Regulation of Steady-state β-Amyloid Levels in the Brain by Neprilysin and Endothelin-converting Enzyme but Not Angiotensin-converting Enzyme*

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The deposition of β-amyloid in the brain is a pathological hallmark of Alzheimer disease (AD). Normally, the accumulation of β-amyloid is prevented in part by the activities of several degradative enzymes, including the endothelin-converting enzymes, neprilysin, insulin-degrading enzyme, and plasmin. Recent reports indicate that another metalloproteinase, angiotensin-converting enzyme (ACE), can degrade β-amyloid in vitro and in cellular overexpression experiments. In addition, ACE gene variants are linked to AD risk in several populations. Angiotensin-converting enzyme, neprilysin and endothelin-converting enzyme function as vasopeptidases and are the targets of drugs designed to treat cardiovascular disorders, and ACE inhibitors are commonly prescribed. We investigated the potential physiological role of ACE in regulating endogenous brain β-amylloid levels for two reasons: first, to determine whether β-amylloid degradation might be the mechanism by which ACE is associated with AD, and second, to determine whether ACE inhibitor drugs might block β-amylloid degradation in the brain and potentially increase the risk for AD. We analyzed β-amylloid accumulation in brains from ACE-deficient mice and in mice treated with ACE inhibitors and found that ACE deficiency did not alter steady-state β-amylloid concentration. In contrast, β-amylloid levels are significantly elevated in endothelin-converting enzyme and neprilysin knock-out mice, and inhibitors of these enzymes cause a rapid increase in β-amylloid concentration in the brain. The results of these studies do not support a physiological role for ACE in the degradation of β-amylloid in the brain but confirm roles for endothelin-converting enzyme and neprilysin and indicate that reductions in these enzymes result in additive increases in brain amyloid β-peptide levels.

The accumulation of amyloid β-peptide (Aβ)² in the brain occurs in all forms of Alzheimer disease (AD), but increased production of the peptide due to trisomy 21 or mutations in the amyloid β protein precursor (APP) or the presenilins accounts for only a very small fraction of AD cases (1). In the vast majority of late-onset AD cases there is no evidence of increased production. The underlying cause of Aβ accumulation in these patients remains unknown, and this has sparked intense investigation by geneticists, biochemists, and epidemiologists to identify risk factors contributing to the development of AD in the elderly population. Many genetic loci have been reported to be linked to late-onset AD in certain populations, but the only widely accepted genetic risk factor for late-onset AD across many populations is APOE genotype (2). ApoE complexes with Aβ and appears to facilitate its clearance through the low density lipoprotein receptor. The strong association of APOE genotype with AD demonstrates the significance of Aβ clearance in the development of the disease and underscores the importance of elucidating the multiple enzymes and pathways responsible for removing the peptide from the brain.

Recently, considerable data have emerged indicating that neprilysin (NEP), endothelin-converting enzymes 1 and 2 (ECE-1 and ECE-2), insulin-degrading enzyme, and plasmin each play a role in regulating the accumulation of Aβ in the brain (3). Experimental deletion of each of these enzymes in mice perturbs Aβ degradation, and steady-state Aβ levels are elevated in the brains of mice deficient in NEP, the ECEs, and insulin-degrading enzyme (4–10). Angiotensin-converting enzyme (ACE) is also capable of degrading Aβ in vitro assays and in cellular overexpression experiments, but its physiological role in the brain has not yet been explored (11–13).

ACE, NEP, and ECE can be categorized as vasopeptidases based on their ability to generate or inactivate vasoactive peptides (14). ACE generates angiotensin II and inactivates bradykinin, NEP degrades the natriuretic peptides, bradykinin, and endothelin, and ECE generates endothelin and potentially degrades bradykinin. Because of these functions, the vasopeptidases are attractive targets for drugs designed to treat hypertension, heart failure, and other cardiovascular disorders. The

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² The abbreviations used are: Aβ, amyloid β-peptide; AD, Alzheimer disease; APP, β-amylloid precursor protein; NEP, neprilysin; ECE, endothelin-converting enzyme; ACE, angiotensin-converting enzyme; icv, intracerebroventricular(ly); DEA, diethylamine; ELISA, enzyme-linked immunosorbent assay.
first vasopeptidase inhibitors put into clinical use were pure ACE inhibitors, and many of these are now FDA-approved for the treatment of hypertension and heart failure (15). Dual ACE-NEP inhibitors were later developed to potentiate the effect of ACE inhibition on bradykinin, as well as to promote the vasodilatory effects of the natriuretic peptides. In pre-clinical and clinical studies, the antihypertensive, cardioprotective, and renal effects of dual inhibitors have been shown to be superior to pure ACE inhibitors (14, 16). Because NEP degrades and inactivates endothelins, the vasorelaxant effects of dual ACE-NEP inhibitors may be reduced by the accumulation of these vasoconstrictive peptides, suggesting that triple inhibition of NEP, ACE, and ECE may produce even more potent antihypertensive effects. A recent study of chronic heart failure in rats indicated that triple ACE-ECE-NEP inhibition was superior to ACE inhibition or ECE-NEP inhibition with respect to left ventricular hemodynamics and remodeling (17).

The common prescription of ACE inhibitors, and the development of NEP and ECE inhibitors, is concerning particularly if ACE activity regulates the steady-state concentration of Aβ in the brain. Although in vivo evidence of Aβ degradation by ACE has not been reported, a common insertion/deletion polymorphism in the ACE gene has been found to be associated with AD in several populations (18). The mechanism behind this association is not yet clear. In the present study we examined the ability of ACE to regulate Aβ concentration in vivo by analyzing Aβ levels in mice genetically engineered to lack ACE expression in the brain and in mice treated with clinically relevant ACE inhibitors. We also further investigated the roles of NEP and ECE by generating dual knock-out mice and by treating mice with ECE and ECE-NEP inhibitors that are being developed for the treatment of cardiovascular disease.

EXPERIMENTAL PROCEDURES

Animals—Outbred Hsd:ICR(CD-1) mice were obtained from Harlan (Indianapolis, IN). Tg2576 mice, which express the familial AD-linked Swedish mutation (βAPPK670N/M671L) under control of the prion promoter (19), were obtained from Charles River Laboratories. ACE-8 mice, in which the endogenous ACE gene is under control of the α-mysin heavy chain promoter (20), were maintained at Emory University. ECE-1 and ECE-2 knock-out mice (21, 22) were a gift from Dr. Masashi Yanagisawa and were maintained at the Mayo Clinic, Jacksonville, FL. NEP knock-out mice (23) were a gift from Dr. Takami Saito and were maintained at Mayo Clinic Jacksonville. NEP−/− and ECE-1−/− or ECE-2−/− mice were crossed to generate double-heterozygous mice, which were then intercrossed to generate offspring with the genotypes shown in Fig. 6 (ECE-1 homozygous knock-out mice are not viable). Littermates from the heterozygous crossings were sacrificed by CO2 asphyxiation at 4 weeks of age, and brains were removed, quickly frozen on dry ice, and stored at −80 °C prior to analysis of Aβ concentration. Littermates from heterozygous crosses of ACE-8 mice were similarly analyzed at 3 weeks of age. All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Metalloprotease Inhibitors—Phosphoramidon (>95% by high-performance liquid chromatography) was obtained from Roche Applied Science and was dissolved in 0.9% NaCl. Enalapril maleate (∼98% by TLC) and captopril (USP) were obtained from Sigma-Aldrich and dissolved in 0.9% NaCl. Perindopril erbumine solution was prepared by grinding Aceon® tablets (Solvay Pharmaceuticals, Marietta, GA) into a fine powder, dissolving in 0.9% NaCl, and removing the insoluble filler material by centrifugation. Enalaprilat was obtained as an intravenous solution in saline containing benzyl alcohol 9 mg/ml (Baxter Healthcare Corp., Deerfield, IL). CGS 26303, CGS 26393, CGS 35066, and CGS 35339 were synthesized by our group using a modification we developed (24) from the original published methods (25, 26). The CGS compounds were fully characterized by H NMR, IR, and electrospray ionization-mass spectrometry data. The purity was judged >97% based on spectral analysis.

Intracerebroventricular Injection of Metalloprotease Inhibitors—Mice were anesthetized with isoflurane (2–5%) and avertin and placed in a stereotaxic frame. The metalloprotease inhibitors or their appropriate vehicles were injected in a total volume of 2 μl, with 1 μl injected into each lateral ventricle at a rate of 0.5 μl/min. The mice were sacrificed 2 h after the second injection, and brains were removed, hemispheres divided, and quickly frozen on dry ice. Injection into the ventricle was verified post mortem by visualizing the needle track.
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FIGURE 2. Oral administration of ACE inhibitors. Non-transgenic CD-1 mice were dosed with ACE inhibitors enalapril (n = 5), perindopril (n = 5), captopril (n = 5), and saline vehicle control (n = 9) by oral gavage. Aβ40 and Aβ42 levels were measured by sandwich ELISA in plasma (A and B) and brain (C and D). Data shown represent the mean ± S.E., with all values normalized to the mean of the vehicle-treated group. *, brain Aβ40 concentration (p = 0.019) and Aβ42 concentration (p = 0.04) were significantly lower in perindopril-treated mice compared with controls.

was performed on one hemisphere, and ACE activity measurement was performed on the other hemisphere, where indicated. Doses used were enalapril (6 nmol), CGS 35066 (3 nmol), CGS 26303 (40 nmol), and phosphoramidon (118 nmol). Because the maximum volume for the icv injections was 2 μl, these doses were determined in part by the availability of concentrated stock solutions. The dose of phosphoramidon employed was similar to that used in reports of the physiologic effects of neprilysin inhibition in the brain (27, 28).

Oral Administration of Metalloprotease Inhibitors—The metalloprotease inhibitors were administered to CD-1 mice by oral gavage in a volume of 0.2 ml. The doses of the ACE inhibitors used were captopril (2 mg/kg), enalapril (0.6 mg/kg), and perindopril (0.2 mg/kg) in saline. These doses are similar to the maximum human dose for each drug. CGS 26393 (prodrug of CGS 26303) was dissolved in Me2SO and diluted in 3% cornstarch (final concentration of Me2SO was 8%). The compound was administered at 30 mg/kg, a dose reported to significantly lower mean arterial pressure in the DOCA-salt rat (25). CGS 35339 (prodrug of CGS 35066) was administered as a suspension in 0.5% methylcellulose. Although CGS 35066 is selective for ECE-1 over NEP (IC50 NEP/IC50 ECE = 104), the drug will inhibit both enzymes if given at a high enough dose (26). Therefore, we dosed mice with 0.45, 4.5, and 45 mg/kg to evaluate the effect of selective ECE inhibition versus dual ECE/NEP inhibition. Studies of intravenous CGS 35066 administration in rats suggest that NEP is not inhibited at the lowest dose, partially inhibited at the 4.5 mg/kg dose equivalent, and significantly inhibited at the 45 mg/kg dose equivalent (29). Except for mice treated with captopril, all mice were sacrificed 4 h after dosing. Because captopril does not require prodrug conversion and has a shorter duration of action, mice dosed with this compound were sacrificed after 3 h. Following sacrifice, blood was withdrawn by cardiac puncture and collected in EDTA-coated tubes for plasma preparation (for Aβ analysis), or standard tubes for serum preparation (for analysis of ACE activity). The mice were perfused with saline to eliminate residual plasma Aβ in the brain. Brains were then removed, quickly frozen on dry ice, and stored at −80 °C prior to Aβ analysis. Aβ concentration did not differ in saline control mice sacrificed after 3 or 4 h, and the data were combined in Fig. 2.

Analysis of Aβ Concentration—Brains from mice expressing endogenous Aβ were extracted with 0.2% diethylamine (DEA) as previously described (5). DEA extracts were neutralized and stored at −80 °C prior to analysis of Aβ40 using either the BNT77/BA27 or 32.4.1/13.1.1 sandwich ELISA system, and Aβ42 using the BNT77/BC05 sandwich ELISA system (30). The BNT77 antibody (Takeda) was raised against amino acids 11–28 and thus may recognize N-terminally modified or truncated peptides (31). The 32.4.1 antibody (generated at Mayo Clinic) is directed against the N-terminal region of rodent Aβ and fails to capture the Aβ42-40 putative ACE cleavage product. Brains from Tg2576 mice were extracted with 70% formic acid, and Aβ was analyzed using the BAN50/BA27 and BAN50/BC05 sandwich ELISA systems (19). BAN50 is directed against the N terminus of human Aβ, requiring position 1. Plasma Aβ was analyzed without further extraction using the BNT77/BA27 and BNT77/BC05 sandwich ELISA systems. In all figures the data were normalized to the mean of the control group and analyzed using the unpaired t test.

Analysis of ACE Activity—ACE activity was measured in serum and in brain homogenates as previously described (32) using the ACE-REA kit from American Laboratory Products Co. (Windham, NH).

RESULTS

Combined Inhibition of Multiple Metalloproteases Causes a Rapid Increase in the Accumulation of Aβ in the Brain—We have previously shown that phosphoramidon, an inhibitor of metalloproteases including NEP and the ECEs, causes a rapid increase in the accumulation of Aβ in the medium of
ACE Inhibition Fails to Elevate Aβ Concentration—To determine the extent of ACE inhibition in oral perindopril-treated mice (see Fig. 2), ACE activity was measured in serum (n = 3, A) and brain (n = 6, B) 4 h after administration. Data shown represent the mean ± S.E.

FIGURE 3. ACE inhibition in perindopril-treated mice. To determine the extent of ACE inhibition in oral perindopril-treated mice (Fig. 2), ACE activity was measured in serum (n = 3, A) and brain (n = 6, B) 4 h after administration. Data shown represent the mean ± S.E.

ACE Inhibition Fails to Elevate Aβ Concentration in Brain or Plasma—Phosphoramidon is capable of inhibiting ACE only at high concentrations, but more potent and selective ACE inhibitors are available and commonly prescribed. To determine whether ACE inhibition was partly responsible for the rapid increase in the levels of both Aβ40 and Aβ42, with Aβ40 levels increasing greater than 5-fold in wild type mouse brains within 2 h (Fig. 1).

ACE inhibition in the brain without the added variables of very low blood pressure, impaired renal function, and anemia. Aβ40 and Aβ42 levels were measured in brain extracts from ACE 8/8 mice and age-matched heterozygous and wild-type controls using the well characterized BNT77/BA27 and BNT77/BC05 sandwich ELISA systems (30). These systems have been previously used by our group and others to demonstrate significant elevations in Aβ concentration in mice deficient in the ECEs, NEP, and insulin-degrading enzyme (4–7). Consistent with the results of our studies with ACE inhibitors, no difference in steady-state Aβ concentration was found in mice lacking ACE expression in the brain (Fig. 4).

The major cleavage site of human Aβ by ACE is reported to be between amino acids Asp and Ser (11, 12). If Aβ40 were a major species of the peptide in wild-type, but not ACE-deficient mice, it is possible that differences in Aβ concentration could be obscured by the use of the BNT77 capture antibody, which was raised against amino acids 11–28 (31). Although we feel that this is unlikely, because Aβ42 is a potential substrate of other Aβ-degrading enzymes, we re-analyzed Aβ40 concentration in the ACE 8/8 brains using a rodent Aβ40 sandwich ELISA system that fails to recognize Aβ42. The results obtained with this antibody system were consistent with the results of the BNT77/BA27 assay, indi-
cating no difference in steady-state Aβ concentration between wild-type and brain ACE-deficient mice (Fig. 4C).

ACE Inhibition Does Not Alter Aβ Levels in Young Tg2576 APP Transgenic Mice—Because mouse Aβ differs from the human sequence at amino acid positions 5, 10, and 13, and the major cleavage site of human Aβ by ACE is reported to be between amino acids Asp7 and Ser8, there is a possibility that mouse Aβ may not be an equally effective substrate for ACE. If this were true, it could account for the fact that Aβ levels are unchanged in ACE-deficient mice and in wild-type mice treated with ACE inhibitors. To determine whether ACE regulates steady-state Aβ concentration in the brains of mice expressing human Aβ, we injected young Tg2576 APP transgenic mice iv with enalaprilat and, for comparison, with phosphoramidon. As expected, there was a significant elevation in Aβ levels in Tg2576 mice treated with phosphoramidon. However, consistent with the results in wild-type mice, Aβ levels were unchanged in Tg2576 mice treated with the ACE inhibitor enalaprilat (Fig. 5).

Dual NEP/ECE Knock-out Mice Show Additive Effects on Aβ Accumulation—Unlike brain ACE-deficient mice, mice deficient in NEP, ECE-1, or ECE-2 have increased accumulation of both Aβ40 and Aβ42 in the brain (4, 5). The increases in these mice are relatively modest when compared with the effect of inhibiting multiple metalloproteases with phosphoramidon. Given that NEP and the ECEs are members of the same protease family with overlapping substrates, it is possible that in the knock-out models these enzymes are able to partially compensate for the loss of one Aβ degrading pathway. However, NEP, ECE-1, and ECE-2 differ in regional and subcellular localization in the brain and their activities may be complementary rather than redundant with respect to Aβ degradation. To investigate the effect of removing two pathways of Aβ degradation in the brain, we generated crosses of NEP and either ECE-1- or ECE-2-deficient mice. The elevations in Aβ concentration in the brains of the dual NEP/ECE-1 and NEP/ECE-2 knockouts were approximately additive of the effects of the single knockouts, suggesting that NEP and the ECEs do not compensate for one another with respect to Aβ degradation (Fig. 6).

Vasopeptidase Inhibitors That Target NEP and ECE Cause Rapid Increases in Aβ Concentration—We have shown that treatment with commonly prescribed ACE inhibitors is unlikely to directly affect Aβ levels (Fig. 1–4). However, vasopeptidase inhibitors that target ACE-NEP, ECE, or even triple inhibitors of NEP-ECE-ACE, are currently in development for the treatment of hypertension and heart failure (14, 17, 34). We treated mice both orally and iv with a selective ECE inhibitor and a dual NEP-ECE inhibitor to evaluate the potential of these preclinical drugs to elevate plasma and brain Aβ levels. The iv injections were carried out to evaluate the potential of these compounds to elevate Aβ should they reach the brain following their normal route of administration. As predicted by our experiments with phosphoramidon, both CGS 35066, an ECE inhibitor (29), and CGS 26303, a dual NEP-ECE inhibitor (35), significantly elevated Aβ levels 2 h following a single iv injection (Fig. 7). After a single dose of the orally active prodrug of each
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Inhibitor, plasma Aβ levels were also significantly elevated (Fig. 8). A low dose of CGS 35339, which causes inhibition of ECE but not NEP, resulted in a significant elevation of Aβ40 and a slight elevation of Aβ42. Higher doses of this compound, which have been shown to inhibit both ECE and NEP, resulted in larger increases in the concentration of both Aβ40 and Aβ42 in plasma. The concentration of Aβ in the brain was not increased after any dose of CGS 35339, suggesting that the compound did not cross the blood-brain barrier in sufficient quantity to inhibit ECE or NEP in the brain. However, oral administration of the dual NEP-ECE inhibitor CGS 26393 did result in a small increase in Aβ concentration in the brain (Fig. 8). Further studies are required to determine whether long term administration of NEP and ECE inhibitors alters Aβ accumulation in the brain.

**DISCUSSION**

Efficient clearance of Aβ is essential to prevent its accumulation in the brain. Under normal conditions Aβ is removed by multiple clearance pathways, including direct proteolysis by degradative enzymes. Decreased activity of any of the Aβ-degrading enzymes might increase the risk for AD by elevating Aβ levels in the brain. Recent reports demonstrating the ability of ACE to degrade Aβ both in assays in vitro and in cell overexpression experiments raised the possibility that the observed linkage of the ACE gene with AD could be due to alterations in Aβ degradation. Furthermore, these reports raised the concern that commonly prescribed ACE inhibitors could increase Aβ accumulation in patients and potentially promote or accelerate the development of AD.

We have found no evidence that ACE directly regulates endogenous steady-state Aβ levels in vivo. Mice lacking ACE expression in the brain show no alterations in Aβ concentration, and treatment with ACE inhibitors does not cause a detectable increase in Aβ concentration in brain or in plasma even when ACE activity is substantially inhibited. Because the mouse Aβ sequence differs from the human sequence near the putative ACE cleavage site, it is possible that the mouse peptide may not be an equally effective substrate for the enzyme. To address this, we determined the effect of short term ACE inhibition in human APP transgenic mice and similarly found no effect on human Aβ concentration. However, because young, non-depositing, Tg2576 mice were used in these experiments, we cannot rule out an effect of chronic ACE inhibition on amyloid deposition in these mice.

Consistent with our results, epidemiological evidence suggests that the use of ACE inhibitors does not increase the risk for developing AD. In a large study of elderly patients in the United States taking various medications for hypertension or cardiovascular disease, AD prevalence in patients treated with the ACE inhibitor captopril was not higher than in the total study population (36). In a study of elderly hypertensive patients in Japan, the incidence of AD was also similar in
individuals treated with ACE inhibitors compared with those treated with other types of antihypertensive drugs (37).

Interestingly, a subgroup analysis of patients in the Japanese study taking blood-brain barrier-penetrating ACE inhibitors (captopril and perindopril) versus those taking brain-impermeable ACE inhibitors (imidapril and enalapril) showed that AD incidence was actually significantly reduced in the population taking blood-brain barrier-crossing drugs. In a subsequent study, the authors analyzed the rate of decline of cognitive function in elderly hypertensive patients with mild-to-moderate AD. Compared with patients taking calcium channel blockers or brain-impermeable ACE inhibitors, AD patients treated with blood-brain barrier-crossing ACE inhibitors had a significantly lower 1-year decline in Mini-Mental State Examination score (38). Blood pressure control was similar in the three treatment groups over the course of the study. Thus it appears that ACE inhibition in the brain may actually have a cognitively beneficial effect. The apparent cognitive benefits of ACE inhibition in the brain may be due to increased cholinergic activity, because ACE activity is increased in AD brain (39–41) and angiotensin II has been shown to inhibit acetylcholine release (42).

In contrast to ACE, there is a growing body of evidence that NEP and ECE activity are critical for limiting Aβ accumulation in the brain. These enzymes differ in their regional and subcellular localization (22, 43, 46–49), suggesting that their activities may be complementary with respect to Aβ degradation (44). For example, NEP has been shown to be important in the clearance of extracellular Aβ (4, 45), whereas the ECEs degrade Aβ predominately intracellularly (33). In the brain, NEP and the ECEs show a distinct pattern of expression (22, 43). For example, ECE-1 is highly expressed in the pyramidal cell layers of the hippocampus and in the granular layer of the dentate gyrus, areas where the level of NEP mRNA expression is considerably lower. ECE-1 expression is also abundant in the thalamus and areas of the hypothalamus, whereas NEP is highly expressed in the nucleus accumbens, caudate putamen, and olfactory bulb (43). Although elevations in steady-state Aβ levels are apparent in mice deficient in each of the Aβ degrading enzymes, it is not known whether decreased Aβ degradation by a single enzyme can be partially compensated for by the activities of the other enzymes. The combined NEP and ECE knock-out mice generated in this study demonstrate for the first time that these enzymes likely act independently with respect to Aβ clearance in the brain.

We have shown that oral administration of a selective ECE inhibitor and a dual ECE-NEP inhibitor causes a rapid increase in plasma Aβ concentration, with the latter also causing a small but significant increase in brain Aβ concentration. These compounds can more than double the total concentration of Aβ in mouse brain when administered icv, warning of a potentially serious side-effect should these and similar compounds reach the clinic for use in human patients. Whether elevations in plasma Aβ can alter the course of amyloid plaque deposition or cerebral amyloid angiopathy is unknown and warrants further study. The effect of chronic administration of NEP and ECE inhibitors on both plasma and cerebral Aβ levels should be carefully evaluated in animal models and in human clinical trial participants.

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