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Amyloid β-Protein Is Degraded by Cellular Angiotensin-converting Enzyme (ACE) and Elevated by an ACE Inhibitor*

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Human genetic data have associated angiotensin-converting enzyme (ACE) with Alzheimer disease (AD), and purified ACE has been reported to cleave synthetic amyloid β-protein (Aβ) *in vitro*. Whether deficiency in ACE activity, arising from genetic alteration or pharmacological inhibition, can decrease Aβ degradation and allow Aβ accumulation in intact cells is unknown. We cloned ACE from human neuroblastoma cells and showed that it had posttranslational processing and enzymatic activity typical of the endogenous protease. Cellular expression of ACE promoted degradation of naturally secreted Aβ40 and Aβ42, leading to significant clearance of both species. Using site-directed mutagenesis, we determined that both active sites within ACE contribute to Aβ clearance, and an ACE construct bearing mutations in each catalytic domain had no effect on Aβ levels. Pharmacological inhibition of ACE with a widely prescribed drug, captopril, promoted the accumulation of cell-derived Aβ in the media of β-amyloid precursor-protein expressing cells. Together, these results show that ACE can lower the levels of secreted Aβ in living cells and that this effect is blocked by inhibiting the protease's activity with an ACE inhibitor. This work, combined with the genetic studies, supports the hypothesis that ACE may modulate the susceptibility to and progression of AD via degradation of Aβ. Our data encourage further analyses of the *ACE* gene for disease association and raise the question of whether currently prescribed ACE inhibitors could elevate cerebral Aβ levels in humans.

An early and pathogenically important feature of Alzheimer disease (AD)[2] is the progressive accumulation and deposition of the amyloid β-protein (Aβ) in brain regions serving memory and cognition. Biochemical, cell biological, animal modeling, genetic, and emerging clinical data all suggest that Aβ is an upstream initiator of the disease process and its associated neuropathology (1–4). Although no proven disease-modifying treatments are currently available, recent efforts to treat AD have focused on both decreasing the production of Aβ and enhancing its clearance from the brain. One little studied approach to Aβ clearance is augmenting the degradation of the peptide by various proteases expressed in the brain. Thus far, the metalloproteases neprilysin (NEP) (5), insulin-degrading enzyme (IDE) (6), and the endothelin-converting enzymes 1 and 2 (7) have each been implicated as Aβ-degrading proteases in the mammalian brain. The serine protease plasmin has been implicated in Aβ degradation *in vitro* (8), although genetic plasmin deficiency did not promote accumulation of murine Aβ *in vivo* (9). Supporting a role for therapeutic regulation of Aβ-degrading proteases, the overexpression of IDE or NEP in a murine model of AD decreased cerebral Aβ levels and produced significant attenuation of Aβ-associated neuropathology (10).

Somatic angiotensin-converting enzyme (ACE) is a zinc metalloprotease containing two homologous regions, termed the N- and C-domains, each of which is proteolytically active (11). ACE has a single transmembrane domain and is cleaved from the cell surface at a low rate, resulting in the secretion of its ectodomain into the media of cultured cells and plasma (12, 13). ACE has been intensively characterized for its role in the regulation of blood pressure by cleaving angiotensin I to angiotensin II and inactivating bradykinin (14). This function of ACE is modulated pharmacologically by ACE inhibitors, which bind competitively to the active site zinc to prevent substrate hydrolysis. This commonly prescribed class of drugs is used for the treatment of hypertension and other disorders. In addition to regulating vasoactive peptides, ACE shows a broad substrate specificity, including several neuropeptides, and it is able to metabolize substrates using both exopeptidase and endopeptidase activities (15). Interestingly, despite their homology, the N- and C-domains display differential capacity to degrade some substrates and can be inhibited selectively by certain ACE inhibitors (16, 17). Recently, ACE was found to participate in the c-Jun N-terminal kinase signal transduction pathway, apparently independently of its proteolytic function (18).

A potential relationship between ACE and AD was first suggested by human genetic studies, which reported that an insertion (*I*)/deletion (*D*) polymorphism within intron 16 of the *ACE* gene associates with AD (19). Specifically, the *I* allele was associated with an increased risk for AD, whereas the *D* allele was associated with protection (20, 21). Of potential mechanistic relevance, inheritance of the *D* allele has been associated with increased plasma ACE levels (22). Regarding disease specificity, the *I* allele has been found to associate positively with AD but not with vascular dementia or vascular pathology (23, 24). The *I*/*I* genotype has also been linked to smaller volumes of the hippocampus and the amygdala (24). Importantly, post-mortem analyses of AD patients determined that those with the *I*/*I* genotype had a trend toward increased brain Aβ42 load compared with the *D*/*D* genotype (25). Single nucleotide polymorphisms in the *ACE* gene have also been shown to associate with AD, and there is a decrease in the prevalence of the AD-susceptible genotype with increased age, consistent with a modulation of longevity (26).

Post-mortem studies of patients with AD have found elevated levels of ACE in the temporal cortex and specifically within pyramidal cortical neurons (27, 28) as well as significantly increased ACE activity in the

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[2] The abbreviations used are: AD, Alzheimer disease; ACE, angiotensin-converting enzyme; APP, β-amyloid precursor protein; CHO, Chinese hamster ovary; HEK293, human embryonic kidney 293; Aβ, amyloid β-protein; IDE, insulin-degrading enzyme; NEP, neprilysin; ELISA, enzyme-linked immunosorbant assay; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; *I*, ACE insertion; *D*, ACE deletion; wtACE, wild-type ACE; HA, hemagglutinin.

medial hippocampus, parahippocampal gyrus, frontal cortex, and caudate nucleus (29). A mechanistic link between ACE and AD was suggested when affinity-purified ACE was shown to degrade synthetic Aβ-(1–40) between the Asp^7-Ser^8 bond *in vitro*, producing a truncated 33-residue peptide that exhibited decreased aggregation and cytotoxic potential (30).

A plausible interpretation of the above genetic, neuropathological, and biochemical findings is that ACE is implicated in AD via direct proteolysis of the Aβ peptide, modulating Aβ levels within the brain. This hypothesis suggests that reduced ACE activity, either by genetic mechanisms or chronic pharmacological inhibition, could increase cerebral levels of Aβ40 and/or Aβ42 and thus presumably increase the risk of developing AD and/or contribute to its progression. To address this hypothesis in intact, living cells, we cloned and characterized human neural ACE and determined its role in the clearance of secreted Aβ. We demonstrate that ACE promotes the clearance of naturally produced Aβ40 and Aβ42 and leads to secondary degradation of both Aβ species. Further, by using site-directed mutagenesis in the context of the full-length enzyme, we show that both the N- and C-domains of ACE are capable of promoting Aβ degradation with similar capacity. Finally, we demonstrate that treatment of Aβ-secreting cells with a commonly prescribed ACE inhibitor prevents ACE-mediated Aβ clearance and results in accumulation of the Aβ peptide.

MATERIALS AND METHODS

Cloning of ACE and Creation of Active Site Mutants—The 4.0-kb ACE cDNA was obtained by reverse transcription-PCR of RNA isolated from the human neuroblastoma cell line SK-N-SH. 5′- and 3′-end primers used to amplify the sequence were (5′-GGAAGCTTGCCGAGCACCGCGCACCGC-3′) and (5′-CAGTGTTCCCATCCCAGTCT-3′), respectively. This coding region contains the full-length ACE protein, including signal peptide, N- and C-catalytic domains, transmembrane domain, and the cytoplasmic C terminus (Fig. 1*B*). The neurally derived ACE cDNA was cloned into the pcDNA5/FRT expression vector and confirmed by DNA sequencing to be identical to the reported human ACE cDNA sequence. The full-length ACE cDNA was used as the template for oligonucleotide-directed mutagenesis with the QuikChange® XL site-directed mutagenesis kit (Stratagene) to generate the catalytically inactive ACE constructs. The two ACE zinc metalloprotease active site glutamates (amino acids 362 in the C-domain and 960 in the N-domain) were changed to aspartates using the following primers: for E362D, 5′-CCACAGTGCACCATGACATGGGCCATATACAG-3′ (forward) and 5′-ACTGTATATGGCCCATGTCATGGTGCACTGTGG-3′ (reverse); for E960D, 5′-GGCCCACCACGACATGGGCCACATC-3′ (forward) and 5′-GATGTGGCCCATGTCGTGGTGGGCC-3′ (reverse). Mutant constructs were cloned into pcDNA5/FRT and screened by DNA sequencing to ensure that no other mutations had occurred. Four constructs were thus generated: the N-domain mutant, the C-domain mutant, the combined N- and C-domain mutant, and the wild-type enzyme. All expression plasmids were characterized by both restriction digestion and DNA sequencing of the entire ACE cDNA. The HA-tagged IDE construct in the pcDNA5/FRT vector has been previously described (31).

Cell Culture—Chinese hamster ovary (CHO) and human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells stably expressing APP_{695} bearing the K595N/M596L ("Swedish") AD-causing mutation (32) were selected in medium containing 200 μg/ml G418. CHO cells stably transfected with APP_{751} with the V717F AD-causing mutation and a pFRT/lacZeo construct enabling Flp-In™ (Invitrogen) competence were grown in 200 μg/ml G418 and 100 μg/ml Zeocin. Once successful genomic recombination of pcDNA5/FRT constructs occurred, the Flp-In host cells lost Zeocin resistance and were selected with 350 μg/ml hygromycin B. Effects of Capoten (captopril; Sigma) were assayed by a 24-h preincubation of cells with the drug, followed by an 18-h conditioning period in which cells were grown in serum-free Dulbecco's modified Eagle's medium (Invitrogen) with captopril.

Expression of Cloned Constructs in CHO and HEK293 Cells—Transient transfections in CHO and HEK293 cell lines were performed using GenePorter 2 (GTS), and expression was assayed 24–42 h posttransfection. CHO cell lines stably expressing APP_{751} with the V717F mutation and either empty vector, ACE, or mutant ACE constructs were generated using the Flp-In™ system (Invitrogen). This allowed stable integration of each construct into the same genomic locus of a single cell line.

Immunoblotting—Cells and tissue were lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture (Roche Applied Science), 2 mM 1,10-phenanthroline, and 5 mM EDTA, and the extracts were centrifuged at 1,000 × *g* for 10 min to remove nuclei. Protein concentrations were determined using a bicinchoninic acid-based assay (Pierce). Samples were then subject to SDS-PAGE and Western blotting. ACE was detected using a polyclonal antibody raised to amino acids 1–170 of human ACE (sc-20791; Santa Cruz Biotechnology, Inc.). Full-length human APP was detected using 8E5, reactive to APP-(444–592) of APP_{695} (gift of P. Seubert). APP C99 and C83 C-terminal fragments were detected using the polyclonal antibody C9, specific for residues 676–695 of APP_{695}. Total cellular IDE was detected using the polyclonal IDE-1 raised to amino acids 62–73 of human IDE (33). HA-tagged IDE was detected using the anti-HA monoclonal 3F10 (Roche Applied Science). Deglycosylation was performed using peptide: *N*-glycosidase F to remove *N*-linked sugars and a mixture of the *O*-deglycosylating enzymes sialidase A, *O*-glycanase, β(1–4)-galactosidase, and β-*N*-acetylglucosaminidase (Prozyme). Western blots were probed with anti-mouse, anti-rat, or anti-rabbit secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes, Inc., Eugene, OR) or IRdye 800 (Rockland Immunochemicals). Blots were detected, and bands were sized and quantified using the Odyssey infrared imaging system (LI-COR).

ACE Activity Assay—ACE proteolytic activity was assayed using the substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu; Sigma) as described previously (34), with several modifications. Cells were lysed in 50 mM Tris (pH 7.4) containing 0.5% Nonidet P-40, and nuclei and cell debris was pelleted at 1,000 × *g* for 10 min. To assay ACE activity, 2.5 μg of cell lysate was incubated with 1 mM Hip-His-Leu in 0.4 M sodium borate buffer (pH 8.3) with 0.3 M NaCl in a total volume of 35 μl for 5–60 min at 37 °C. When assaying samples with captopril, a 15-min preincubation with the drug was performed at 4 °C before substrate addition. Time 0 values were calculated by the addition of EDTA to a final concentration of 10 mM prior to the addition of Hip-His-Leu. Enzymatic reactions were terminated by the addition of EDTA (10 mM final concentration). Samples were developed first by the addition of 150 μl of 0.34 M NaOH, followed by a 10-min room temperature incubation with 20 μl of 20 mg/ml *o*-phthaldialdehyde (Sigma). This reaction was terminated by acidification with 50 μl of 3 N HCl. Fluorescence of liberated and *o*-phthaldialdehyde-modified His-Leu was assayed in a 96-well plate format using a Victor2 multilabel plate reader (excitation, 355; emission, 535) (PerkinElmer Life Sciences). 100% degradation was defined as the maximal fluorescence signal achieved by digestion of 1 mM Hip-His-Leu with 2.5 μg of ACE-transfected cell lysate for 60 min.

Enzyme-linked Immunosorbant Assay—Conditioned medium samples were harvested by removing cellular debris by centrifugation at 700 × *g* for 10 min, and protease inhibitors were added (final concentration of 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 μg/ml pepstatin, 2 mM 1,10-phenanthroline, and 5 mM EDTA). ELISAs for Aβ were performed as previously described (35), with the following modifications. 96-Well ELISA plates (Costar) were coated with 3.5 μg/ml of the capture antibody. Aβ-(1–40) and Aβ-(1–42) were measured by capturing with antibodies specific to the Aβ C-terminal 40 (2G3) or 42 (21F12) residues. Captured Aβ was detected with 0.1 μg/ml biotinylated 3D6, specific to residues 1–5 of the Aβ N terminus. ELISAs measuring X-40 or X-42 forms of Aβ were captured identically with 2G3 or 21F12 and detected with 0.1 μg/ml biotinylated 266, specific to residues 13–28 of Aβ. Total Aβ was measured by capture with antibody 266 and detection with 0.1 μg/ml biotinylated 3D6 (all antibodies gift of Elan Pharmaceuticals, San Francisco, CA). ELISA development was accomplished by incubating the Aβ-bound biotinylated detection antibody with Avidin-horseradish peroxidase (Vector), followed by tetramethylbenzidine-ELISA (Pierce). Plates were washed three times for 1 min after each incubation with Tris-buffered saline, 0.05% Tween 20.

Statistical Analyses—The data were analyzed using a one-way analysis of variance and Tukey's *post hoc* comparison or a two-way analysis of variance and Bonferroni *post hoc* comparison, where appropriate. Calculated comparisons of $p < 0.05$ were considered significant.

RESULTS

Expression and Characterization of Transfected Human ACE—To determine the regional distribution of ACE within the brain, we first dissected several murine brain and peripheral tissues to assess relative ACE content, particularly in AD-relevant brain regions. As previously reported, we found that ACE was highly expressed in kidney and lung but absent in the liver (36). In the brain, we observed similar expression of ACE protein in the cerebral cortex, hippocampus, cerebellum, and basal ganglia/brainstem (Fig. 1*A*). ACE in each of these brain regions was found to electrophorese as two distinct bands, consisting of the typical full-length ~180-kDa species and a higher molecular weight species, the latter reported to arise from alternative glycosylation of ACE in the brain (37).

To characterize ACE at a cellular level, we cloned the full-length 4.0-kb cDNA from the human neuroblastoma cell line SK-N-SH. ACE cDNA was then cloned into the mammalian expression vector pcDNA5/FRT and shown to conform to the reported human ACE sequence by both restriction mapping and DNA sequencing. Transient transfection of the ACE construct into CHO and HEK293 cells yielded indistinguishable results in all assays performed (Fig. 2). Neither cell line expressed detectable endogenous levels of ACE protein by Western blotting, although ACE mRNA was detected in HEK293 cells by reverse transcription-PCR (data not shown).

ACE is predicted to contain 17 *N*-type glycosylation sites and no *O*-type glycosylation sites. To determine whether transfected ACE protein was processed similarly to tissue-derived ACE, we compared glycosylation patterns of mouse kidney tissue ACE and ACE transfected into either CHO or HEK293 cells. As expected, deglycosylation with several enzymes specific to *O*-linked sugars produced no shift in ACE size, whereas removal of *N*-linked modifications yielded a shift in the ACE protein from ~182 to ~154 kDa (Fig. 2*A*). ACE is known to be secreted both *in vivo* and *in vitro* from the cell surface at low levels in an α-secretase-dependent manner (38). The concentration of conditioned medium from HEK293 and CHO cells transiently transfected with ACE cDNA was found to contain low levels of ACE protein, whereas control cells transfected with the empty vector had no corresponding signal (Fig. 2*B*).

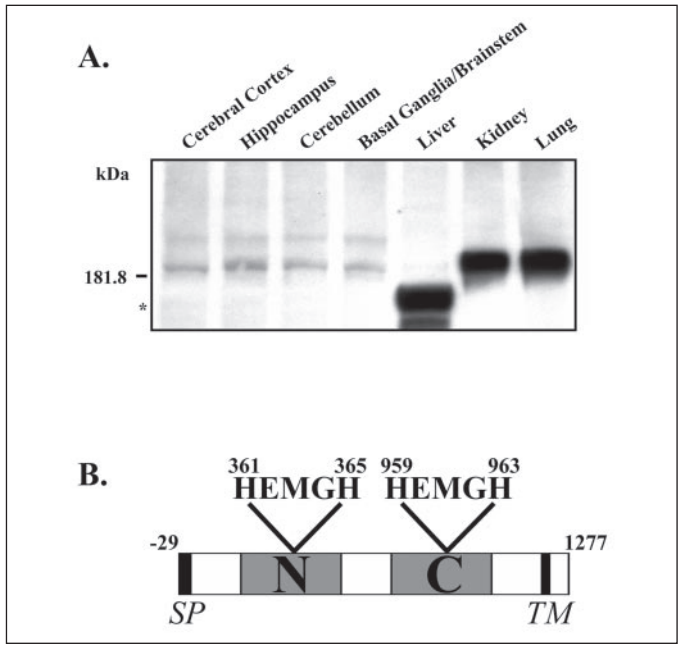

FIGURE 1. **Brain distribution and schematic diagram of angiotensin-converting enzyme.** *A*, 20 μg of murine tissue homogenates from the designated brain regions, as well as peripheral tissues known to lack or highly express ACE, were probed by Western blotting with an ACE-specific antibody. Note that brain ACE occurs as a doublet, arising from alternate glycosylation. The *asterisk* indicates a nonspecific band detected only in liver, as has been previously reported (46). *B*, schematic representation of the human full-length ACE protein. The wild-type enzyme contains a signal peptide (*SP*), two homologous catalytic domains (*gray boxes*), a single transmembrane segment (*TM*), and a small C-terminal cytoplasmic tail. The metalloprotease catalytic sequence of each domain is indicated, with the corresponding amino acid numbering from the first N-terminal residue of the mature protein.

To determine whether the transfected ACE was enzymatically active, we incubated lysates from cells transiently transfected with ACE or empty vector with the commonly used synthetic ACE substrate, Hip-His-Leu, under standard assay conditions (1 mM substrate, 2.5 μg of cell lysate, 0.4 M sodium borate buffer, and 0.3 M NaCl, pH 8.3) for 30 min at 37 °C. Lysates from cells transfected with ACE exhibited robust degradation of Hip-His-Leu, and this was completely blocked by a 1 μM concentration of the competitive ACE inhibitor, captopril. In contrast, lysates from cells transfected with empty vector had no detectable ACE activity (Fig. 2*C*). Taken together, the above results demonstrate that the cloned and transfected ACE construct is processed similarly to tissue ACE and retains proteolytic activity and sensitivity to ACE inhibitors.

Transiently Expressed ACE Promotes Clearance and Secondary Degradation of Aβ—To determine whether ACE can modulate Aβ levels in intact, living cells, we transiently transfected equal DNA amounts of plasmids encoding either empty vector, IDE, or ACE in pcDNA5/FRT into HEK293 cells stably expressing human APP_{695} bearing the Swedish AD-causing mutation and measured the levels of Aβ in the resulting conditioned medium. Transfection of HA-tagged IDE served as a positive control for Aβ proteolysis, since this has been shown to lower Aβ in cultured cells (6, 39). Using an ELISA specific to Aβ-(1–40) and Aβ-(1–42), IDE was found to reduce cell-derived Aβ levels to 55 and 47%, respectively, of those in control cells transfected with empty vector ($p < 0.001$ for both comparisons). Transfections with ACE yielded similar results, reducing Aβ-(1–42) levels to 70% and Aβ-(1–42) levels to 61% of control ($p < 0.05$ for Aβ-(1–40) and $p < 0.01$ for Aβ-(1–42)) (Fig. 3*A*). Thus, both ACE- and IDE-transfected cells had significantly

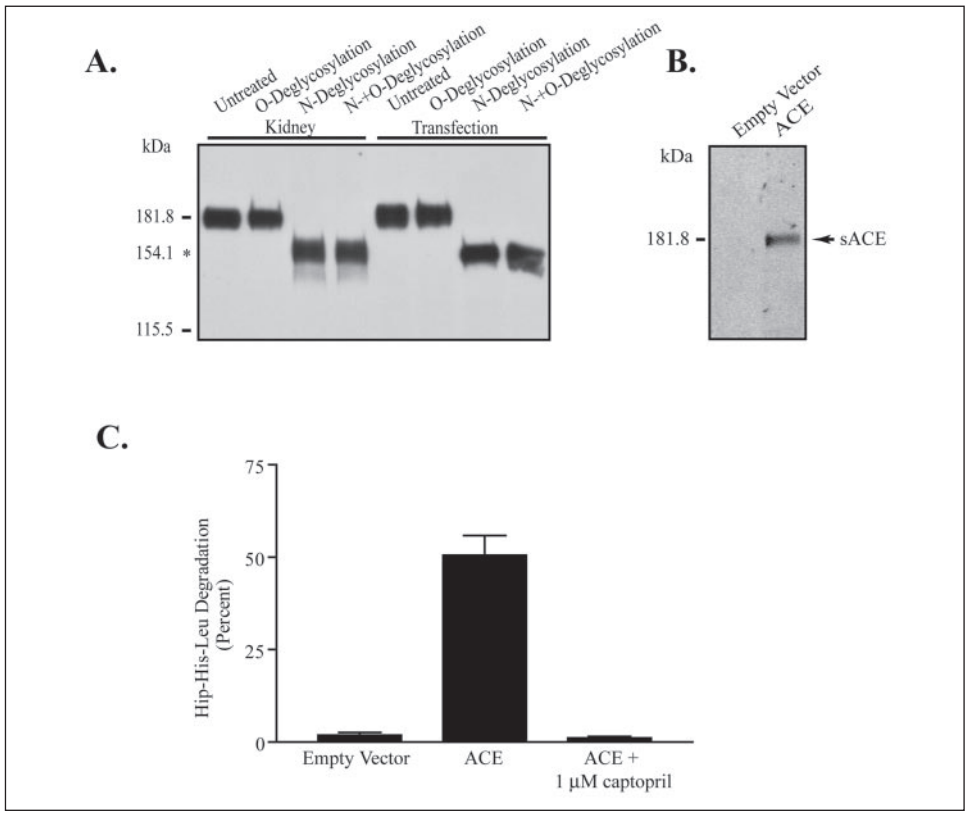

FIGURE 2. **Characterization of the processing and enzymatic activity of cloned human ACE.** *A*, lysates from mouse kidney or CHO cells transiently transfected with the ACE cDNA construct were subjected to deglycosylation with enzymes specific to *N*- or *O*-linked sugars and subject to Western blot analysis with an ACE-specific antibody. The *asterisk* indicates the calculated molecular weight of the deglycosylated band. *B*, conditioned medium from CHO cells transiently transfected with ACE or empty vector was concentrated and analyzed by Western blot, reflecting a low rate of ACE ectodomain secretion (*sACE*), as previously reported (12). *C*, ACE enzymatic activity was measured by incubating 2.5 μg of lysate from HEK293 cells transfected with the indicated constructs with 1 mM of the substrate Hip-His-Leu for 30 min at 37 °C. 100% degradation was defined as the maximal fluorescence produced by 60 min of substrate incubation with 2.5 μg of lysate from cells transfected with the ACE cDNA. Values in *C* represent the means ± S.E. obtained from four independent experiments. Each blot in *A* and *B* is representative of at least four independent experiments. Deglycosylation, secretion, and enzymatic activity assays were identical for transfected CHO and HEK293 cell lines.

reduced levels of both principal Aβ species compared with control but were not significantly different from each other.

Because ACE has been shown to cleave Aβ between Asp^7 and Ser^8 *in vitro* (30), we hypothesized that the remaining Aβ fragment, beginning at Ser^8, might either accumulate or undergo secondary degradation in a cellular context. To discriminate between these possibilities, we used an ELISA specific to internal residues 13–28 of Aβ for capture and to either the 40 or 42 C termini for detection; the measured species are denoted Aβ-(X-40) and Aβ-(X-42). We found IDE to decrease Aβ-(X-40) levels to 78% and Aβ-(X-42) levels to 68% of those of control cells ($p < 0.05$ for Aβ-(X-40) and $p < 0.001$ for Aβ-(X-42)). ACE again mirrored this reduction, with Aβ-(X-40) decreased to 79% and Aβ-(X-42) decreased to 71% of controls ($p < 0.05$ for Aβ-(X-40) and $p < 0.001$ for Aβ-(X-42)) (Fig. 3*B*). Both Aβ-(X-40) and Aβ-(X-42) values for IDE and ACE were significantly different from control but not different from each other. Thus, in a human cell line transfected with human cDNAs encoding both APP and either IDE or ACE, Aβ levels were significantly reduced. Further, cell-derived Aβ in ACE-transfected cells showed greater secondary degradation of the peptide than has been reported *in vitro* (30). This result suggests that ACE degrades Aβ at additional sites when in a cellular context and/or that Aβ species cleaved by ACE are subsequently degraded by other cellular proteases. Transfection efficiency was assayed by Western blot analysis (Fig. 3*C*). Whereas both transfected constructs resulted in robust expression, total IDE levels were only increased ~2.5-fold over endogenous IDE (Fig. 3*C*, *bottom panel*). In contrast, no detectable endogenous ACE was expressed by the HEK293 cells (Fig. 3*C*, *top panel*).

Generation of Catalytically Inactive ACE Mutants—ACE contains two homologous catalytic regions, termed the N- and C-domains, each containing a canonical zinc metalloprotease active site (Fig. 1*B*). To determine which active site mediates Aβ clearance, we generated three ACE mutant constructs: two containing only one functional catalytic domain and one catalytically inactive enzyme bearing mutations in both sites. Mutations were made by site-directed mutagenesis to change the active site sequence HEMGH to HDMGH, a conservative mutation previously shown to inactivate ACE proteolysis (11). The C-domain was inactivated by mutating ACE glutamate residue 362 to aspartate (termed E362D), and the N-domain was similarly mutated by changing glutamate residue 960 to aspartate (termed E960D). Singly mutated constructs were combined by restriction digestion and ligation to form the catalytically inactive double mutant (E362D/E960D). Each construct was analyzed by restriction digestion and DNA sequencing to ensure that no mutations were made outside of those produced by the site-directed mutagenesis. Each was cloned into pcDNA5/FRT to achieve identical levels of cellular expression.

A CHO cell line stably expressing both human APP_{751} bearing the AD-causing V717F mutation, and a Flp-In acceptor locus was used to make stable cell lines expressing wild-type ACE (wtACE), E362D, E960D, E362D/E960D, or empty vector. Thus, each stable cell line contained APP as well as one of the transfected constructs integrated into the same genomic locus by site-directed recombination of the pcDNA5/FRT vector (cell lines are designated as *APP + X*, where *X* is the integrated construct). The resulting ACE stable lines produced identical levels of the wtACE or mutant ACE proteins as well as indistinguishable levels of human APP (Fig. 4*A*). Notably, the APP + empty vector line expressed an estimated 11% higher level of APP than the four APP + ACE lines (Fig. 4*A*, *middle panel*), presumably the result of modest cytomegalovirus promoter competition between the APP and ACE constructs.

The E362D, E960D, and E362D/E960D mutant proteins were all posttranslationally modified and secreted at similar levels as the wtACE protein (Fig. 4, *A* and *B*). Canonical enzymatic activity of the mutant constructs was confirmed by degradation of the substrate Hip-His-Leu (Fig. 4*C*). The wtACE and E362D proteins were found to degrade this

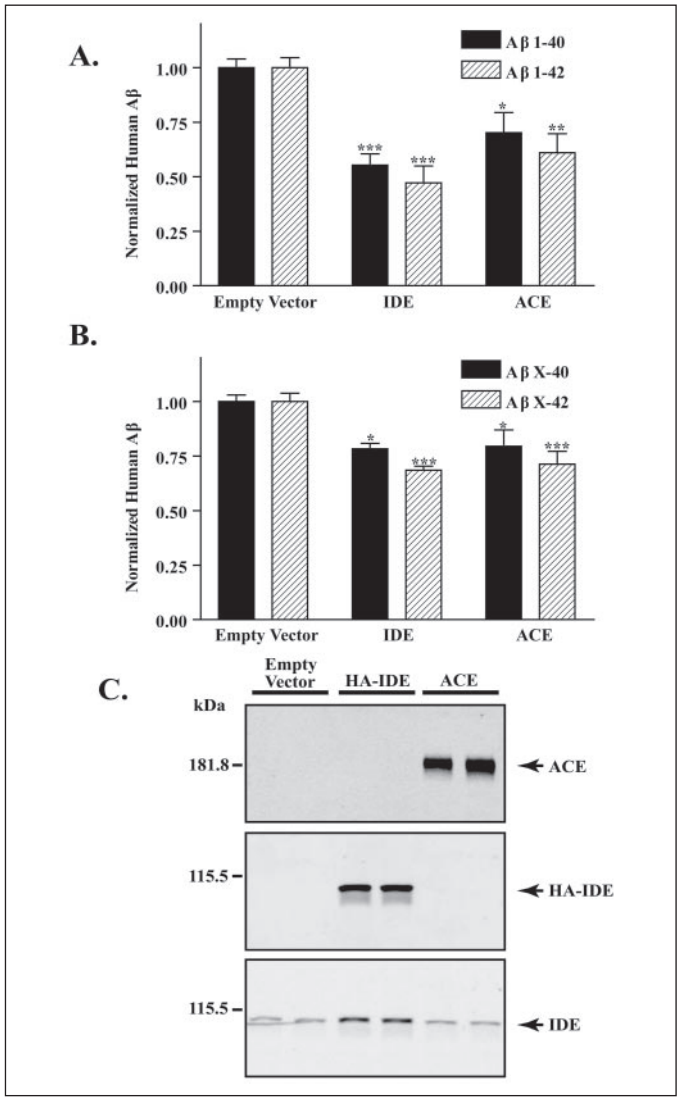

FIGURE 3. **Transfected human ACE promotes clearance and secondary degradation of cell-derived human Aβ40 and Aβ42.** HEK293 cells stably transfected with human APP_{695} bearing the AD-causing Swedish mutation were transiently transfected with empty vector, the well characterized Aβ-degrading protease IDE, or ACE. Media were conditioned on the cells for 18 h, and their Aβ levels were determined by ELISA specific to intact Aβ species beginning at residue 1 and ending at either residue 40 (*solid bars*) or 42 (*hatched bars*) (*A*) or ELISA specific to the middle region of the Aβ peptide (residues 13–28, denoted as *X*) and either residue 40 (*solid bars*) or 42 (*hatched bars*) (*B*). Data represent the means ± S.E. of 6–8 independent experiments measured in duplicate. Values were normalized to empty vector to allow combination of data sets, compared with empty vector (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *C*, immunoblots of transient transfections into HEK293 cells. *ACE* and *IDE panels* indicate total cellular expression of the protein, whereas the *HA-IDE panel* indicates expression of the tagged IDE construct only. Each immunoblot is representative of at least four independent experiments.

substrate at similar rates, whereas E960D had a far reduced efficiency of hydrolysis, conforming to the published kinetic parameters of each active site for Hip-His-Leu (11). Both the enzymatically inactive E362D/E960D and the wtACE protein incubated with 1 μM captopril produced no detectable degradation product (Fig. 4*C*).

Both the N- and C-domains of ACE Promote Aβ Degradation—To determine the effects of each ACE active site on Aβ levels, conditioned medium from each doubly stable line was analyzed for total Aβ content by ELISA. Normalizing Aβ values of the APP + empty vector condition to APP content, there was no significant difference in Aβ levels between APP + empty vector and the catalytically inactive APP + E362D/E960D. The N- and C-catalytic domains of ACE were each found to decrease cell-derived Aβ levels to a quantitatively similar degree as wtACE. E362D reduced Aβ levels to 52%, E960D to 43%, and wtACE to 34% of the APP + empty vector condition (Fig. 4*D*). Both single mutants and the wtACE enzymes were significantly different from APP + empty vector ($p < 0.001$) but not significantly different from each other. These differences in Aβ content could not be ascribed to the ACE enzyme altering the levels of the α- or β-secretase-generated APP C-terminal fragments, since both C99 and C83 were not significantly changed compared with APP + empty vector (Fig. 4*A*, *bottom panel*). Thus, using conservative mutations to inactivate the ACE catalytic domains, these experiments demonstrate that both the N- and C-domains are capable of mediating clearance of naturally produced, cell-derived Aβ in intact cells.

Aβ Levels Are Increased by ACE Inhibition—To determine whether decreasing ACE activity with a small molecule inhibitor could elevate cell-derived Aβ levels, we preincubated cells for 24 h in the presence of the prototypical ACE inhibitor, captopril, and then conditioned the media for 18 h in the presence of the drug. Increasing doses of captopril were tested, and the resulting conditioned media were analyzed by ELISA for total Aβ content. Aβ values were normalized to those of the same cell line with no drug treatment. Captopril was found to have no significant effect on Aβ levels in the catalytically inactive APP + E362D/E960D cell line, as expected. In contrast, the captopril-treated APP + wtACE cells accumulated nearly 2-fold more Aβ than untreated cells at drug concentrations above 1 μM ($p < 0.01$). At 0.1 μM captopril, the lowest concentration tested, Aβ levels were elevated 1.5-fold in APP + wtACE cells compared with the same cell line without drug ($p < 0.05$). These results demonstrate that a widely prescribed ACE inhibitor can promote accumulation of natural, cell-derived Aβ by blocking ACE proteolytic activity.

DISCUSSION

Our experiments demonstrate that ACE expression leads to cleavage of the amyloid β-protein in a cellular context and that ACE promotes the degradation of both naturally produced Aβ40 and Aβ42. Investigating the role of the N- and C-domains of ACE in Aβ clearance by site-directed mutagenesis, we used a conservative but inactivating mutation of the ACE metalloprotease active site and found that both domains are responsible for Aβ clearance. Our data do not support a role for the putative signal transduction mechanism of ACE (18) in its lowering of Aβ, since overexpressing the proteolytically inactive form of ACE produced no change in Aβ levels compared with vector alone. Finally, we show that captopril, a widely prescribed ACE inhibitor, promotes Aβ accumulation in the media of cells expressing human APP and ACE.

It has been reported recently that the N-domain of ACE, but not its C-domain, is responsible for the degradation of synthetic Aβ40 *in vitro* (40). In that study, the ACE catalytic domains were cloned in isolation, producing recombinant truncation proteins bearing one ACE active site. In contrast, we find that both the N- and C-domains of ACE are able to degrade naturally produced Aβ in a cellular context. In our studies, we used conservative mutations to inactivate each catalytic domain in the same molecule, conserving the overall structure and sequence of the full-length protein. It is possible that the C-domain is only able to degrade Aβ in intact cells, not *in vitro*. There is evidence that each catalytic domain of ACE regulates the activity of the other (41), suggesting that the full-length protein is required for normal substrate recognition and degradation. The C-domain, when expressed outside of the context of the full-length enzyme, may not retain the tertiary structure required to degrade larger substrates such as Aβ, whereas much smaller substrates are still processed (40). It is unlikely

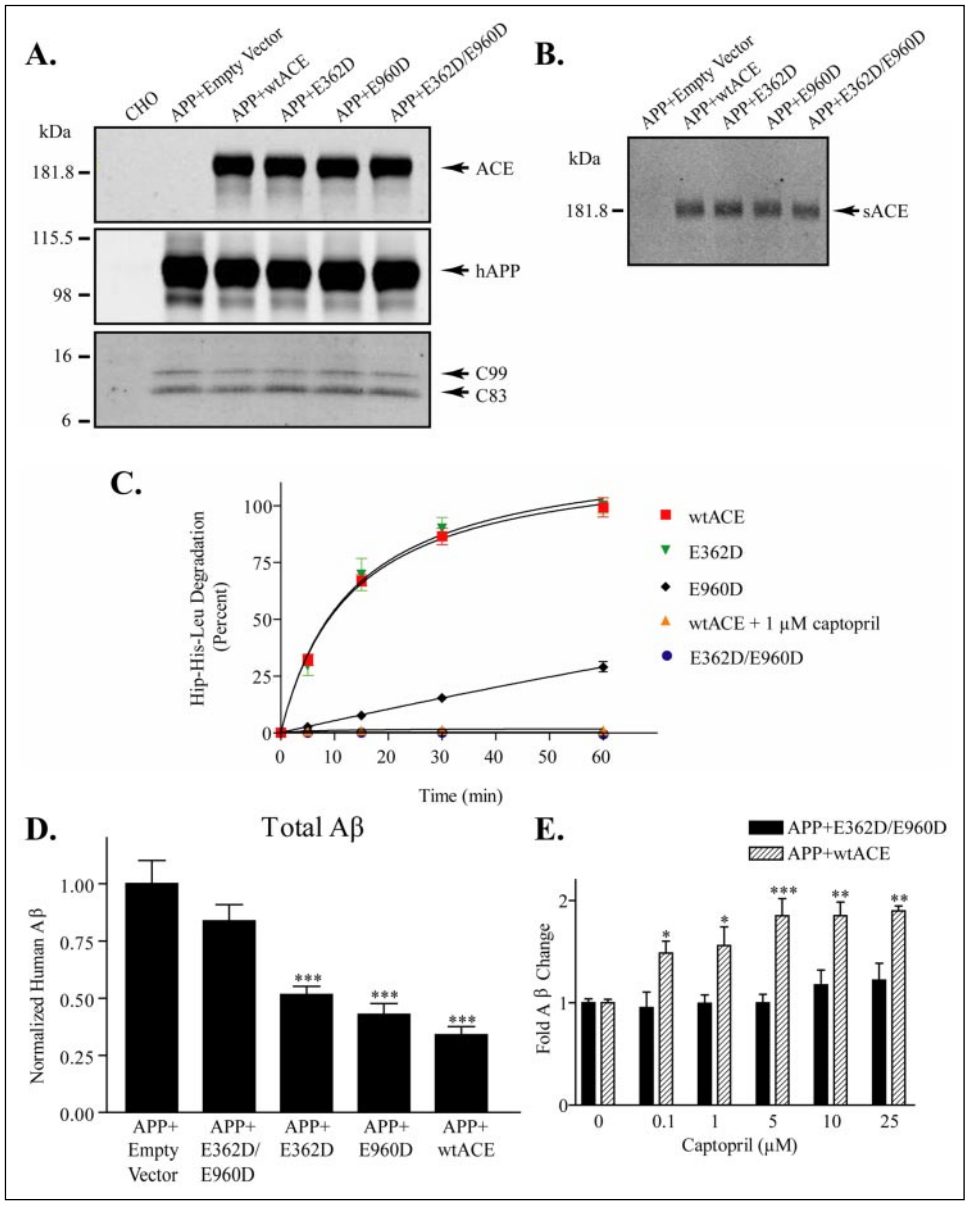

FIGURE 4. **Cell-derived Aβ is degraded by both the N- and C-domains of ACE and elevated by ACE inhibition.** CHO cells were stably transfected with both human APP_{751} bearing the V717F AD-causing missense mutation and either empty vector, human wild-type ACE, or the indicated ACE mutant constructs. *A*, immunoblots showing expression of ACE, APP, and APP C-terminal fragments in the stable cell lines. Note the slightly higher expression of human APP (*hAPP*) in the APP + empty vector line, resulting presumably from modest cytomegalovirus promoter competition between the APP and ACE constructs. Immunoblots are representative of at least four independent determinations. *B*, conditioned medium of the stable lines was concentrated and probed for the presence of secreted ACE (*sACE*) protein. *C*, ACE activity assay incubating 2.5 μg of cell lysate with 1 mM Hip-His-Leu for the indicated time points at 37 °C. Values represent the means ± S.E. obtained from 3–5 independent experiments. *D*, cell lines were conditioned for 18 h, and the media were harvested and probed by ELISA for total Aβ content. Due to elevated APP expression, Aβ values in the APP + empty vector condition were normalized by APP expression to the APP and ACE doubly stable lines. Data represent the means ± S.E. of seven independent experiments measured in duplicate. Values were normalized to APP + empty vector to allow the combination of data sets; ***, $p < 0.001$, compared with empty vector. *E*, fold change in Aβ content of conditioned medium of APP + wtACE and APP + E362D/E960D cell lines after 24-h pretreatment and 18-h conditioning in the presence of captopril. Each data point was normalized to untreated cells (0 μM captopril) of the same doubly transfected cell line. Data represent the means ± S.E. of four independent experiments measured in duplicate; ACE compared with E362D/E960D at each dose: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

that incomplete inactivation of the N-domain in our E362D mutant enzyme could account for the observed decrease in Aβ, because when both domains are inactivated in the E362D/E960D ACE construct, we observe no change in Aβ levels compared with the empty vector control.

Growing genetic evidence suggests ACE as a potential risk factor for AD. At this writing, approximately 40 published studies have examined this relationship, with the majority finding a significant association of *ACE* genotype to AD. Further, post-mortem analysis of AD brain tissue has shown significant elevation of ACE protein compared with control (28), perhaps representing an up-regulation of an Aβ clearance mechanism. Interestingly, initiation of ACE-mediated signal transduction by its substrates has been shown to up-regulate the enzyme's own expression via the c-Jun N-terminal kinase pathway (18, 42). This feedback system provides a hypothetical mechanism by which ACE could modulate its own expression depending on levels of the Aβ substrate, leading to elevated levels of ACE in AD cortex.

Our observation that ACE inhibition in cells expressing both human APP and ACE promotes accumulation of secreted Aβ may bear clinical relevance. Given that neurons produce both APP and ACE and neuronal ACE is up-regulated in AD (27), we hypothesize that chronic pharmacological inhibition of ACE could increase levels of brain Aβ by reducing ACE-dependent proteolysis. No studies have yet examined the consequence of chronic ACE inhibition on levels of brain or plasma Aβ in humans. Very few clinical trials of ACE inhibitors have analyzed their effects on cognitive decline in AD subjects, and results to date are inconclusive (43–45).

No studies on the effects of ACE deficiency or overexpression on Aβ metabolism *in vivo* have been reported. Such studies may be problematic in interpretation, because endogenous murine Aβ has amino acid differences from the human peptide immediately surrounding the putative site of cleavage by ACE, and these could affect its properties as a substrate. Future *in vivo* studies that examine ACE inhibition, deficiency, or overexpression in mice expressing human APP may be most instructive regarding the role of ACE in Alzheimer disease.

In the context of our new findings and the earlier studies reviewed above, we hypothesize that decreased activity of ACE in the aged human brain, originating either from genetic polymorphisms or pharmacolog-

ical inhibition, may promote Aβ accumulation and thus modulate the likelihood of the development of Alzheimer disease and/or its neuropathological severity. Our findings suggest the need for further analyses of the biological effects of ACE genetic deficiency and ACE pharmacological inhibition, both in experimental models of AD and in the large human population currently experiencing chronic ACE inhibition.

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