Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8098–8102, September 1993
Medical Sciences

Binding of human apolipoprotein E to synthetic amyloid β peptide: Isoform-specific effects and implications for late-onset Alzheimer disease

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Communicated by Wolfgang K. Joklik, June 1, 1993

ABSTRACT **Apolipoprotein E (apoE), a plasma apolipoprotein that plays a central role in lipoprotein metabolism, is localized in the senile plaques, congophilic angiopathy, and neurofibrillary tangles of Alzheimer disease. Late-onset familial and sporadic Alzheimer disease patients have an increased frequency of one of the three common apoE alleles, ε4, suggesting apoE4 is associated with increased susceptibility to disease. To follow up on this suggestion, we compared the binding of synthetic amyloid β (β/A4) peptide to purified apoE4 and apoE3, the most common isoform. Both isoforms bound synthetic β/A4 peptide, the primary constituent of the plaque and angiopathy, forming a complex that resisted dissociation by boiling in SDS. Oxygen-mediated complex formation was implicated because binding was increased in oxygenated buffer, reduced in nitrogen-purged buffer, and prevented by reduction with dithiothreitol or 2-mercaptoethanol. Binding of β/A4 peptide was saturable at 10^{-4} M peptide and required residues 12–28. Examination of apoE fragments revealed that residues 244–272 are critical for complex formation. Both oxidized apoE4 and apoE3 bound β/A4 peptide; however, binding to apoE4 was observed in minutes, whereas binding to apoE3 required hours. In addition, apoE4 did not bind β/A4 peptide at pH < 6.6, whereas apoE3 bound β/A4 peptide from pH 7.6 to 4.6. Together these results indicate differences in the two isoforms in complexing with the β/A4 peptide. Binding of β/A4 peptide by oxidized apoE may determine the sequestration or targeting of either apoE or β/A4 peptide, and isoform-specific differences in apoE binding or oxidation may be involved in the pathogenesis of the intra- and extracellular lesions of Alzheimer disease.**

Apolipoprotein E (apoE) is synthesized by many organs, primarily the liver, and is involved in lipid transport and metabolism (1). The protein is also produced and secreted in the brain (2, 3) and is implicated in neuronal regeneration. The synthesis of apoE markedly increases after neural injury in both the peripheral (4) and central (5) nervous systems. In addition, apoE regulates the outgrowth and sprouting of cultured dorsal root ganglia neurons *in vitro* (6). Recent studies have implicated apoE in the pathogenesis of Alzheimer disease with the protein accumulating extracellularly in the senile plaque and congophilic angiopathy of Alzheimer disease and intracellularly in the neurofibrillary tangle (7, 8, 44). In cerebrospinal fluid, apoE avidly binds to synthetic amyloid β (β/A4) peptide (7), the primary constituent of the senile plaque and congophilic angiopathy. The mRNA for apoE is increased in the brains of Alzheimer disease patients (9). One of the apoE alleles, ε4, is highly associated with late-onset familial (7) and sporadic (10) Alzheimer disease. Three major protein isoforms of apoE (apoE2, E3, and E4) are the products of three alleles (ε2, ε3, and ε4) at a single gene locus on the proximal long arm of chromosome 19q13.2 (1), within the region previously associated with linkage of late-onset familial Alzheimer disease (11). In these patients, the ε4 allele frequency was 0.50 ± 0.06, compared with age-matched controls, 0.16 ± 0.03 (7). In follow-up studies, we found that ε4 was also highly associated with patients with sporadic Alzheimer disease, with an allele frequency of 0.40 ± 0.026 (10). In a study of the comparative neuropathology of brain tissue from Alzheimer disease patients homozygous for ε4 or ε3, congophilic staining of amyloid in senile plaques and blood vessels was greatly increased in ε4 patients (42). In addition, β/A4 immunoreactivity in plaques and blood vessels was also increased in ε4 homozygotes.

The presence of apoE in the extracellular senile plaques and angiopathy and in the intracellular neurofibrillary tangles of Alzheimer disease suggests that apoE may be involved in the formation of these lesions. The association of apoE with both late-onset familial and sporadic Alzheimer disease suggests that the function of this isoform may be important in the pathogenesis of the disease. The demonstration that apoE avidly binds β/A4 peptide *in vitro*, resisting dissociation by SDS or guanidine hydrochloride (7), provides an *in vitro* system for examining molecular interactions. Differences in the interactions of specific isoforms of apoE with β/A4 peptide may affect molecular mechanisms or targeting of these molecules. The localization of apoE to both characteristic extra- and intracellular lesions of Alzheimer disease implies that apoE may be central to a unifying hypothesis.

The most common isoform of apoE in the general population, apoE3, is secreted as a 299-amino acid protein containing a single cysteine residue at position 112. The other two common isoforms, apoE2 and apoE4, differ at one of two positions (residues 112 and 158) from apoE3 by cysteine–arginine interchanges: apoE2 contains a cysteine at position 158, and apoE4 contains an arginine at 112. The single cysteine in apoE3 permits disulfide-bond formation with other molecules, including itself. Heterodimer formation with apolipoprotein A-II and homodimers with another apoE3 molecule have been reported (12, 13). The apoE4 isoform lacks a cysteine and cannot form these disulfide complexes. The isoforms of apoE also differ in their interactions with the low density lipoprotein (LDL) receptor:

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Abbreviations: apoE, apolipoprotein E; apoE2, apoE3, and apoE4, apoE isoforms 2, 3, and 4, respectively; β/A4, amyloid β; LDL, low density lipoprotein; HDL, high density lipoprotein.

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apoE3 and apoE4 bind normally, and apoE2 binds at ≈1% of normal binding. This defective binding of apoE2 is associated with type III hyperlipoproteinemia, a disorder in which plasma cholesterol and triglycerides are elevated, and subjects are prone to an increased susceptibility to cardiovascular disease (1). It has been demonstrated that apoE contains two independently folded domains that can be modeled by the thrombolytic fragments residues 1–191 and 216–299 (14, 15). The region of apoE that binds to the LDL receptor is contained in the amino-terminal domain (residues 1–191), whereas the carboxyl-terminal domain contains the major lipid-binding region of the protein. Recently it was demonstrated that residues that are carboxyl-terminal to residue 244 play a major role in lipoprotein binding (16). Although the cysteine–arginine interchange at position 112 (which distinguishes apoE3 from apoE4) is not contained in the major lipid-binding region of apoE, this position influences the distribution of these isoforms among the various classes; apoE3 preferentially binds to the high density lipoproteins (HDL), whereas apoE4 strongly binds the triglyceride-rich lower density lipoprotein particles, both very low-density lipoprotein and intermediate density lipoprotein (17–19). A domain–domain interaction has been suggested to account for the distribution effect (19). The recent elucidation of the three-dimensional structure of the receptor-binding domain of apoE has helped model isoform-specific functional properties, including differences in LDL-receptor binding (20). We therefore characterized the binding of synthetic β/A4 peptide to purified apoE4 and apoE3, with the rationale that binding of β/A4 peptide by apoE may be important in targeting these molecules to their extracellular and intracellular destinations in Alzheimer disease.

MATERIALS AND METHODS

The apoE3 and apoE4 isoforms were isolated from the plasma of fasting subjects with the E3/3 and E4/4 homozygous phenotypes, using described techniques (21). Proteolytic fragments of apoE (22) and recombinant-expressed truncated apoE were produced as described (16). Synthetic β/A4 peptides were from Bachem. One milligram of β/A4 peptide was dissolved in 60 μl of distilled water and then diluted in phosphate-buffered saline, pH 7.30, to the indicated concentrations.

One microgram of purified apoE was incubated 5 hr at 37°C with β/A4 peptide in phosphate-buffered saline, pH 7.30, in a total volume of 20 μl. Incubation was terminated by the addition of 20 μl of 2× Laemmli buffer (4% SDS, with no 2-mercaptoethanol) and boiled 5 min. Samples were stored at −80°C. Proteins were electrophoresed on either a 7.5 or 12% polyacrylamide gel with 2% SDS and transferred to Immobilon P (Millipore), as described (7). The Immobilon membrane was washed and incubated in primary antibody overnight, as described (7). Rabbit anti-human apoE antibody was used at 1:5000. Rabbit anti-β/A4 amyloid peptide antibody (Boehringer Mannheim) was used at 1:80. The Immobilon membrane was incubated with horseradish peroxidase-conjugated secondary antibody, and chemoluminescence (Enhanced Chemoluminescence kit; Amersham) was visualized by exposure to Hyperfilm (Amersham). Quantitative scanning densitometry was on a Hoeffer gel scanner and was analyzed with the included GS370 software.

RESULTS

Incubation of purified, delipidated apoE4 or apoE3 with synthetic β/A4 peptide [β/A4-(1–28)] resulted in the formation of an apoE-β/A4 peptide complex with an apparent molecular weight greater than apoE alone that was recognized by both an apoE antibody and by a β/A4 peptide antibody. This complex was maintained even after boiling in 2% SDS for 5 min (Fig. 1). In contrast, boiling apoE before incubation with β/A4 peptide prevented binding (data not shown). The apoE3–β/A4 peptide complex was first detectable after 2-hr incubation and increased over the next 24 hr. In contrast, the apoE4–β/A4 complex was easily detected after 5-min incubation. β/A4 peptide bound to both the monomer of apoE3 and to the disulfide-linked homodimer of apoE3 (see Figs. 2 and 4). After incubation for 12 hr, an additional, higher-molecular-weight apoE–β/A4 complex was seen. Only a small percentage (<10%) of the total amount of apoE in the incubation bound β/A4 peptide after 24 hr, despite a large molar excess of β/A4 peptide (β/A4 peptide at 2.5×10^{-4} molar, apoE at 1.8×10^{-6} molar).

The incomplete formation of the apoE–β/A4 peptide complex could be due either to the slow association of these molecules or to modification of protein before binding. Addition of the reducing agents dithiothreitol or 2-mercaptoenthanol, either before or after incubation of apoE and β/A4 peptide, prevented SDS-stable binding (Fig. 2), suggesting that oxidation may be required. No differences were detected in the amount or relative molecular weight of immunoreactive apoE during these incubations (data not shown). As a further test of the hypothesis that only oxidized apoE bound β/A4 peptide, the incubation buffer was saturated with either oxygen or nitrogen. Fig. 3 shows that oxygen increased and nitrogen decreased the rate of SDS-stable binding. Incubation of apoE3 or apoE4 alone in oxygenated buffer increased the amount of β/A4 peptide bound subsequently (data not shown). Prolonged incubation of apoE3 or apoE4 alone at 37°C resulted in the gradual loss of the ability of apoE to bind β/A4 peptide. The amount of

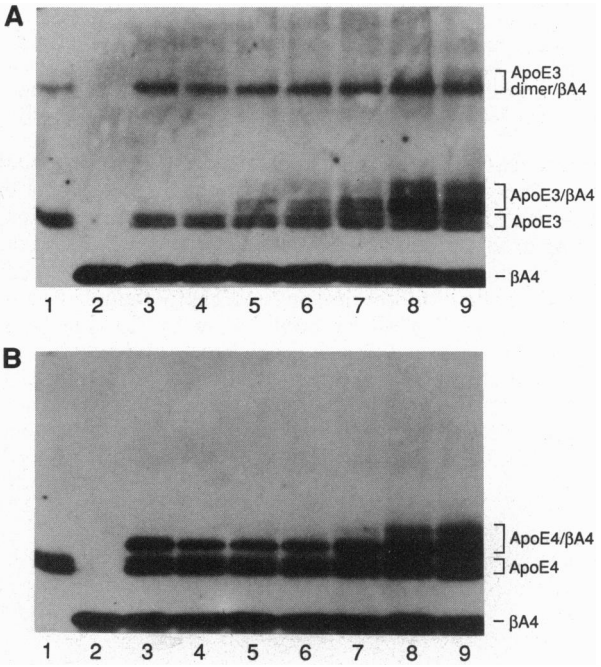

FIG. 1. Time course of SDS-stable binding of β/A4 peptide by apoE3 and apoE4. One microgram of apoE3 (*A*) or apoE4 (*B*) was incubated with β/A4-(1–28) (2.5×10^{-4} M) in a total volume of 20 μl between 5 min and 24 hr at 37°C. The incubation was ended by adding 20 μl of 2× Laemmli buffer (without 2-mercaptoethanol) and boiling 5 min. Proteins were electrophoretically separated on a 7.5% polyacrylamide gel and transferred to Immobilon P membrane. Both apoE and β/A4 peptide were detected with the anti-apoE antibody and the anti-β/A4 peptide antibody. Lanes: 1, apoE alone; 2, β/A4 peptide alone. 3–9, incubation of apoE with β/A4 peptide for 5 min (lane 3), 20 min (lane 4), 2 hr (lane 5), 4 hr (lane 6), 6 hr (lane 7), 12 hr (lane 8), and 24 hr (lane 9).

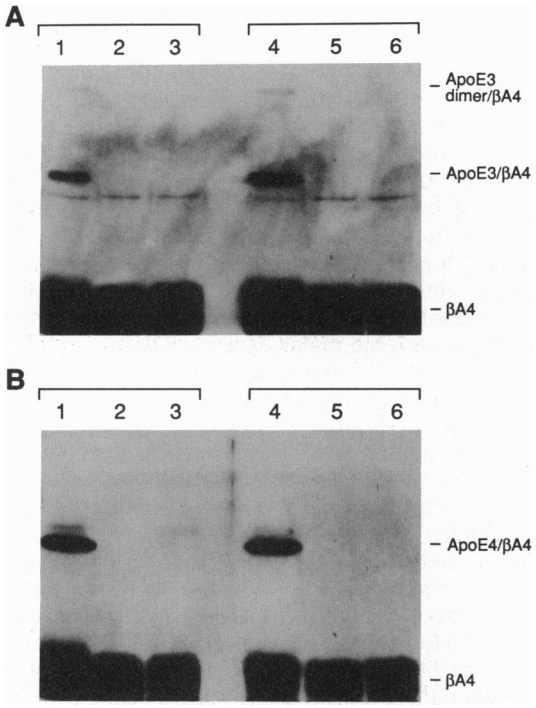

FIG. 2. Effect of reducing agents 2-mercaptoethanol or dithiothreitol on SDS-stable binding of β/A4 by apoE. One microgram of apoE3 (*A*) or apoE4 (*B*) was incubated with β/A4-(1–28) (2.5×10^{-4} M) for 5 hr at 37°C. Incubation was stopped by the addition of an equal volume of 2× Laemmli buffer (without 2-mercaptoethanol) and boiled 5 min. Lanes: 1 and 4, apoE and β/A4 peptide without reducing agents; 2, 0.2% (vol/vol) 2-mercaptoethanol during incubation; 3, 30 mM dithiothreitol during incubation; 5, 2-mercaptoethanol was added after incubation; 6, dithiothreitol was added after incubation. Proteins were electrophoresed on a 12% polyacrylamide gel and transferred to Immobilon P membranes; the β/A4 peptide–apoE complexes were detected by anti-β/A4 peptide antibody.

immunoreactive apoE was unchanged during these incubations. These results show that both apoE3 and apoE4 bind β/A4 peptide, forming a complex that resists dissociation by boiling in SDS. Binding of β/A4 by apoE appears to require the oxidation of apoE and can be prevented or reversed by reduction with dithiothreitol or 2-mercaptoethanol. The more rapid binding of β/A4 by apoE4 than by E3 (Fig. 1) was observed in several different preparations and may be due to an increased rate of oxidation of E4 or other factors leading to differences in the oxidation of these isoforms. Whether isoform-specific differences in the sites, rates, or extent of oxidation alter the ability to bind β/A4 peptide requires study of the oxidation of apoE. Initial experiments in which apoE3 and apoE4 were first reduced with 2-mercaptoethanol and then subsequently dialyzed to remove the 2-mercaptoethanol suggest that the differences in complex formation between E3 and E4 with β/A4 are isoform specific. Differences in the rates of oxidation or in denaturation of these apoE isoforms may account for the longer duration apoE4 maintains the ability to subsequently bind β/A4.

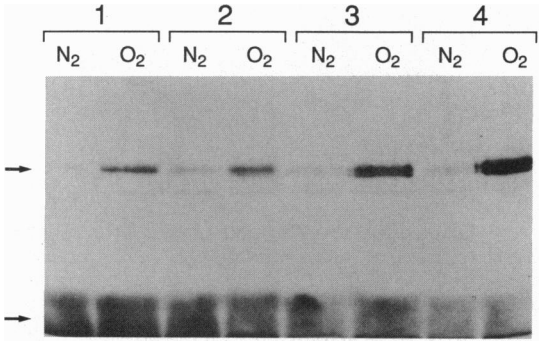

FIG. 3. Effect of O_2 and N_2 on the rate of SDS-stable binding of β/A4 by apoE3. Phosphate-buffered saline was saturated with O_2 or N_2 before incubating apoE3 with β/A4-(1–28) for 30 min (lane 1); 2 hr (lane 2); 4 hr (lane 3); 6 hr (lane 4). β/A4 peptide was detected with the anti-β/A4 peptide antibody. Upper arrow indicates β/A4-(1–28)–apoE complex; lower arrow indicates free β/A4.

The SDS-stable binding of β/A4 peptides by apoE4 and by apoE3 was dose dependent. As shown in Fig. 4, apoE3 and apoE4 bound β/A4-(1–40), β/A4-(1–28), and β/A4-(12–28) and was maximal at 10^{-4} molar peptide in all three cases, and half-maximal binding was $\approx 10^{-5}$ molar. Binding of β/A4 peptide by apoE was pH dependent (Fig. 5). Binding of β/A4 peptide by apoE3 and apoE4 was observed at pH 7.5 and decreased at lower pH. Virtually no apoE4–β/A4 peptide complex was observed at pH lower than 6.6. In contrast, apoE3–β/A4 peptide complex was still detectable at pH 4.6, suggesting isoform-specific effects.

The domain of apoE that binds β/A4 peptide was determined by examining various apoE fragments. Proteolysis of apoE by thrombin produces a 22-kDa fragment containing amino acid residues 1–191 (22). β/A4 peptide did not bind to the 22-kDa apoE3 fragment (Fig. 6). Binding of β/A4 to

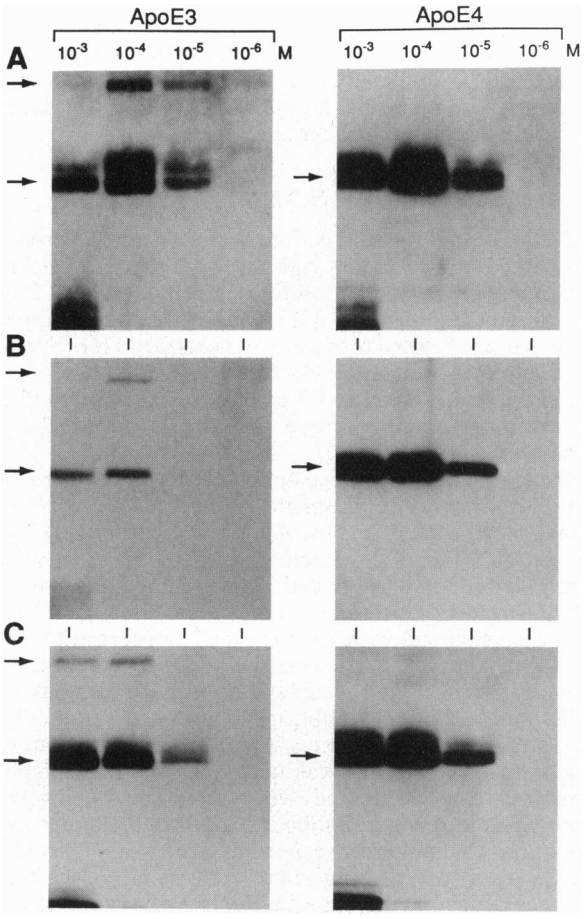

FIG. 4. SDS-stable binding of various β/A4 peptides to apoE3 and apoE4. ApoE3 and apoE4 were incubated with the indicated concentrations of β/A4-(1–40) (*A*), β/A4-(1–28) (*B*), or β/A4-(12–28) (*C*) for 5 hr. Incubation was stopped by the addition of an equal volume of 2× Laemmli buffer (without 2-mercaptoethanol) and boiled 5 min. β/A4 was detected with the anti-β/A4 peptide antibody. (*Left*) Upper arrows indicate apoE3 dimer–β/A4 complex; lower arrows indicate apoE3 monomer–β/A4 complex. (*Right*) Arrows indicate apoE4–β/A4 complex.

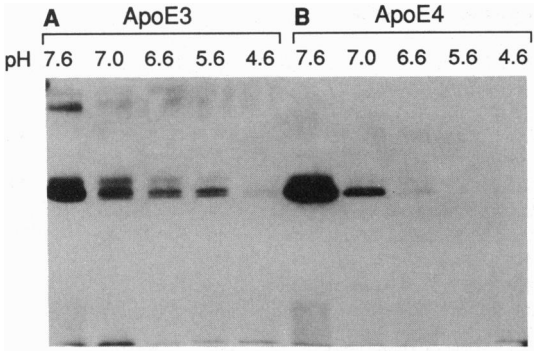

FIG. 5. pH dependence of SDS-stable β/A4 binding by apoE3 and apoE4. ApoE3 and apoE4 were incubated with β/A4-(1–28) in citric acid/Na_2HPO_4 buffer at the indicated pH for 5 hr. β/A4 peptide was detected with the anti-β/A4 peptide antibody.

recombinant-expressed truncated apoE mutants (16) was also investigated. Binding of β/A4 peptide to apoE3-(1–244) was very low or minimal. In contrast, apoE3-(1–266) did form SDS-stable β/A4 peptide complex, which was further increased with apoE-(1–272) (Fig. 6). Therefore, β/A4 binding by apoE appears to require the domain of apoE between amino acids 244 and 272 within the region previously demonstrated to mediate binding to lipoprotein particles (16).

DISCUSSION

β/A4 peptide is the primary constituent of the senile plaque and angiopathy in Alzheimer disease (23). β/A4 peptide is formed by proteolytic processing of the amyloid precursor protein, and recent studies demonstrate that it is not only produced by Alzheimer disease patients but is also produced by normal individuals and by cultured cells (24, 25). Therefore, the subsequent metabolism of the β/A4 peptide in normal and Alzheimer disease patients may be important in the mechanism of the disease. We have shown that oxidized apoE3 and apoE4 bind β/A4 peptide. β/A4-(1–40) and smaller β/A4 fragments, including β/A4-(12–28), form complexes with apoE that are stable to boiling in SDS. Noncovalent protein–protein interactions that resist dissociation by boiling in SDS are extremely rare and imply extremely avid binding. The apoE–β/A4 complex is probably noncovalent because the binding is prevented by dithiothreitol, and apoE4 contains no cysteine. Binding of β/A4 peptide by apoE appears to require the region of the carboxyl terminus previously shown as important in lipoprotein association—i.e., lipid binding.

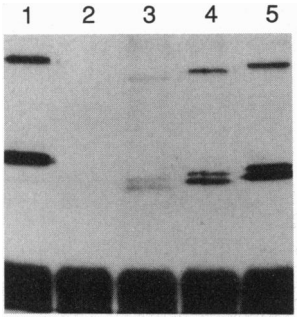

FIG. 6. SDS-stable binding of β/A4 by truncated apoE3. β/A4-(1–28) was incubated with truncated apoE3 (1 μg) for 5 hr, and the incubation was ended by boiling in Laemmli buffer (without 2-mercaptoethanol) 5 min. Lanes: 1, apoE3-(1–299) (full length); 2, apoE3-(1–191); 3, apoE3-(1–244); 4, apoE3-(1–266); 5, apoE3-(1–272). β/A4 peptide was detected with the anti-β/A4 peptide antibody.

In many organs and tissues, including the central nervous system, apoE binds and targets lipoprotein particles to cells (1). Isoform-specific interactions of apoE with the LDL receptor, and isoform-specific interactions of apoE with HDL and very high density lipoprotein particles, have profound consequences on the targeting and metabolism of cholesterol and, in part, determine an individual's susceptibility to atherosclerosis and cardiovascular disease. In certain instances, individuals homozygous for apoE2 have increased vulnerability to atherosclerosis compared with individuals homozygous for apoE3 because of reduced binding to the LDL receptor. Individuals homozygous for apoE4 may also be at increased risk for atherosclerosis due to the preference of apoE4 for triglyceride-rich lipoproteins, possibly resulting in more effective clearance that leads to a down-regulation of hepatic LDL receptors and a resulting increase in LDL levels (26, 27). Atherosclerosis also depends, in part, on posttranslational modifications of apolipoproteins. Posttranslational proteolysis or oxidation of apoB in LDL particles accelerates atherogenesis by permitting binding to an alternate LDL receptor, the acetyl-LDL or scavenger receptor, found on smooth muscle cell macrophages and the subsequent evolution of macrophages into foam cells (28).

Oxidation of other apolipoproteins (apoA-I, -A-II, -C-II, and -C-III) results in site-specific methionine sulfoxide formation and profoundly alters their ability to bind lipids (29, 30). Because apoE4 (which contains no cysteines) must be oxidized to bind β/A4 peptide, oxidation of methionine is one possibility in apoE–β/A4 binding. Oxidation of the reactive-site methionine in a_1-antitrypsin, producing methionine sulfoxide, causes a large decrease in protease inhibitor activity of a_1-antitrypsin (31) and plays a critical role in the pathogenesis of rheumatoid arthritis and emphysema. Oxidation of a_1-antitrypsin is mediated by peroxidases produced by resident macrophages and neutrophils (32). Similarly, oxidation of apoE locally in the region of brain blood vessels or senile plaques by resident microglia might be important in Alzheimer disease. Evidence for increased protein oxidation in normal aging (33, 34) and in Alzheimer disease (34) has been reported. Overexpression of a cytochrome oxidase gene has been demonstrated in Alzheimer disease brain (35). Cu/Zn superoxide dismutase was recently indentified as the locus for genetic mutations in some families with familial amyotrophic lateral sclerosis (36), as well as implicated in the neuropathology of Down syndrome (37). The possible role of apoE oxidation in these diseases needs to be explored.

The apoE4 allele appears to be a possible risk factor or susceptibility gene in late-onset familial (7) and sporadic (10) Alzheimer disease. The mechanism of disease expression may involve metabolic effects that are isoform specific. Differential posttranslational modifications of apoE isoforms may determine the differences in metabolism contributing to the pathogenesis of the disease. Oxidation of apoE may confer several isoform-specific, biochemically distinct properties. Oxidation of apoE may permit binding of β/A4 peptide to apoE, thereby altering metabolism of lipids needed for neuronal growth and survival or providing a carrier for β/A4 for intracellular transport. Because β/A4 peptide binds apoE in the lipoprotein-binding domain of the protein and does not bind apoE in the receptor-binding domain, apoE could target bound β/A4 peptide to neurons via the LDL receptor (38), the a_2-macroglobulin receptor (also referred to as the LDL receptor-related protein; refs. 39 and 40), or the very low-density lipoprotein receptor (41), all of which bind apoE. Internalization of the apoE–β/A4 peptide complex into the cell, by the same route as the apoE-containing lipoproteins, would result in incorporation into primary lysosomes and pH-dependent dissociation. Binding of β/A4 peptide by oxidized apoE4 is more sensitive to pH changes than binding

by oxidized apoE3. This isoform-specific difference in binding as well as the differences in disulfide interactions illustrate only part of the differential repertoire that could lead to disease pathogenesis. The appearance of apoE in neurofibrillary tangles (7, 8) suggests that apoE is targeted to intracellular locations in neurons. The mechanism for apoE targeting into the neuron and the formation of neurofibrillary tangles may be critical to the loss of neurons central to Alzheimer disease. Autopsied brain of homozygous *APOE4* Alzheimer disease patients have much greater Congo red angiopathy and senile-plaque staining, as well as increased β/A4 staining, than do *APOE3* homozygotes (42). This result suggests that patients with Alzheimer disease may have heterogenous pathogeneses that can be reflected in subtly different neuropathologies. These data are consistent with the view that late-onset sporadic Alzheimer disease can be viewed as a complex genetic disease, similar to the interaction of multiple contributing factors in coronary artery disease and atherosclerosis (43).

We thank Margaret Martin for excellent technical assistance. We thank Dr. Joseph C. Greenfield, Jr., for his support. This work was conducted with the support of National Institutes of Health Leadership and Excellence in Alzheimer's Disease Award 5R35 AG-07922 to A.D.R., National Institutes of Health Alzheimer's Disease Research Center 5P50 AG-05128, and numerous contributions to the Joseph and Kathleen Bryan Research Center. The L. K. Whittier Foundation, South Pasadena, CA, provided support to K.H.W.

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