重合体細胞内蓄積を予防していることと、AT8 陽性神経原線維変化の形成が抑制されていることが明らかとなった。26 ヶ月齢のアルツハイマー病モデルマウス(3X-Tg AD)脳内には 100%神経原線維変化が形成されることが報告されており、今回観察した AT8 陽性リン酸化タウは pre-tangle と PHF の両者を含んであると考えられる。静脈投与された 6H4 抗体の作用点が細胞外や細胞膜上であることを考慮すると、今回観察された病理所見から、細胞外 Aβ重合体が細胞内へと蓄積し、神経原線維変化形成を誘導する神経変性発症メカニズムの存在が示唆された。

E. 結論

1) Aβ重合体の神経変性機序として、細胞内 Aβ重合体蓄積が神経原線維変化形成を誘導する可能性が示唆された。
2) in vivo で Aβ重合体を選択的に抗体療法で制御すると神経変性予防と記憶障害改善効果が得られることが明らかとなった。

F. 健康危険情報

なし。

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H. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金(長寿科学総合研究事業)

(分担)研究報告書

Aβ分解調節に関する研究

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研究要旨

アミロイドβペプチド（Aβ）の分解や代謝を制御する因子として、カルパイン-カルパスタチン系を同定した。カルパインはカルシウムによって活性化される細胞内プロテアーゼであり、カルパスタチン（CS）はその阻害タンパク質である。アミロイド前駆体タンパク（APP）を過剰発現するトランスジェニック（Tg）マウスにCS-KOマウスを交配したところ、ネプリライシン活性が低下し、Aβ病理が増悪化した。一方、CS-Tgの場合は、Aβ病理が減弱した。これにより、カルパイン過剰活性化がAβ蓄積を促進することが初めて明らかになった。さらに、APP-Tg x CS-KOマウスでは、タウタンパク質のリン酸化が昂進し（神経原線維変化は認められなかった）、これはAPP-Tg x CS-Tgマウスでは抑制されていた。同様に、前者ではperipheral benzodiazepine receptorによって表される炎症反応が増悪化し、後者ではこれが抑制されていた。また、APP-Tgマウス単独では神経細胞の変性は認められないが、APP-Tg x CS-KOマウスでは、嗅内野において細胞体のatrophyおよび樹状突起のdystrophyが観察された。アルツハイマー病患者の脳においてもカルパイン活性化が認められることから、アルツハイマー病の発症機構において、カルパイン-カルパスタチン系が重要な働きを有すること、また、カルパイン特異的阻害剤はアルツハイマー病の予防薬・治療薬となる可能性が示唆された。

A. 研究目的

アルツハイマー病の発症機構におけるカルパイン-カルパスタチン系の役割を明らかにすることを目的とした。

B. 研究方法

APP-TgマウスとCS-KOマウスを交配し、神経病理学的・生化学的表現型を解析した。

(倫理面への配慮)

本研究は、当該施設の倫理委員会の承認を受けて行った。特に動物実験については当施設の実験規則ならびに動物愛護の精神に則って行い苦痛の防止にも留意した。

C. 研究結果と D. 考察

APP-Tgマウスと比較して、APP-Tg x CS-KOマウスでは、Aβ病理が増悪化し、タウタンパク質リン酸化が上昇した。さらに、炎症反応が増強され、嗅内野における神経細胞体および樹状突起における変性が観察された。一方、APP-Tg xCS-Tgでは、これらの現象が抑制されていた。これらのことは、アルツハイマー病の発症機構にお

いてカルパイン-カルパスタチン系が重要な役割を果たすことを示すと考えられる。

E. 結論

カルパイン特異的阻害剤はアルツハイマー病の予防薬・治療薬となる可能性が示唆された。

F. 健康危険情報

なし。

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第28回日本認知症学会学術集会, 仙台, 2009年11月 20 日

H. 知的財産権の出願・登録状況
なし

厚生労働科学研究費補助金(認知症対策総合研究事業)
(分担)研究報告書

Aβ産生分子機構の解明と特異的制御による治療法の開発

研究分担者: 富田泰輔　東京大学大学院薬学系研究科

研究要旨

βおよびγセクレターゼは、脳におけるAβ産生を担う酵素であり、その活性の特異的制御はADの根本治療法となることが期待されている。申請者は特に両セクレターゼが膜結合型プロテアーゼであることに注目し、その酵素活性と膜脂質との関連を解明し、「脂質環境の変化によるセクレターゼ活性制御」という新たなコンセプトに基づいたAD創薬標的候補分子群の同定と活性制御法の開発を行った。γセクレターゼについては、Notchシグナル遮断による副作用を回避する可能性を持つγセクレターゼモジュレーターについて、新規骨格を持つ化合物群の開発に成功した。一方、昨年度までにβセクレターゼ活性を制御することを見出していたスフィンゴシン-1-リン酸（S1P）の合成酵素であるSphingosine kinase（Sphk）阻害剤を用い、準慢性経口投与実験により脳内アミロイド蓄積が減少することをモデル動物において確認した。また初代培養神経細胞に対するAβ線維処理によりSphk活性が上昇することを見出し、アミロイドによる神経細胞ストレスがSphk活性上昇を惹起し、さらなるAβ産生上昇を導くVicious Cycleの存在を明らかにした。

A. 研究目的：本研究では、セクレターゼ活性阻害による脳内Aβ量の制御により、副作用のないアルツハイマー病の予防・治療法の確立を目指す。本年度は、具体的に以下の3点について解明を進めた。1）γセクレターゼモジュレーターの同定と構造活性相関解析、2）S1Pによるβセクレターゼ活性の制御機構解明

B. 研究方法

1）有機化学研究者らによって作出されたオリジナルライブラリーに含まれる化合物のγセクレターゼ活性制御効果について、培養細胞および*in vitro*アッセイ系を用いて検討した。

2）Sphk阻害剤のモデル動物への投与実験を行った。またSphk活性測定系を樹立し、各種サンプルでのSphk活性変化を検出した。

(倫理面への配慮)

本研究は、当該施設の倫理委員会の承認を受けて行った。特に動物実験については当施設の実験規則ならびに動物愛護の精神に則って行い苦痛の防止にも留意した。

C. 研究結果と D. 考察

①脳内移行性を考慮し、脂質をリガンドとする核内受容体やGPCRに注目し、これらのアゴニスト・アンタゴニストおよび誘導体を含んだフォーカストライブラリーを用

いて検討した。その結果 Notch 切断を保ちながらＡβ42 産生のみ、若しくはＡβ40/42 産生を低下させる各種γセクレターゼモジュレーターを同定した。

②蛍光 Sphingosine を用い、*in vitro* Sphk assay を確立した。各種サンプルについて検討を行い、マウス脳および初代培養神経細胞においても Sphk 活性が十分存在し、Sphk 阻害剤である SKI II により活性が抑制されることを確認した。また合成Ａβを凝集させたアミロイド線維を初代培養神経細胞に投与したところ、Sphk 活性の上昇が観察された。

③SKI II を当研究室において樹立された AD モデルマウス A7 に対して、脳内アミロイド蓄積の見られる前の 8 ヶ月齢個体に対して 50mg/kg/day で 6 日経口投与し、大脳半球内のＡβ量を測定したところ、20 数%の有意な減少を確認した。

E. 結論

1）脂溶性が高く、新規骨格を持つ Aβ42 産生を低下させる GSM の同定に成功した。

2）Sphk 活性が神経細胞に存在し、アミロイド線維処理によって上昇することが明らかとなった。

3）Sphk 阻害剤である SKI II の準慢性投与により脳内Ａβ量の低下が観察された。

4）以上の結果から、神経細胞特異的な脂質環境を利用したセクレターゼ活性制御システムの理解により、副作用の軽減された治療薬開発が見込めることを提案する。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況

なし

III.研究成果の刊行に関する一覧表

別紙 4

研究成果の刊行に関する一覧表

著書

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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IV.研究成果の刊行物・別刷

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Aβ42-to-Aβ40- and Angiotensin-converting Activities in Different Domains of Angiotensin-converting Enzyme*

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Amyloid β-protein 1–42 (Aβ42) is believed to play a causative role in the development of Alzheimer disease (AD), although it is a minor part of Aβ. In contrast, Aβ40 is the predominant secreted form of Aβ and recent studies have suggested that Aβ40 has neuroprotective effects and inhibits amyloid deposition. We have reported that angiotensin-converting enzyme (ACE) converts Aβ42 to Aβ40, and its inhibition enhances brain Aβ42 deposition (Zou, K., Yamaguchi, H., Akatsu, H., Sakamoto, T., Ko, M., Mizoguchi, K., Gong, J. S., Yu, W., Yamamoto, T., Kosaka, K., Yanagisawa, K., and Michikawa, M. (2007) *J. Neurosci.* 27, 8628–8635). ACE has two homologous domains, each having a functional active site. In the present study, we identified the domain of ACE, which is responsible for converting Aβ42 to Aβ40. Interestingly, Aβ42-to-Aβ40-converting activity is solely found in the N-domain of ACE and the angiotensin-converting activity is found predominantly in the C-domain of ACE. We also found that the *N*-linked glycosylation is essential for both Aβ42-to-Aβ40- and angiotensin-converting activities and that unglycosylated ACE rapidly degraded. The domain-specific converting activity of ACE suggests that ACE inhibitors could be designed to specifically target the angiotensin-converting C-domain, without inhibiting the Aβ42-to-Aβ40-converting activity of ACE or increasing neurotoxic Aβ42.

Angiotensin-converting enzyme (ACE)[4] plays a key role in the renin-angiotensin system (RAS), which is involved in the long-term regulation of blood pressure and blood volume in the human body. Recent genetic, pathologic, and biochemical studies have associated ACE with onset of Alzheimer disease (AD) (1, 2). The I allele of the *ACE* gene, which results in a reduced serum ACE level, has been demonstrated to be associated with AD (3–5). Hypertension is a risk factor for AD and ACE inhibitors for treatment of hypertension were shown to be the only drug class among the antihypertensives to potentially be associated with a slight increased incidence of AD (adjusted hazard ratio 1.13) (6, 7). A mechanistic link between ACE and AD was suggested when ACE was shown to degrade Aβ40 and Aβ42 (8, 9). Overexpression of Aβ40 in transgenic mice does not cause brain amyloid deposition, the major pathological hallmark of AD, whereas expression of Aβ42 is shown to be essential for amyloid deposition (10, 11). In addition, Aβ40 has an inhibitory effect on amyloid deposition *in vitro* and *in vivo* and has neuroprotective effects (12–14). These lines of evidence suggest that converting Aβ42 to Aβ40 may be a potential strategy for development of an AD therapy. In our previous study, we identified ACE as an Aβ42-to-Aβ40-converting (Aβ-converting) enzyme and showed that ACE inhibitor enhances brain Aβ42 deposition in transgenic mice (15). Clarifying the molecular base of ACE domain-specific enzymatic activity on Aβ42 to Aβ40 conversion, Aβ degradation, and angiotensin conversion emerges to be important for development of a strategy for hypertension and AD treatment.

ACE is a type I integral membrane glycoprotein, and there are two isoforms of ACE in mammals that arise from the use of alternative promoters in a single gene: somatic ACE and testicular ACE. ACE also has one mammalian relative, ACE2, which consists of a single active site domain that, by sequence comparison, more closely resembles the N-domain than the C-domain of somatic ACE. ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor, and inactivates bradykinin, a vasodilator (16). Given the central role ACE plays in regulation of blood pressure, ACE inhibitors are widely used for the treatment of hypertension in the elderly population. ACE also hydrolyzes a wide range of polypeptide substrates, including substance P, luteinizing hormone-releasing hormone, acetyl-Ser-Asp-Lys-Pro (AcSDKP), and neurotensin (16). The mammalian somatic ACE contains two homologous domains, the N-terminal domain (N-domain) and C-terminal domain (C-domain), each bearing a zinc-dependent active site. The pres-

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[4] The abbreviations used are: ACE, angiotensin-converting enzyme; Aβ, amyloid β-protein; F-ACE, full-domain ACE; N-ACE, N-terminal domain ACE; C-ACE, C-terminal domain ACE; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; AD, Alzheimer Disease.

ence of two active sites in ACE has stimulated many attempts to establish whether they differ in function. For example, AcSDKP, a peptide suggested to inhibit bone marrow maturation, is found to be preferentially cleaved by the N-domain of ACE *in vitro* (17). In contrast, the ACE C-domain is demonstrated to be the main site of angiotensin I cleavage *in vivo* (18). The *N*-linked glycosylation of testicular ACE, a homologue of the somatic ACE N-domain, is essential for its enzymatic activity and for preventing degradation (19).

In our current study, we determined the contributions of each ACE domain, toward Aβ42-to-Aβ40- and/or angiotensin-converting activity. We postulated that the dipeptidyl carboxypeptidase activity of ACE, which converts angiotensin I to angiotensin II and Aβ42 to Aβ40, is located in its C-domain. Surprisingly, we found that the Aβ42-to-Aβ40-converting activity is specifically in the N-domain of ACE, and the angiotensin-converting activity is predominantly in the C-domain of ACE. We also found that both Aβ42-to-Aβ40- and angiotensin-converting activities require the *N*-linked glycosylation of ACE. The finding of domain-specific Aβ42-to-Aβ40-converting activity of ACE may help design a domain-specific ACE inhibitor for treatment of hypertension, without inhibiting the N-domain-specific Aβ42-to-Aβ40-converting activity of ACE.

EXPERIMENTAL PROCEDURES

Truncated ACE Expression and Purification—Expression and purification of ACE recombinant proteins were carried out as described previously (20). Mutated ACE cDNAs containing two active domains (F-ACE) or only the N-terminal active domain or C-terminal active domain (N-ACE or C-ACE) were cloned into pcDNA3.1(-) vectors (Invitrogen). Six histidine residues were introduced at the C-terminal end of each cDNA. The C-terminal transmembrane domain was removed from all of the recombinant ACE proteins to allow them to be secreted into the culture medium. COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Transfections of the ACE pcDNA3.1(-) vectors in COS7 cells were performed using Lipofectamine 2000 (Invitrogen), and COS7 cells stably expressing F-, N-, and C-ACE were selected in DMEM containing 10% fetal bovine serum and 1 mg/ml Geneticin (Wako, Japan). Culture media were harvested 3 days after the cells reached confluence, and recombinant ACE proteins were purified using a TALON purification kit (Clontech). The purified proteins were then dialyzed in 50 mM HEPES, 50 mM NaCl, 1 μM $ZnCl_2$, pH 7.5 and concentrated with Centricon YM-50 (Millipore). Protein concentrations of the ACE proteins were determined using a BCA protein assay kit (Pierce).

Western Blot Analysis and Determining Conversion of Aβ42 to Aβ40—COS7 cells were lysed in radioimmune precipitation assay buffer (10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.2% sodium deoxycholate, containing a protease inhibitor mixture (Roche Applied Science)). The expression of ACE recombinant proteins was detected by Western blotting using a polyclonal anti-ACE antibody (R&D). Aβ1–42 (Peptide Institute) was freshly dissolved in 0.1% $NH_3 \cdot H_2O$ at 200 μM for each experiment. 80 μl of F-, N-, and C-ACE at a concentration of 0.5 μM were mixed with synthetic Aβ42 to a final concentration of 40 μM and incubated at 37 °C. 10 μl of the mixture was subjected to SDS-PAGE and blotted on a nitrocellulose membrane. To enhance the reactivity to an anti-Aβ40 antibody, the membrane was boiled in PBS for 3 min after blotting, probed with an anti-Aβ40 monoclonal antibody (1A10) (IBL), and visualized with SuperSignal (Pierce). Because of the high level of exogenous Aβ42, the membrane was not boiled before the reaction with a polyclonal anti-Aβ42 antibody. The quantitation of Aβ40 generation and Aβ42 degradation was carried out using Image J 1.41 software (NIH).

ACE Activity Assay—F-ACE, N-ACE, and C-ACE were dialyzed in 50 mM HEPES, 50 mM NaCl, 1 μM $ZnCl_2$, pH 7.5, and their activities against the synthetic substrate *N*-hippuryl-l-histidyl-l-leucine (Hip-His-Leu) were determined using an ACE colorimetric kit (Buhlmann Laboratories, Schonenbuch, Switzerland). 10 μl of ACE proteins at a concentration of 0.5 μM were mixed and incubated with ACE substrate at 37 °C. The reaction time was 15 min. All samples were measured in triplicate.

Mass Spectrometry Analysis—Purified F-ACE, N-ACE, or C-ACE was incubated with 80 μM Aβ42 at 37 °C for 2 h. Captopril (10 μM) was added to stop digestion, and the sample was frozen in −80 °C until use. The samples were mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (Wako, Japan) as a matrix, and then subjected to matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (AXIMA-CFR, SHIMADZU, Kyoto, Japan) to detect the generation of Aβ40 and other Aβ fragments. The same amount of F-ACE, N-ACE, C-ACE, or Aβ42 incubated alone under the same conditions as described above was used as control.

Expression of ACE Active Site Mutants and Determining Their Domain-specific Activities—The pcDNA5/FRT expression vectors bearing the catalytically inactive full-length ACE were kindly provided by Dr. Dennis J. Selkoe (9). The two ACE zinc metalloprotease active site glutamates (amino acids 362 in the N-domain and 960 in the C-domain) were changed to aspartates. Mouse embryonic fibroblasts at 90% confluence were transiently transfected with the vectors bearing ACE full-length protein with active site mutations using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were lysed in in 50 mM Tris/HCl (pH 7.5) containing 0.5% Nonidet P-40, and nuclei and cell debris was pelleted at 10,000 × *g* for 10 min at 4 °C. To assay ACE activity, 5 μg of protein of cell lysate was incubated with Hip-His-Leu. For the Aβ42-to-Aβ40-converting activity assay, ACE in each cell lysate was immunoprecipitated using a polyclonal anti-ACE antibody (R&D) and protein G-Sepharose (GE Healthcare). Immunoprecipitated ACE was then incubated with 40 μM synthetic Aβ42 at 37 °C for 15 h. Captopril (10 μM) was added to the mixture to stop the reaction and the conversion of Aβ40 from Aβ42 was detected by Western blot.

Deglycosylation of ACE Proteins—To assess the type of glycosylation of human kidney ACE and recombinant ACE proteins, the ACE proteins were treated with PNGase F, *O*-glycanase, or sialidase A using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme, San Leandro, CA). To evaluate the enzymatic activities of deglycosylated ACE proteins, non-denaturing protocol was used, and ACE proteins

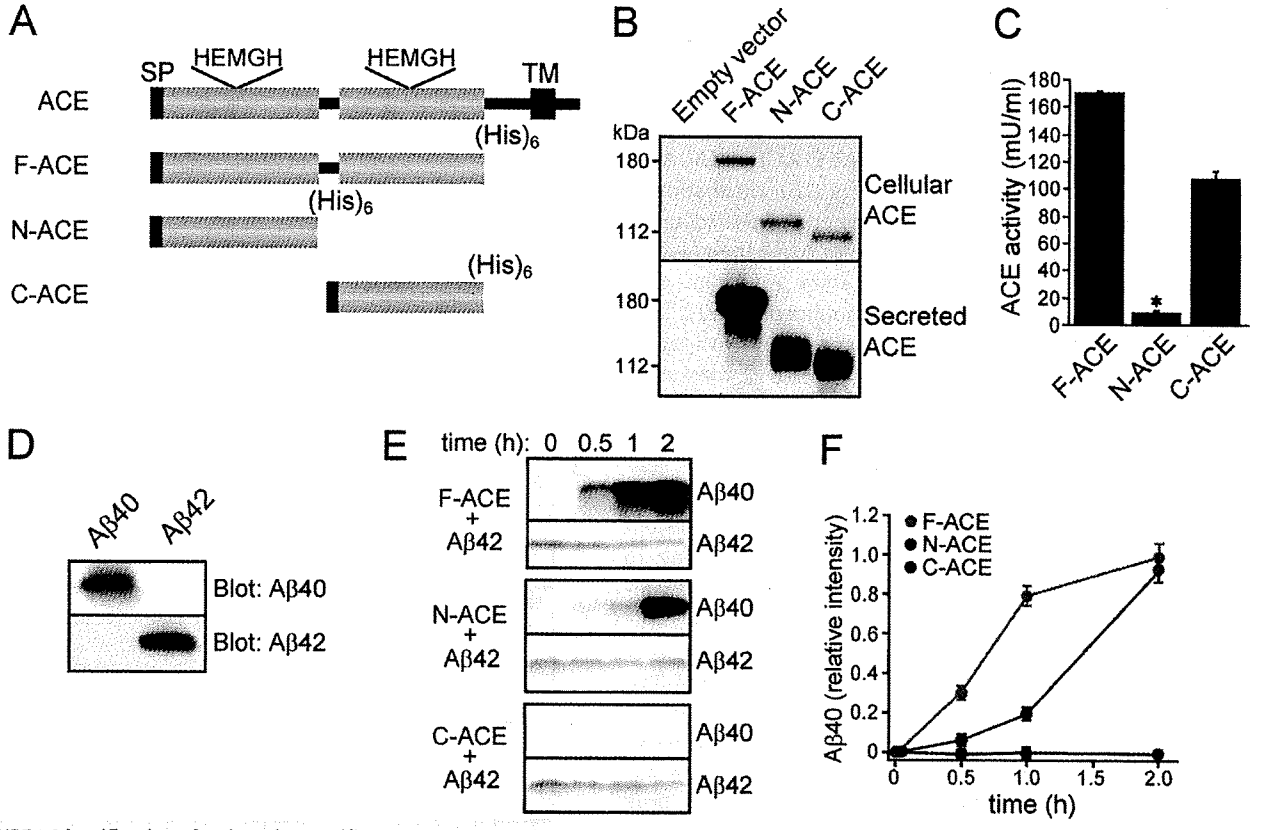

FIGURE 1. **Identification of N-domain-specific Aβ42-to-Aβ40-converting activity of ACE.** *A*, schematic representation of the human ACE and recombinant ACE proteins. The wild-type ACE protein contains a signal peptide (*SP*), a single transmembrane domain (*TM*), and two homologous catalytic domains (*light blue box*). Recombinant ACE proteins, F-ACE, N-ACE, and C-ACE, contain 6 histidine residues (*yellow box*) at the C terminus and a signal peptide at the N terminus. *B*, COS7 cells transfected with empty vector or cells stably expressing F-ACE, N-ACE, or C-ACE were lysed in radioimmune precipitation assay buffer. Western blots of 20 μg of total protein from the cells or 2 μg of ACE isolated from the culture medium were probed with a polyclonal anti-ACE antibody. *C*, ACE activity was measured by incubating 0.5 μM F-ACE, N-ACE, or C-ACE with the substrate Hip-His-Leu for 15 min at 37 °C. N-ACE has markedly reduced ACE activity compared with C-ACE. Values represent the means + S.E.; $n = 3$; *, $p < 0.001$, Bonferroni/Dunn test. *D*, specificities of monoclonal anti-Aβ40 (1A10) and polyclonal anti-Aβ42 antibodies were confirmed by Western blot of 0.1 μg of Aβ40 and Aβ42. *E*, F-, N-, and C-ACE were mixed with synthetic Aβ42 and incubated at 37 °C for 0.5, 1, or 2 h. Western blots of the mixture were probed with anti-Aβ40 and anti-Aβ42 antibodies. In contrast to the ACE activity, the Aβ42-to-Aβ40-converting activity was solely detected in N-ACE. *F*, generation of Aβ40 and the degradation of Aβ42 were determined by densitometry.

were deglycosylated at 37 °C for 1 h. The non-deglycosylated ACE proteins were mixed with the same incubation buffer provided by the manufacturer and incubated except that glycosidases were not added.

RESULTS

ACE N-domain, but Not C-domain, Converts Aβ42 to Aβ40—To explore which domain of ACE has Aβ42-to-Aβ40-converting activity, we prepared 3 kinds of recombinant ACE proteins, which were transfected into COS7 cells. F-ACE contains both the N-domain and C-domain active sites. N-ACE contains only the N-terminal active site, and C-ACE only contains the C-terminal active site. All three kinds of mutated ACE were fused with a 6-histidine tag at the C-terminal for the isolation from the culture medium (Fig. 1*A*). Cell lines stably expressing F-ACE, N-ACE, or C-ACE were selected by Geneticin and the expression of the ACE-mutated proteins were confirmed by Western blot. The endogenous ACE was not detected in the cell lysate of COS7 cells transfected with empty vectors. F-, N-, and C-ACE showed molecular masses at 180, 130, and 110 kDa, respectively (Fig. 1*B*). The secreted ACE recombinant proteins were isolated from the culture medium by immobilized metal chromatography. The proteins were then dialyzed and concentrated. The apparent molecular mass of each secreted ACE recombinant protein did not differ from each of the cellular ACE recombinant proteins (Fig. 1*B*). ACE enzymatic activity of the F-ACE, N-ACE, and C-ACE was confirmed by degradation of the substrate Hip-His-Leu (Fig. 1*C*). F-ACE was found to have the highest Hip-His-Leu-degrading activity and C-ACE had 63% ACE activity compared with F-ACE, whereas N-ACE had a significantly reduced ACE activity, confirming the finding of the ACE C-domain as the main site of angiotensin I cleavage *in vivo* (18) (Fig. 1*C*).

We have found that ACE releases two amino acids from the C terminus of Aβ42 and generates Aβ40. Aβ1–41 was not found during the degradation of Aβ42 by ACE, suggesting that the Aβ42-to-Aβ40-converting activity of ACE is a dipeptidyl carboxypeptidase enzymatic activity (15). To determine which domain is responsible for the Aβ42-to-Aβ40-converting activity, we incubated Aβ42 with F-ACE, N-ACE, or C-ACE and examined the generation of Aβ40 from Aβ42 by Western blot using anti-Aβ40- and anti-Aβ42-specific antibodies. The specificity of the two antibodies was examined by Western blotting of synthetic Aβ40 and Aβ42, and cross reaction between these

two antibodies was not found (Fig. 1*D*). Unexpectedly, in contrast to the angiotensin-converting activity, the Aβ42-to-Aβ40-converting activity was found in F-ACE and N-ACE, but not in C-ACE, indicating that N-domain of ACE has the Aβ42-to-Aβ40-converting activity. Although C-ACE showed a similar Aβ42-degrading activity compared with F-ACE and N-ACE, it did not generate Aβ40 from Aβ42 (Fig. 1*E*). Incubation of C-ACE with Aβ42 up until 16 h did not generate Aβ40 (data not shown). F-ACE generated more Aβ40 from Aβ42 than N-ACE at the time points of 0.5 and 1 h, whereas at the time point of 2 h F-ACE and N-ACE generated similar amounts of Aβ40 (Fig. 1, *E* and *F*). F-ACE had similar activities compared with native human kidney ACE regarding the Aβ42-to-Aβ40-converting activity and the Hip-His-Leu-degrading activity (Figs. 1*E* and 4*C* and data not shown).

To determine other products other than Aβ40 that were generated by ACE from Aβ42 and to confirm the result from Western blot, we performed mass spectrometry analysis. Consistent with our immunological studies, a peak corresponding to Aβ1–40 was detected in the F-ACE- and N-ACE-digested samples; in addition, F-ACE and N-ACE also generated peaks corresponding to Aβ1–33, Aβ1–28, Aβ1–24, and Aβ1–21 from Aβ42, whereas Aβ1–40 was not formed by C-ACE. However, C-ACE generated four other Aβ fragments, Aβ1–33, Aβ1–28, Aβ1–24, and Aβ1–21 (Fig. 2*A*). Mass spectrometry analysis for incubated F-ACE, N-ACE, or C-ACE alone did not show any Aβ peptide signal, and synthetic Aβ42 only showed one peak with a mass at 4514, which matched the predicted mass of Aβ1–42 (Fig. 2*B* and data not shown). These results from mass spectrometry confirmed that the Aβ42-to-Aβ40-converting activity is restricted to the ACE N-domain.

N-domain-inactive ACE Mutant Loses Aβ42-to-Aβ40-converting Activity—Three ACE mutants were generated by site-directed mutagenesis to change the active site sequence HEMGH to HDMGH in N-, C-, or both N- and C-domain. The N-domain active site was inactivated by mutating glutamate 362 to aspartate (termed E362D), and the C-domain active site was similarly inactivated by mutating glutamate 960 to aspartate (termed E960D). E362/960D has double mutations in its N- and C-domain active sites (Fig. 3*A*). The fibroblasts were transiently transfected with empty vector or ACE mutant constructs. ACE was not detected in the fibroblasts transfected with empty vector, and wtACE and ACE mutants were expressed in the cells at a similar level (Fig. 3*B*). To determine the effects of each ACE active site on ACE activity, cell lysate from each cell lines was analyzed for ACE activity. Consistent with our results from purified truncated ACE proteins, E362D containing only the C-domain active site has the similar ACE activity compared with wtACE, whereas E960D has an extremely low ACE activity. ACE inhibitor, captopril, completely inhibited this ACE activity (Fig. 3*C*). To determine which active site in each domain is responsible for the Aβ42-to-Aβ40-converting activity, wtACE and ACE mutant proteins were immunoprecipitated and incubated with Aβ42. A similar amount of immunoprecipitated ACE was confirmed by Western blotting (Fig. 3*D*, *upper panel*). E960D without the C-domain activity generated similar amount of Aβ40 from Aβ42 compared with

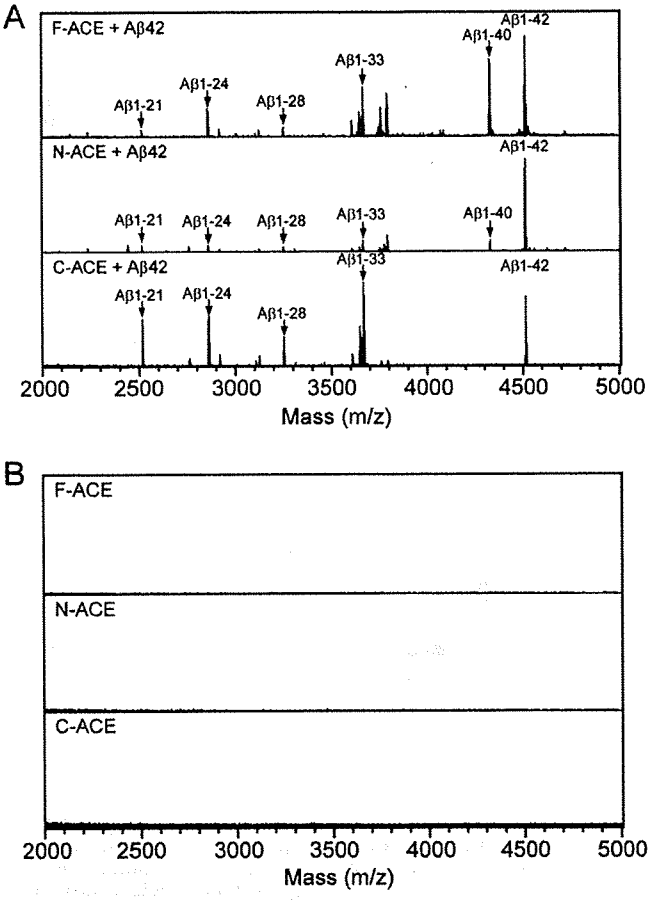

FIGURE 2. **MALDI-TOF-MS analysis for Aβ42 degradation by F-ACE, N-ACE, or C-ACE.** *A*, Aβ42 (80 μM) was incubated with 0.5 μM purified F-ACE, N-ACE, or C-ACE at 37 °C for 2 h, then captopril (10 μM) was added after incubation to stop the digestion. 1 μl of the mixture was subjected to MALDI-TOF-MS analysis. F-ACE and N-ACE generated Aβ1–40, whereas C-ACE did not. *B*, 1 μl of F-ACE, N-ACE, or C-ACE alone incubated at 37 °C for 2 h was subjected to MALDI-TOF-MS analysis, and a peptide signal was not detected.

wtACE, whereas E362D and E362/960D without N-domain activity did not convert Aβ42 to Aβ40 (Fig. 3*D*, *middle panel*).

N-Glycosylation Is Essential for Aβ42-to-Aβ40- and Angiotensin-converting Activities—ACE is a glycoprotein, and the *N*-linked glycosylation of testicular ACE has been shown to be essential for its angiotensin-converting activity. Human ACE has 17 putative Asn*X*(Ser/Thr) *N*-linked glycosylation sites distributed throughout both the N-domain and C-domain (21). To determine the role of glycosylation of ACE in its enzymatic activities and to compare the glycosylation of natural human ACE with that of recombinant F-ACE, N-ACE, and C-ACE, we examined the type of glycosylation of these ACE proteins by the treatment with PNGase F, *O*-glycanase, and sialidase A. Treatment with PNGase F, *O*-glycanase, and sialidase A remarkably reduced the molecular weight of human kidney ACE, F-ACE, N-ACE, and C-ACE (Fig. 4*A*, *lanes 1* and *2*). Removal of *N*-linked glycosylation using PNGase F alone produced similar molecular weight shifts, whereas *O*-glycanase did not produce any shift in ACE size (Fig. 4*A*, *lanes 3* and *4*). The sensitivity of N-ACE to PNGase F indicates that N-ACE is modified by *N*-linked glycosylation. All the ACE proteins showed a slight decrease in the molecular weight after sialidase A digestion,

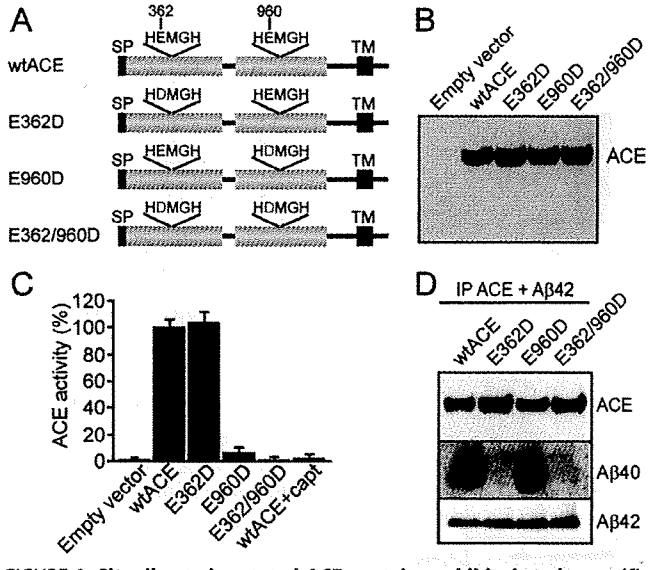

FIGURE 3. **Site-directed mutated ACE proteins exhibit domain-specific Aβ42-to-Aβ40- and angiotensin-converting activity.** *A*, schematic representation of human ACE and the mutant positions. The two ACE zinc metalloprotease active site glutamates (amino acids 362 in the N-domain and 960 in the C-domain) were changed to aspartates. *B*, fibroblasts were transiently transfected with empty vector, wtACE or mutant ACE plasmids and the expression of ACE proteins was detected by Western blotting using a polyclonal anti-ACE antibody. *C*, ACE activity was measured by incubating 5 μg of protein of cell lysate with the substrate Hip-His-Leu for 10 min at 37 °C. ACE activity in cell lysate was clearly detected in wtACE and E362D. C-domain inactive ACE protein, E960D, showed an extremely low ACE activity; and double mutants in both domains of ACE, E362/960D, did not show ACE activity. ACE activity was clearly inhibited by captopril (1 μM) treatment. *D*, ACE in cell lysate (4 mg of protein) from each transfected cell line was immunoprecipitated by 5 μg of polyclonal anti-ACE antibody and 100 μl of protein G-Sepharose. Immunoprecipitated ACE was then incubated with synthetic Aβ42 and the generation of Aβ40 was detected by Western blotting. *SP*, signal peptide; *TM*, transmembrane.

indicating the sialylation of their *N*-glycans (Fig. 4*A*, *lane 5*). These results suggest that the glycosylation type of natural human kidney ACE and recombinant ACE proteins produced by COS7 cells are identical. Because ACE is modified by *N*-linked glycosylation and *O*-linked glycosylation was not detected, we used PNGase to remove its *N*-glycans and studied the ACE activity. As expected, PNGase-treated human kidney ACE showed a 96% reduced ACE activity in degradation of Hip-His-Leu compared with untreated ACE (Fig. 4*B*).

To determine the role of *N*-linked glycosylation of ACE in its Aβ42-to-Aβ40-converting activity, we incubated Aβ42 with PNGase F-treated or untreated human kidney ACE and examined Aβ40 generation by Western blot. Deglycosylated human kidney ACE showed a decreased molecular mass at ~150 kDa and was degraded by itself after 2 h of incubation. After incubation for 16 h, ~150-kDa deglycosylated ACE was completely degraded (Fig. 4*C*, *upper panel*). Aβ40 was generated from Aβ42 by ACE after incubating the mixture of Aβ42 and ACE for 15 min. The level of Aβ40 increased in a time-dependent manner and reached a peak after incubation for 2 h, whereas deglycosylated ACE did not generate Aβ40 from Aβ42, although it showed a similar Aβ42-degrading activity compared with non-deglycosylated ACE (Fig. 4*C*, *middle and bottom panels*). This glycosylation-required Aβ42-to-Aβ40-converting activity was also confirmed in recombinant ACE proteins. PNGase F-deglycosylated F-ACE, N-ACE, and C-ACE have similar Aβ42-degrading activity. However, deglycosylated F-ACE and N-ACE failed to generate Aβ40 from Aβ42, suggesting that the *N*-linked glycosylation in the ACE N-domain is essential for its Aβ42-to-Aβ40-converting activity (Fig. 4*D*). Sialidase A treatment did not change the Aβ42-to-Aβ40-converting activity and the ACE activity of human kidney ACE, indicating that sialylation is not required for its activities (data not shown).

Captopril and Enalaprilat Showed Different IC_{50} on Aβ42-to-Aβ40-converting Activity—The feature of ACE inhibitors has been well studied in terms of their angiotensin-converting inhibitory effect. To explore whether ACE inhibitors differentially inhibit the Aβ42-to-Aβ40-converting activity, we determined the IC_{50} of captopril, perindopril, lisinopril, and enalaprilat toward the angiotensin- and Aβ42-to-Aβ40-converting activity of F-ACE. All four ACE inhibitors showed a similar IC_{50} on the inhibition of angiotensin-converting activity of F-ACE, whereas enalaprilat exhibited a 10-fold lower IC_{50} (0.003~0.01 μM) on Aβ42-to-Aβ40-converting activity than captopril (0.03~0.1 μM) (Table 1).

DISCUSSION

Most mammalian tissues contain ACE with two catalytic domains. Evolutionary conservation of the ACE N- and C-domains suggests important distinct functions of these domains. Recent genetic studies have associated the I allele of the *ACE* gene, which results in a reduced serum ACE level, with onset of AD (1, 3). We have shown previously that ACE converts Aβ42 to Aβ40, and its inhibition predominantly enhances brain Aβ42 deposition (15). To investigate which domain of ACE is responsible for Aβ42-to-Aβ40-converting activity and whether ACE inhibitors inhibit this activity, we generated three kinds of ACE proteins, containing both N- and C-domains or containing either single active domain. We also used selective site-directed mutagenesis of ACE to study the domain-specific activity of full-length ACE. The present study shows that the Aβ42-to-Aβ40- and angiotensin-converting activities were located in different ACE domains and that *N*-linked glycosylation was essential for the two ACE enzymatic activities. The N-domain of ACE clearly showed an Aβ42-to-Aβ40-converting activity, whereas it has an extremely low angiotensin-converting activity. In contrast, the C-domain of ACE showed angiotensin-converting activity, whereas the Aβ42-to-Aβ40-converting activity was not detected in this domain.

In a cellular context, both the N-domain and C-domains of ACE are able to degrade Aβ40 and Aβ42 (9). In our studies, we also found that the N- and C-domains were indistinguishable as regarding degrading Aβ42, suggesting that both N- and C-domains of ACE have endopeptidase activity for Aβ42. In the overall scheme of Aβ42 processing, the full-length ACE cleaving into many fragments may be important for therapeutic treatment of AD. We showed that Aβ40, but not Aβ41, was generated from Aβ42 (Fig. 2*A*). However, the Aβ42-to-Aβ40-converting activity was solely found in the N-domain of ACE (Figs. 1, 2, and 3). These results suggest that the dipeptidyl carboxypeptidase activity converting Aβ42 to Aβ40 is restricted to its N-domain. The N-domain specific dipeptidyl activity was

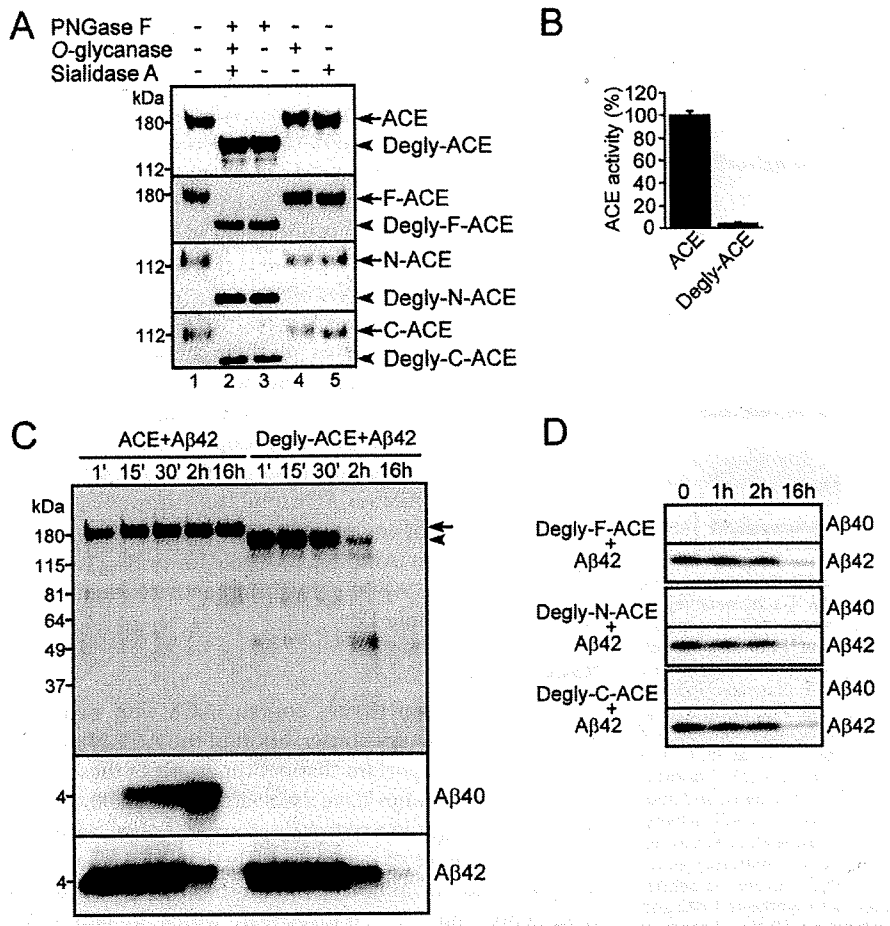

FIGURE 4. **Characterization of ACE glycosylation and role of the glycosylation in ACE activity and Aβ42-to-Aβ40-converting activity.** *A*, 5 μg of purified human kidney ACE, F-ACE, N-ACE, and C-ACE were deglycosylated with 1 μl of PNGase F, *O*-glycanase, and/or sialidase A for 1 h at 37 °C. PNGase F alone was able to remove all glycosylation of ACE. *B*, ACE activity of PNGase F-deglycosylated human kidney ACE was measured immediately after deglycosylation using an ACE colorimetric kit. ACE activity was almost completely abolished by *N*-deglycosylation. *C*, 80 μl of human kidney ACE (0.5 μM) with or without *N*-deglycosylation was mixed with synthetic Aβ42 (40 μM) and incubated at 37 °C. 10 μl of the mixture were collected at various incubation time points and subjected to Western blot analysis. Deglycosylated ACE showed no Aβ42-to-Aβ40-converting activity, whereas the Aβ42-degrading activity remained. *D*, 40 μl of recombinant F-, N-, and C-ACE proteins (0.5 μM) were deglycosylated and mixed with Aβ42 and incubated at 37 °C for 1, 2, or 16 h. Aβ42-to-Aβ40-converting activity was not detected in either deglycosylated F-ACE or deglycosylated N-ACE, whereas all the deglycosylated ACE showed an Aβ42-degrading activity.

TABLE 1

ACE inhibitors inhibited Aβ42-to-Aβ40-converting activity with different IC_{50}

ACE activity of 10 μl of F-ACE (0.5 μM) was measured using an ACE colorimetric kit, and Aβ42-to-Aβ40-converting activity was measured by Western blotting and densitometry. 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM ACE inhibitors were added to determine the IC_{50} for Aβ42-to-Aβ40-converting activity.

ACE inhibitors	ACE activity IC_{50}	Aβ-converting activity IC_{50}[a]
	μM	μM
Captopril	0.01–0.03	0.03–0.1
Enalaprilat	0.01–0.03	0.003–0.01
Lisinopril	0.01–0.03	0.01–0.03
Perindopril	0.03–0.1	0.01–0.03

[a] Aβ-converting activity, Aβ42-to-Aβ40-converting activity.

also found in the degradation of AcSDKP, which is involved in the control of hematopoietic stem cell proliferation. The molecular basis in which the N-domain of ACE accesses AcSDKP and Aβ42 remains to be elucidated. The N- and C-domains of ACE have reduced Aβ42-to-Aβ40-converting activity and angiotensin-converting activity, respectively, compared with full domain ACE (Fig. 1, *C* and *F*), suggesting that each catalytic domain of ACE regulates the activity of the other, and both domains are required for normal substrate recognition and degradation. Mice with a selective inactivation of either the N- or C-domain of ACE were generated, and the C-domain was demonstrated to be the main site of angiotensin I cleavage (18, 22), which is consistent with our *in vitro* finding. However, the role of the N-domain of ACE toward Aβ42 to Aβ40 conversion *in vivo* needs to be addressed.

It has been previously reported that testicular ACE, the C-domain isoform of ACE, without *N*-linked glycosylation has no enzyme activity and was rapidly degraded (19). We confirmed that deglycosylation of human kidney ACE abolished its angiotensin-converting activity, whereas the endopeptidase activity for degrading itself and Aβ42 was not affected. Moreover, the N-domain-specific Aβ42-to-Aβ40-converting activity was abolished by the deglycosylation, indicating that the *N*-linked glycosylation is also essential for maintaining the N-domain-specific enzymatic activity of ACE. Deglycosylated ACE was retained as an intact protein 30 min after deglycosylation. ACE activity and Aβ42-to-Aβ40-converting activity were clearly detected in non-deglycosylated ACE within 30 min (Fig. 4, *B* and *C*). Thus, the loss of ACE activity and Aβ42-to-Aβ40-converting activity of deglycosylated ACE may not result from its self-degradation, but likely result from the deglycosylation. These results suggest that *N*-linked glycosylation is required to maintain the ACE structure and its dipeptidyl carboxypeptidase activity in both N- and C-domains. Presenilins have been shown to be involved in the maturation of membrane proteins, whether presenilin mutants in familial AD affect ACE glycosylation and its Aβ42-to-Aβ40-converting activity need to be clarified in future (23). Finally, we showed that ACE inhibitors inhibited the N-domain-specific Aβ42-to-Aβ40-converting activity each with a different IC_{50}. Among the examined ACE inhibitors, enalaprilat has the strongest inhibitory effect on Aβ42-to-Aβ40-converting activity. This result may provide a mechanism underlying the finding that non-centrally active ACE inhibitors, such as enalapril, are associated with a greater risk of incident dementia (24). In

our previous *in vivo* study, captopril treatment enhanced predominantly brain Aβ42 deposition in 17-month-old amyloid precursor protein (APP) transgenic mice and led to a tendency of increased brain Aβ42/40 ratio (15). Taking the anti-amyloid and antioxidant effects of Aβ40 into account, our findings suggest that ACE inhibitors could be designed specifically to target the C-domain of ACE without inhibiting its N-domain-specific Aβ42-to-Aβ40-converting activity.

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