  $17,000 \times g$  for 60 min, the supernatant was removed, and the precipitate was suspended in 1 ml of 5  $\mu M$  thioflavin-T in 50 mM glycine-NaOH, pH 8.5. Steady-state fluorescence intensities for each sample were determined as described previously (Zou et al., 2002).

**Matrix-assisted laser desorption/ionization-time of flight mass spectrometry.** Purified human ACE (2 U/ml) (Millipore, Billerica, MA) was added to 30  $\mu M$   $A\beta_{1-42}$  dissolved in 0.1%  $NH_3 \cdot H_2O$  and incubated at 37°C for 6 h. Captopril (2 mM) was added to stop the digestion, and the sample was frozen in  $-80^\circ C$  until use. The sample was subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) to detect the generation of  $A\beta_{1-40}$ . The same amount of ACE or  $A\beta_{1-42}$  incubated alone under the same conditions as described above was used as control.

**Biotinylation of  $A\beta_{1-42}$  and determination of  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting activity in human brain.**  $A\beta_{1-42}$  was biotinylated using a ProTectOn biotin labeling kit (Vector Laboratories, Burlingame, CA). In brief, 0.5 mg of  $A\beta_{1-42}$  was dissolved in DMSO and diluted with distilled water to 100  $\mu l$ .  $A\beta_{1-42}$  was biotinylated with a biotin-labeling reagent, and free biotin was removed using gel filtration slurry provided with the kit. The human frontal cortex tissue sample used was from a 76-year-old autopsied non-AD subject. The brain tissue was homogenized in a fourfold volume (w/v) of PBS, and the homogenate was mixed with biotinylated  $A\beta_{1-42}$  at a concentration of 0.5  $\mu g/\mu l$ . The mixtures were incubated at 37°C for 8 h with or without an ACE inhibitor (1  $\mu M$ ), namely captopril or enalapril. After incubation, 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate was added to extract  $A\beta$ s. The samples were centrifuged at 4°C and  $10,000 \times g$  for 10 min, and the supernatant was applied to avidin agarose provided with the kit to purify biotinylated  $A\beta$ s. Avidin agarose was washed with PBS four times, and biotinylated  $A\beta$ s were eluted with 50 mM DTT in SDS-PAGE sample buffer. Biotinylated  $A\beta_{1-40}$  converted from biotinylated  $A\beta_{1-42}$  was detected by Western blot analysis using an anti- $A\beta_{1-40}$  monoclonal antibody (1A10).

**Immunohistochemistry.** The left hemispheres of the brains of Tg2576 mice were sagittally cut into 30  $\mu m$  sections using a freezing microtome (RM 2145; Leica, Wetzlar, Germany). Thioflavin-S staining was performed as described previously (Wyss-Coray et al., 2001). For each mouse, thioflavin-S-positive plaques were counted in four to five sections per left hemisphere of the brain at a magnification of 40 $\times$ . Serial sections were immunostained with anti- $A\beta_{1-40}$  and anti- $A\beta_{1-42}$  end-specific polyclonal antibodies, namely, RIB40 and RIB42, respectively (2  $\mu g/ml$ ; IBL), after a brief formic acid pretreatment, and immunopositive signals were visualized using an ABC elite kit (Vector Laboratories). Images of the cerebral cortex and hippocampus were captured using a digital camera attached to a microscope and analyzed using simple PCI software (Compix Imaging Systems, Lake Oswego, OR).  $A\beta_{1-40}$  and  $A\beta_{1-42}$  plaques were estimated as the percentage of immunostained area (positive pixels) divided by the examined area (total pixels). The quantification of thioflavin-S-positive plaques and areas attained by anti- $A\beta_{1-40}$  and anti- $A\beta_{1-42}$  antibodies was performed in a double-blind manner.

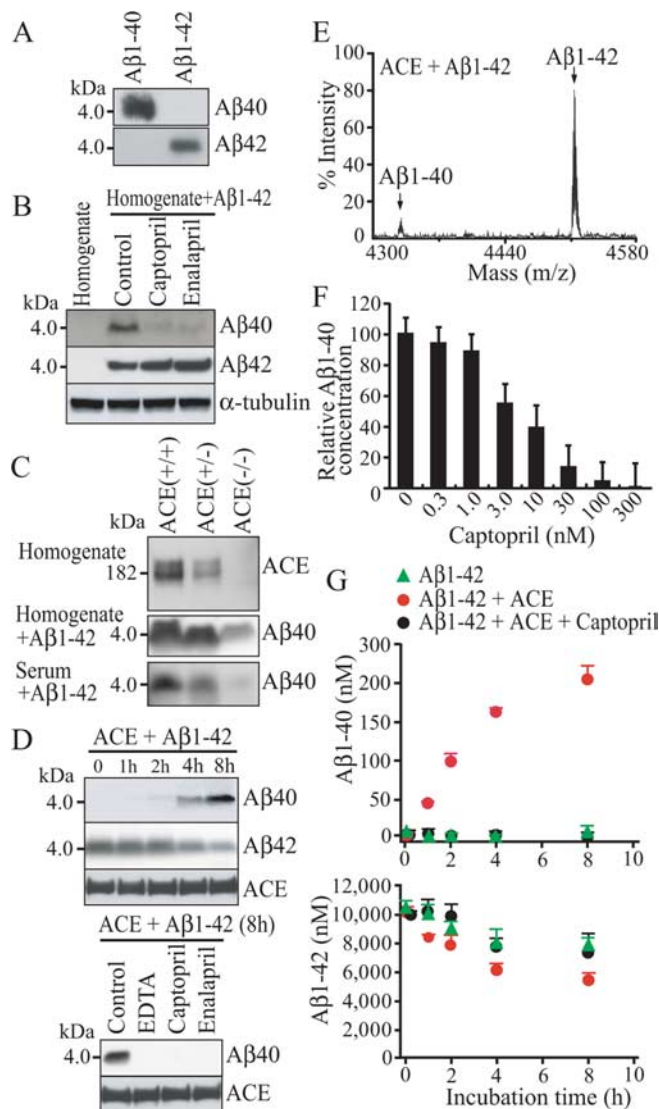
**$A\beta$  ELISA.** Mouse cortices for ELISA were homogenized in 10 volumes of a mixture containing 5.0 M guanidine.HCl and 50 mM Tris.HCl, pH 8.0 (w/v), as described previously (Johnson-Wood et al., 1997). The brain homogenates were further diluted at 1:20 for 13-month-old and 1:500 for 17-month-old mice in a dilution buffer provided with the ELISA kit (Wako, Osaka, Japan).  $A\beta_{1-42}$  and  $A\beta_{1-40}$  standards (Peptide Institute) were prepared such that the final composition included the same concentration of guanidine. Purified human ACE (2 U/ml) with 10  $\mu M$   $A\beta_{1-42}$  was diluted at 1:1000 with a dilution buffer containing complete protease inhibitor mixture (Roche, Mannheim, Germany).  $A\beta_{1-42}$  and  $A\beta_{1-40}$  levels were determined using the ELISA kit. Fibroblasts stably expressing human APP695 (Shiraishi et al., 2004) were grown in DMEM (Invitrogen, Grand Island, NY) containing 10% fetal calf serum. The medium was changed at 100% confluence, and the fibroblasts were treated with or without captopril. The levels of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  in the medium were determined 24 h after the treatment of fibroblasts with captopril. All samples were measured in triplicate.

## Results

We used two specific anti- $A\beta_{1-42}$  and anti- $A\beta_{1-40}$  antibodies to examine  $A\beta_{1-40}$  generation in a tissue homogenate mixed with exogenously added synthetic  $A\beta_{1-42}$ . There was no cross-reaction between the two antibodies, which was confirmed by Western blot analysis (Fig. 1A). The coincubation of  $A\beta_{1-42}$  with mouse cerebral homogenate generated  $A\beta_{1-40}$ , indicating the existence of an  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting enzyme. Also, the generation of  $A\beta_{1-40}$  from synthetic  $A\beta_{1-42}$  was inhibited by the ACE inhibitors captopril and enalapril (Fig. 1B; supplemental Table 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). This converting activity was also found in the mouse cerebellum, kidney, heart, spleen, lung, skeletal muscle, and serum, indicating that the  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting enzyme is distributed widely. In our Western blot system, neither endogenous  $A\beta_{1-40}$  nor  $A\beta_{1-42}$  was detected in these samples (data not shown). Moreover, Western blot analysis showed that the brain homogenate and serum from somatic ACE-deficient mice contain a markedly decreased capacity of generating  $A\beta_{1-40}$  from  $A\beta_{1-42}$  compared with those from wild-type (ACE+/+) and heterozygote (ACE+/-) mice (Fig. 1C). ACE is a dipeptidyl carboxypeptidase that catalyzes the cleavage of C-terminal dipeptides of several substrates and is widely distributed in mammalian tissues. These lines of evidence suggest that ACE in the brain homogenate plays a major role in the conversion of  $A\beta_{1-42}$  to  $A\beta_{1-40}$ .

Thus, we next performed Western blot analysis, ELISA, and MALDI-TOF-MS to examine whether purified human ACE cleaves the 2 aa at the C terminus of synthetic  $A\beta_{1-42}$  to generate  $A\beta_{1-40}$ . After the incubation of  $A\beta_{1-42}$  with ACE,  $A\beta_{1-40}$  level increased in a time-dependent manner, whereas  $A\beta_{1-42}$  level decreased (Fig. 1D, G). Determination of the level of  $A\beta_{1-40}$  generated and  $A\beta_{1-42}$  remaining in the solution containing synthetic  $A\beta_{1-42}$  and ACE shows that the levels of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  were inversely and significantly correlated (supplemental Fig. 1C, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).  $A\beta_{1-40}$  generation was completely inhibited by ACE inhibitors, namely, EDTA, captopril, and enalapril (Fig. 1D; supplemental Table 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Because  $A\beta_{1-42}$  can easily aggregate in aqueous buffers, it is important to understand whether ACE can mediate the cleavage of the aggregated form of  $A\beta_{1-42}$ . We performed thioflavin-T assay to monitor  $A\beta_{1-42}$  aggregation. The thioflavin-T fluorescence of  $A\beta_{1-42}$  incubated with or without ACE or of the mouse brain lysate showed no increase after 8 h of incubation. After 24 h of incubation, the thioflavin-T fluorescence of  $A\beta_{1-42}$  incubated alone and with the brain homogenate markedly increased; however, ACE strongly inhibited the increase in thioflavin-T fluorescence (supplemental Fig. 1A, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). These results suggest that  $A\beta_{1-40}$  is converted from nonfibrillar  $A\beta_{1-42}$  within 8 h of incubation. We previously reported that  $A\beta_{1-42}$  with random structures transformed to a  $\beta$ -sheet structure after 4 h of incubation at 37°C (Zou et al., 2003). Together with the present finding that the level of  $A\beta_{1-40}$  converted from  $A\beta_{1-42}$  increased until 8 h of incubation, it is possible that ACE generates  $A\beta_{1-40}$  from  $A\beta_{1-42}$  with both random and  $\beta$ -sheet structures.

Next we examined whether ACE degrades or converts aggregated  $A\beta_{1-42}$ . ACE did not reduce the thioflavin-T fluorescence of aggregated  $A\beta_{1-42}$  after 24 h of incubation (supplemental Fig. 1B, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material), and no  $A\beta_{1-40}$  converted from  $A\beta_{1-42}$  was detected by Western



**Figure 1.** ACE converts  $A\beta_{1-42}$  to  $A\beta_{1-40}$ . **A**, We confirmed the specificities of monoclonal anti- $A\beta_{1-40}$  (1A10) and polyclonal anti- $A\beta_{1-42}$  antibodies. One microgram each of  $A\beta_{1-40}$  and  $A\beta_{1-42}$  was subjected to SDS-PAGE and then blotted onto a nitrocellulose membrane, and the membrane was probed with anti- $A\beta_{1-40}$  and anti- $A\beta_{1-42}$  antibodies. **B**, Mouse brain homogenate was mixed with or without synthetic  $A\beta_{1-42}$  and incubated at 37°C for 8 h. An anti- $A\beta_{1-40}$  antibody was used for detecting  $A\beta_{1-40}$  generation. Captopril (1  $\mu$ M) or enalapril (1  $\mu$ M) markedly inhibited the generation of  $A\beta_{1-40}$  from  $A\beta_{1-42}$ . **C**, Top, The genotypes of ACE(+/+), ACE(+/-), and ACE(-/-) mice were confirmed by Western blot analysis of the brain homogenate using an anti-ACE polyclonal antibody (AF1513; R & D Systems). Middle, Bottom, Mouse brain homogenate (middle) or serum (bottom) was mixed with 30  $\mu$ M  $A\beta_{1-42}$  and incubated at 37°C for 8 h. The generation of  $A\beta_{1-40}$  was detected by Western blot analysis. **D**, Purified human ACE (2 U/ml) was mixed with synthetic  $A\beta_{1-42}$  (30  $\mu$ M), and the mixture was incubated at 37°C with or without ACE inhibitors. Top,  $A\beta_{1-40}$  was generated in a time-dependent manner. Bottom, EDTA (10  $\mu$ M), captopril (1  $\mu$ M), and enalapril (1  $\mu$ M) completely inhibited the conversion of  $A\beta_{1-42}$  to  $A\beta_{1-40}$ . **E**, MALDI-TOF-MS revealed a new peak with a mass of 4330 (corresponding to that of  $A\beta_{1-40}$ ) after the incubation of  $A\beta_{1-42}$  with ACE, indicating  $A\beta_{1-40}$  generation. **F**, Captopril blocked  $A\beta_{1-40}$  generation in the mixture of  $A\beta_{1-42}$  and purified human ACE in a dose-dependent manner. The density of  $A\beta_{1-40}$  bands was measured by densitometry and normalized to the mean of the bands in the case of incubation without captopril.  $IC_{50}$  was estimated to be  $\sim$ 10 nM. **G**, Time-dependent alterations in  $A\beta_{1-42}$  and  $A\beta_{1-40}$  levels in the solution of  $A\beta_{1-42}$  (10  $\mu$ M) alone;  $A\beta_{1-42}$  (10  $\mu$ M) and ACE (2 U/ml); or  $A\beta_{1-42}$  (10  $\mu$ M), ACE (2 U/ml), and captopril (1  $\mu$ M). Each solution was incubated at 37°C for the time indicated. Ten microliters of each solution were collected at different time points and immediately frozen at  $-80^{\circ}\text{C}$  until analysis. The levels of  $A\beta_{1-42}$  and  $A\beta_{1-40}$  in each sample were determined by ELISA. Data are the mean  $\pm$  SEM of three samples.

**Table 1. Kinetic parameters for conversion of  $A\beta_{1-42}$  to  $A\beta_{1-40}$  by human kidney ACE**

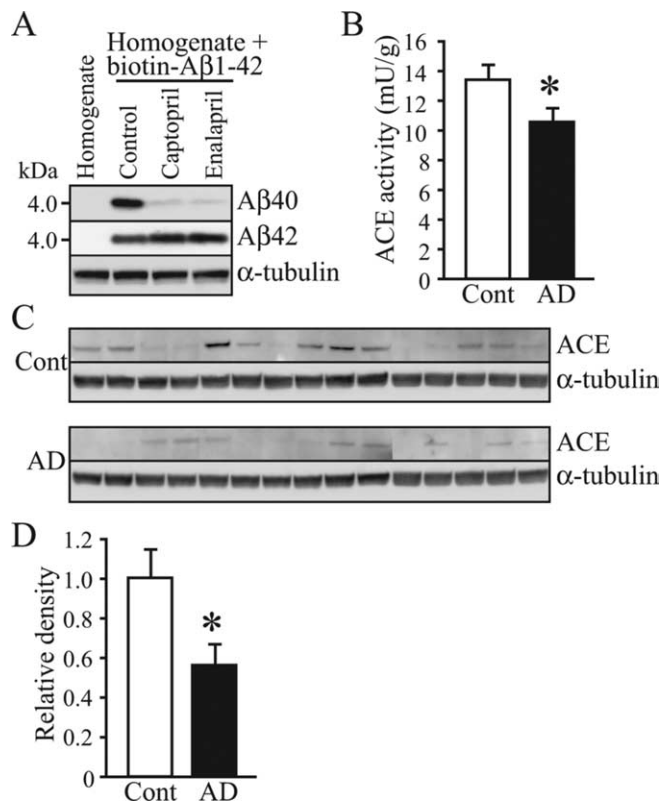
Substrate	Product	$K_m$ ( $\mu\text{M}$ )	$k_{cat}$ ( $\text{s}^{-1}$ )	$k_{cat}/K_m$ ( $\text{s}^{-1}\cdot\text{mM}^{-1}$ )
$A\beta_{1-42}$	$A\beta_{1-40}$	7	4.2	600

$A\beta_{1-42}$  peptides (0, 2.5, 5, 10, 15, 20, 25, 30, and 50  $\mu\text{M}$ ) were incubated at 37°C for 8 h with human kidney ACE (0.45  $\mu\text{M}$ ). The level of  $A\beta_{1-40}$  was determined by ELISA, and  $K_m$ ,  $k_{cat}$ , and  $k_{cat}/K_m$  were then calculated.

blot analysis (data not shown), suggesting that ACE cannot degrade aggregated  $A\beta_{1-42}$  or convert aggregated  $A\beta_{1-42}$  to  $A\beta_{1-40}$ . MALDI-TOF-MS demonstrated peaks with molecular masses of 4330 and 4514 after incubation for 8 h, which matched the predicted masses of  $A\beta_{1-40}$  and  $A\beta_{1-42}$ , respectively (Fig. 1E). MALDI-TOF-MS of the reaction mixture of ACE and  $A\beta_{1-42}$  revealed several peaks with masses corresponding to those of  $A\beta_{1-35}$ ,  $A\beta_{1-34}$ ,  $A\beta_{1-22}$ ,  $A\beta_{1-20}$ , and  $A\beta_{1-19}$ , in addition to  $A\beta_{1-40}$ . Interestingly, however, no  $A\beta_{1-38}$ ,  $A\beta_{1-7}$ , or  $A\beta_{8-42}$  was detected by our MALDI-TOF-MS. No  $A\beta_{1-40}$  was detected by MALDI-TOF-MS in a solution of purified ACE or synthetic  $A\beta_{1-42}$  after incubation at 37°C (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The  $\text{IC}_{50}$  of captopril needed for purified human ACE to convert  $A\beta_{1-42}$  to  $A\beta_{1-40}$  was  $\sim 10$  nM, indicating the specific inhibitory effect of captopril on ACE-mediated  $A\beta_{1-42}$ -to- $A\beta_{1-40}$  conversion (Fig. 1E).

Next we determined the level of  $A\beta_{1-40}$  converted from  $A\beta_{1-42}$  and that of  $A\beta_{1-42}$  remaining in the mixture of  $A\beta_{1-42}$  and ACE using an ELISA kit. To determine the level of  $A\beta_{1-42}$  lost from the solutions as a result of the “sticky” nature of  $A\beta_{1-42}$ , we also determined  $A\beta_{1-42}$  level in the absence or presence of ACE with an ACE inhibitor as control. During the incubation of  $A\beta_{1-42}$  with ACE for 2–8 h,  $\sim 10$ –20% of the degraded  $A\beta_{1-42}$  was converted to  $A\beta_{1-40}$ . These results indicate that  $A\beta_{1-40}$  can be generated from  $A\beta_{1-42}$  through the action of ACE and that ACE cleaves  $A\beta_{1-42}$  at other sites as well (Fig. 1F), which are supported by the results of MALDI-TOF-MS. Moreover,  $A\beta_{40}/A\beta_{42}$  ratio increased during the incubation period, suggesting that ACE decreases  $A\beta_{42}/A\beta_{40}$  ratio via its  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting activity (data not shown). To understand the catalytic efficiency of ACE converting  $A\beta_{1-42}$  to  $A\beta_{1-40}$ , we performed a kinetic assay of this conversion. The  $K_m$ ,  $k_{cat}$ , and  $k_{cat}/K_m$  of the  $A\beta_{1-42}$ -to- $A\beta_{1-40}$  conversion by ACE were determined to be 7  $\mu\text{M}$ , 4.2  $\text{s}^{-1}$ , and 600  $\text{s}^{-1}\cdot\text{mM}^{-1}$ , respectively (Table 1). These values were similar to those of ACE for angiotensin I (Ang I) hydrolysis (Hayakari et al., 2003). Thus, this  $A\beta_{1-42}$ -to- $A\beta_{1-40}$  conversion activity of ACE is likely favorable *in vivo* under normal physiological conditions.

To determine whether the human brain shows this  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting activity and whether the activity is inhibited by ACE inhibitors, we used the frontal cortex from a 76-year-old non-AD subject. To exclude the effect of endogenous  $A\beta_{1-40}$ , we labeled synthetic  $A\beta_{1-42}$  with biotin. The sites of biotinylation were at lysine residues amino acid positions 16 and 28 in  $A\beta_{1-42}$ . The biotinylated  $A\beta$ s were purified with avidin agarose, and biotinylated  $A\beta_{1-40}$  generated from biotinylated  $A\beta_{1-42}$  was detected by Western blot analysis. Similar to the results for the mouse brain homogenate, the incubation of  $A\beta_{1-42}$  with the human brain homogenate resulted in the generation of  $A\beta_{1-40}$ , and this generation was inhibited by an ACE inhibitor, namely captopril or enalapril (Fig. 2A; supplemental Table 3, available at www.jneurosci.org as supplemental material). No endogenous  $A\beta_{1-40}$  was detected in the human brain homogenate that was incubated without biotinylated  $A\beta_{1-42}$  (Fig. 2A). These results indicate that the human brain also shows  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -



**Figure 2.** The human brain shows  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting activity, and this ACE activity decreases in AD brain. **A**, The frontal cortex from a non-AD subject was homogenized in PBS. Biotinylated  $A\beta_{1-42}$  was added to the resulting homogenate, and the mixture was incubated at 37°C for 8 h with or without 1  $\mu\text{M}$  ACE inhibitor, namely captopril or enalapril. Biotinylated  $A\beta$ s were then purified using avidin agarose, and the conversion of  $A\beta_{1-42}$  to  $A\beta_{1-40}$  was detected by Western blot analysis using an anti- $A\beta_{1-40}$  monoclonal antibody (1A10) and an anti- $A\beta_{1-42}$  polyclonal antibody. **B**, The cortices from the frontal gyri of the non-AD subjects and AD patients were homogenized in a lysis buffer. The resulting homogenate was centrifuged, and ACE activity in the supernatant was determined using an ACE colorimetric kit. The ACE activities of 15 non-AD control individuals (Cont) and 15 AD patients were measured. **C**, The ACE expression level in the frontal cortex of those 30 subjects was determined by Western blot analysis, and a representative image is shown. Twenty micrograms of protein from the brain homogenate were subjected to SDS-PAGE and Western blot analysis. The membrane was probed with an anti-ACE polyclonal antibody. **D**, The relative densities of the ACE bands were determined by densitometry. AD cases exhibited a significantly decreased ACE expression level. Data are the mean  $\pm$  SEM of 15 samples. \* $p < 0.05$  versus non-AD control by Mann–Whitney *U* test.

converting activity and that this activity is mainly mediated by ACE. To determine whether ACE activity and ACE expression level are altered in AD brains, we measured ACE activity and determined ACE expression level by Western blot analysis. AD brains showed reduced ACE activity and expression level compared with age-matched non-AD brains (Fig. 2B–D).

To determine whether ACE inhibition promotes AD pathology, we administered captopril, a blood–brain barrier-penetrating ACE inhibitor, to an AD mouse model (hAPPsw, Tg2576) (Hsiao et al., 1996) and assessed its effect on brain amyloid deposition. Tg2576 mice were fed with a captopril-supplemented diet (captopril, 30 mg/kg of body weight/d) from 6 months of age. The mice were killed at 13 and 17 months of age, and their brains were analyzed. In the 13-month-old mice, captopril treatment resulted in a trend toward an increase in the number of thioflavin-S-positive plaques ( $n = 6$  per group; control neocortex,  $27.9 \pm 6.3$ ; neocortex of captopril-treated mice,  $34.9 \pm 7.3$ ;  $p = 0.8728$ ; control hippocampus,  $11.3 \pm 3.2$ ; hip-

pocampus of captopril-treated mice,  $21.3 \pm 5.8$ ;  $p = 0.2623$ , Mann–Whitney  $U$  test). Interestingly, the ELISA of  $A\beta$ s demonstrated a significantly higher  $A\beta_{1-42}$  level in the neocortex of the 13-month-old captopril-treated mice than in that of the control mice (control,  $504.6 \pm 14.1$  pmol/g; captopril-treated mice,  $643.7 \pm 49.0$  pmol/g;  $p = 0.0374$ ); however, the level of  $A\beta_{1-40}$  remained unchanged (control,  $1690.2 \pm 141.4$  pmol/g; captopril-treated mice,  $1700.0 \pm 249.8$  pmol/g;  $p = 0.9361$ , Mann–Whitney  $U$  test). These results suggest that captopril treatment enhances  $A\beta_{1-42}$  deposition to a greater extent than  $A\beta_{1-40}$  deposition in the brain.

The analysis of the 17-month-old captopril-treated mice showed 2.5-fold and twofold increases in the number of thioflavin-S-positive plaques in the cortex and hippocampus, respectively, compared with that of the control mice (Fig. 3B,G). Moreover,  $A\beta_{1-42}$ -immunopositive areas in the neocortex and hippocampus were respectively 2.6-fold and twofold larger in the 17-month-old captopril-treated mice than in the control mice (Fig. 3A,C,H). Captopril treatment resulted in an increase in  $A\beta_{1-40}$ -immunopositive area; however, the increase was not statistically significant (Fig. 3A,D,I). These histological findings are supported by ELISA results.  $A\beta_{1-42}$  level increased significantly higher (1.6-fold higher) in the neocortex of the captopril-treated mice than in that of the control mice, whereas  $A\beta_{1-40}$  level showed no significant increase in the neocortex of the captopril-treated mice (Fig. 3E,F). Consistent with a previous study (Kawarabayashi et al., 2001), the present study also showed that small “diffuse plaques” appear to be labeled preferentially by the anti- $A\beta_{1-42}$  antibody, whereas big “cored plaques” are labeled by the anti- $A\beta_{1-40}$  antibody (Fig. 3A). Captopril treatment decreased serum ACE activity by 40% and neocortex ACE activity by 26% in the 17-month-old captopril-treated Tg2576 mice compared with that in the control mice (Fig. 3J,K). The activity of brain ACE significantly and inversely correlated with enhanced thioflavin-S-positive plaque formation in the neocortex of the mice (Fig. 3L). These findings indicate that ACE inhibition promotes a greater degree of and earlier  $A\beta_{1-42}$  deposition than  $A\beta_{1-40}$  deposition, suggesting that a low ACE activity in the brain promotes AD development by enhancing  $A\beta_{1-42}$  deposition.

To determine whether acute ACE inhibition has a direct effect on the levels of brain  $A\beta$ s, we treated Tg2576 mice with captopril by one-shot oral gavage. The Tg2576 mice used for this experiment were 9 months of age, younger than those used for chronic treatment and supposed to have few  $A\beta$  deposits. The one-shot oral gavage of captopril (30 mg/kg of body weight in a volume of 150  $\mu$ l) to Tg2576 mice resulted in a significant decrease in serum ACE activity (control,  $248 \pm 21$  U/L; captopril-treated mice,  $43 \pm 2$  U/L;  $p < 0.01$ , Mann–Whitney  $U$  test;  $n = 6$  each group). The ACE activity in the neocortex of the captopril-treated mice was significantly lower than that of the control mice (control,  $38 \pm 4$  mU/L; captopril-treated,  $32 \pm 5$  mU/L; Mann–Whitney  $U$  test,  $p < 0.05$ ;  $n = 6$  each group). However, the acute ACE inhibition resulted in no significant increase in the level of  $A\beta_{1-42}$  (control,  $391 \pm 99$  pmol/g; captopril-treated mice,  $619 \pm 94$  pmol/g;  $p = 0.15$ , Mann–Whitney  $U$  test;  $n = 6$  each group) or  $A\beta_{1-40}$  (control,  $929 \pm 164$  pmol/g; captopril-treated mice,  $1270 \pm 156$  pmol/g;  $p = 0.20$ , Mann–Whitney  $U$  test;  $n = 6$  each group) in the Tg2576 mouse brain. These results suggest that ACE inhibition does not significantly alter brain  $A\beta$  degradation in the acute phase.

To exclude the possibility that captopril affects  $\gamma$ -secretase processing, thereby altering  $A\beta_{42}/A\beta_{40}$  ratio, we treated fibroblasts stably expressing hAPP695 with captopril and quantified

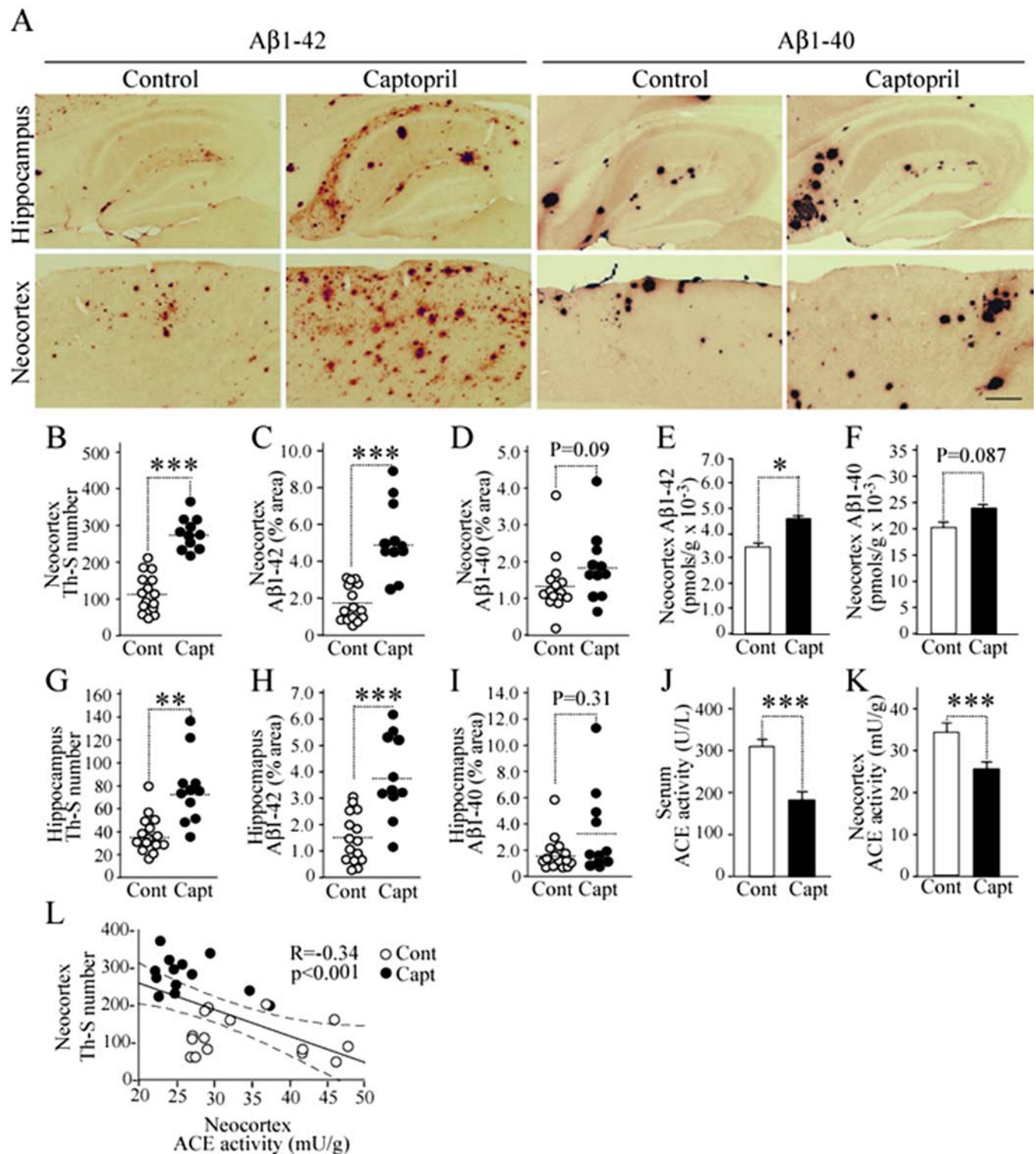
the secreted  $A\beta_{1-40}$  and  $A\beta_{1-42}$ . No cellular ACE in this cell line was detected by Western blot analysis using several anti-ACE antibodies [MAB3500 and MAB3502 (Millipore); ACENabm-9B9 (RDI, Flanders, NJ); 2E2 (Serotec, Oxford, UK); 19501 (QED Bioscience, San Diego, CA); AF1513 (R & D Systems, Minneapolis, MN)] (Fig. 4A) (data not shown). No ACE activity was detected in this cell line either (Fig. 4B). We used a mouse kidney homogenate as a positive control. ACE and ACE activity in the kidney homogenate were clearly detected, and ACE activity was inhibited by captopril (Fig. 4A,B). The treatment with captopril did not alter  $A\beta_{1-40}$  or  $A\beta_{1-42}$  level or  $A\beta_{42}/A\beta_{40}$  ratio in fibroblasts, indicating that captopril has no effect on  $\gamma$ -secretase activity in terms of the shift from  $A\beta_{1-42}$  secretion to  $A\beta_{1-40}$  secretion (Figs. 4C,D). These results suggest that the enhanced predominant  $A\beta_{1-42}$  deposition in the captopril-treated Tg2576 mice is caused by the inhibition of ACE-mediated  $A\beta_{1-42}$ -to- $A\beta_{1-40}$ -converting activity and  $A\beta_{1-42}$  and  $A\beta_{1-40}$  degradations, and not by altering  $\gamma$ -secretase processing.

## Discussion

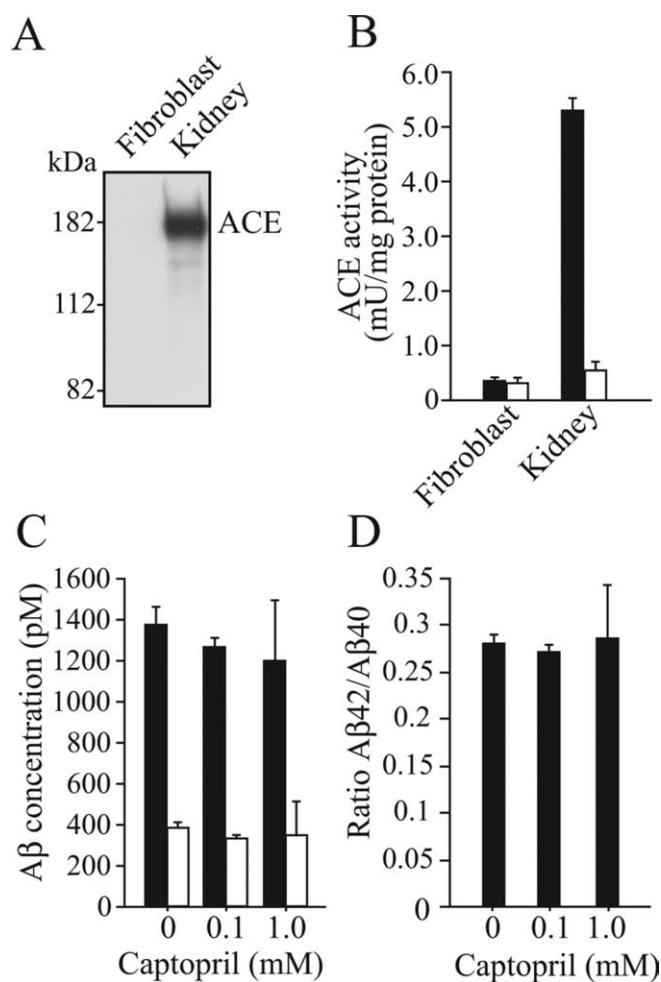
Here, we reported for the first time that ACE converts  $A\beta_{1-42}$  to  $A\beta_{1-40}$  and that a chronic inhibition of ACE enhances predominant  $A\beta_{1-42}$  deposition *in vivo*. A high  $A\beta_{1-42}$  level or a high  $A\beta_{42}/A\beta_{40}$  ratio appears crucial in AD pathogenesis. Although their molecular mechanisms are not yet fully understood,  $A\beta_{1-40}$  and  $A\beta_{1-42}$  generations are supposed to be modulated by the shift of  $\gamma$ -cleavage (Weggen et al., 2001; De Strooper, 2003; Lleo et al., 2004). In addition to the  $\gamma$ -cleavage shift theory,  $A\beta_{1-40}$  and  $A\beta_{1-42}$  have recently been suggested to be generated from a longer form of  $A\beta$  species generated by  $\epsilon$ -cleavage at every three residues in its carboxyl portion; however, the enzyme involved in carboxypeptidyl cleavage has not yet been identified (Qitakahara et al., 2005). Our present study showed a novel catabolism pathway for modulating  $A\beta_{1-42}$  degradation; that is, ACE generates  $A\beta_{1-40}$  from secreted  $A\beta_{1-42}$  by carboxydipeptidyl cleavage in the mouse and human brains.

ACE is a zinc metalloproteinase and a dipeptidyl carboxypeptidase that cleaves 2 aa from the C terminus of Ang I and converts Ang I to the vasoactive and aldosterone-stimulating peptide Ang II (Corvol et al., 1995). ACE is a membrane-bound enzyme in endothelial cells and several types of epithelial and neuroepithelial cells. The active site of ACE is located in the extracellular space, and the unbound form of ACE circulating in biological fluids, such as plasma and CSF, and both types of ACE have enzymatic activity (Zubenko et al., 1985; Rigat et al., 1990; Sibony et al., 1993). These lines of evidence support our findings that ACE converts  $A\beta_{1-42}$  to  $A\beta_{1-40}$  and degrades  $A\beta$ s under physiological conditions, thereby contributing to the prevention of  $A\beta$  deposition in the brain. In this study,  $\sim 10$ – $20\%$  of degraded  $A\beta_{1-42}$  was converted to  $A\beta_{1-40}$  in the presence of ACE.

Previous studies showed that the major cleavage site of  $A\beta_{1-40}$  is between amino acid positions 7 and 8 (Hu et al., 2001; Oba et al., 2005). If the  $A\beta_{1-40}$  generated from  $A\beta_{1-42}$  was subsequently cleaved between amino positions 7 and 8 by ACE, then  $A\beta_{8-40}$  could have been detected by MALDI-TOF-MS. However, no generation of  $A\beta_{1-7}$ ,  $A\beta_{8-40}$ , or  $A\beta_{8-42}$  in the reaction mixture of ACE and  $A\beta_{1-42}$  was detected by MALDI-TOF-MS, indicating that no cleavage between amino positions 7 and 8 occurs in our system. What causes this discrepancy remains undetermined. It is possible that  $A\beta_{1-42}$  and  $A\beta_{1-40}$  have different conformations, which may allow ACE to access these species differently. Another explanation may be that the ACE used in this study contained the plasma membrane domain from the human kidney, and that



**Figure 3.** Long-term inhibition of ACE activity enhances  $A\beta_{1-42}$  deposition in the 17-month-old hAPPsw transgenic mouse (Tg2576) brain. Tg2576 mice at 6 months of age were treated with captopril (30 mg/kg of body weight/d) and killed after 11 months of treatment. *A*, Sagittal brain sections of 17-month-old control diet-fed and captopril-supplemented diet-fed mice were stained with antibodies specific for  $A\beta_{1-42}$  and  $A\beta_{1-40}$  to detect human  $A\beta$  deposition. Representative images of hippocampi and neocortices with or without treatment are shown. The left four panels are images immunostained by the anti- $A\beta_{1-42}$  antibody, and the right four panels are images of brain sections immunostained by the anti- $A\beta_{1-40}$  antibody. Scale bar, 500  $\mu$ m. *B–I*, Determinations of the number of thioflavin-S-positive plaques (*B*, *G*) and immunopositive area demonstrated by anti- $A\beta_{1-42}$  antibody (*C*, *H*) and anti- $A\beta_{1-40}$  antibody (*D*, *I*) in brain neocortex (*B–D*) and hippocampus [*G–I*;  $n = 15$  for control diet (Cont; open circles);  $n = 11$  for captopril diet (Capt; filled circles)]. *E*, *F*,  $A\beta_{1-42}$  (*E*) and  $A\beta_{1-40}$  (*F*) levels determined by ELISA in brain neocortex of 17-month-old mice fed with control and captopril-supplemented diets. The ELISA data included both soluble and insoluble  $A\beta$ s. *J*, *K*, Serum (*J*) and neocortex (*K*) ACE activities in mice fed with control diet and captopril-supplemented diet. *L*, Significant inverse correlation between ACE activity and the number of thioflavin-S-positive plaques in neocortex. Data are the mean  $\pm$  SEM;  $p$  was determined by the Mann–Whitney  $U$  test (*B–K*) and Spearman rank test (*L*). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Figure 4.** Effect of captopril on  $A\beta_{1-42}/A\beta_{1-40}$  ratio in hAPP-expressing fibroblasts. **A**, Fibroblast homogenate and wild-type mouse kidney homogenate (40  $\mu$ g of protein) were subjected to Western blot analysis using an anti-ACE polyclonal antibody. No cellular ACE in fibroblasts was detected, whereas ACE in the mouse kidney homogenate as a positive control was clearly detected. **B**, ACE activities in the fibroblast and kidney homogenates were determined. ACE activity in the fibroblast homogenate was very low, whereas that in kidney homogenate was detectable, which was clearly inhibited by captopril (1  $\mu$ M) treatment (filled bars, no captopril; open bars, captopril). **C**, Fibroblasts stably expressing human APP695 were grown in DMEM containing 10% FBS. The cells were treated with or without captopril at 100% confluence after changing the culture medium. The  $A\beta_{1-40}$  and  $A\beta_{1-42}$  levels in the medium were determined by ELISA after 24 h. Filled bars,  $A\beta_{1-40}$ ; open bars,  $A\beta_{1-42}$ . **D**,  $A\beta_{42}/A\beta_{40}$  ratio was calculated. **C, D**, Note that captopril did not alter the levels of secreted  $A\beta$ s (**C**) or  $A\beta_{42}/A\beta_{40}$  ratio (**D**).

used in previous studies was the secreted form of ACE in human seminal plasma or Cos7-secreted conditioned media. Additional studies are required to clarify this. In this study, MALDI-TOF-MS demonstrated a peak corresponding to  $A\beta_{1-40}$  in the reaction mixture of ACE and  $A\beta_{1-42}$  but no peak corresponding to  $A\beta_{1-38}$ . This suggests that ACE does not cleave the 2 C-terminal amino acids of  $A\beta_{1-40}$ .

In addition, one may question why  $A\beta_{1-40}$  level increases if  $A\beta_{1-40}$  can be digested further. It is possible that the most predominant substrate at the start of the reaction is  $A\beta_{1-42}$  and that the degradation of  $A\beta_{1-40}$  is negligible because the level of  $A\beta_{1-40}$  generated from  $A\beta_{1-42}$  is extremely low (Fig. 1F).

To understand the role of ACE *in vivo*, we induced a chronic ACE inhibition in Tg2576 mice and found that the ACE inhibition enhances  $A\beta$  deposition *in vivo*. However, it is important to determine whether acute and short-term treatment with capto-

pril also increases brain soluble  $A\beta$  level. To exclude the effect of  $A\beta$  deposition, we used younger Tg2576 mice, whose brain shows almost no  $A\beta$  deposition (Kawarabayashi et al., 2001), in this experiment. An acute, one-shot oral administration of captopril to the young Tg2576 mice resulted in a significant decrease in serum and brain ACE activities; however, the level of brain  $A\beta_{1-42}$  or  $A\beta_{1-40}$  was not significantly affected. A recent *in vivo* study also shows that ACE inhibitors at doses similar to those used clinically do not increase the levels of brain  $A\beta$ s (Eckman et al., 2006). Previous studies using purified human seminal plasma ACE and cultured cells showed that ACE degrades  $A\beta$ s and ACE inhibition increases  $A\beta$  levels in APP- and ACE-transfected cells (Hu et al., 2001; Hemming and Selkoe, 2005; Oba et al., 2005). However, these findings seem to disagree with those of *in vivo* studies. One explanation may be that other  $A\beta$ -degrading enzymes, such as neprilysin, the insulin-degrading enzyme, and the endothelin-converting enzyme may compensate for the acute reduction in ACE activity *in vivo*. An important issue to be stressed is that even a high captopril dose induces only a slight decrease in brain ACE activity; however, long-term captopril treatment induces a marked and significant enhancement of  $A\beta$  deposition in the aged mouse brain. Thus, additional studies are required to determine the chronic effect of ACE inhibitors at clinical doses on AD pathology and development.

Numerous studies have shown that the I allele of ACE *D/I* polymorphism is associated with an increased risk of late-onset AD (Hu et al., 1999; Kehoe et al., 1999; Elkins et al., 2004; Lehmann et al., 2005) and that *I* polymorphism is associated with a decreased serum ACE level (Rigat et al., 1990). ACE activity in CSF from patients with a moderately severe senile dementia of the AD type has been shown to decrease to 41% of that in CSF from non-AD patients (Zubenko et al., 1985). Because ACE inhibitors are widely used in patients with hypertension, which is a risk factor for AD, it is important to determine the effects of ACE inhibitors on  $A\beta$  deposition *in vivo*. We have treated an AD mouse model (hAPPsw, Tg2576) (Hsiao et al., 1996) with captopril, a blood–brain barrier-penetrating ACE inhibitor, and found that, consistent with our *in vitro* findings, treatment with captopril enhances the depositions of  $A\beta_{1-42}$  and  $A\beta_{1-40}$ , but more prominently that of  $A\beta_{1-42}$  in mouse brains. This suggests that a certain amount of  $A\beta_{1-40}$  is generated by ACE from secreted  $A\beta_{1-42}$  and that treatment with ACE inhibitors may be a risk factor for AD. There have been very few clinical studies analyzing the effects of ACE inhibitors on AD development and cognitive decline in AD patients, and results to date are inconclusive (Birkenhager et al., 2004; Gard and Rusted, 2004; Ohri et al., 2004; Khachaturian et al., 2006). Therefore, additional studies are required to determine the effects of ACE inhibitors both on  $A\beta$  deposition in the brain of patients during long-term medication with ACE inhibitors and on the cognitive ability of AD patients without hypertension. Together, the results of this study suggest that the upregulation of ACE activity may decrease  $A\beta_{42}/A\beta_{40}$  ratio and the levels of  $A\beta$ s and can be used as a strategy for developing novel therapeutic regimens for AD patients without hypertension.

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