Lipidation of apolipoprotein E influences its isoform-specific interaction with Alzheimer's amyloid β peptides

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The inheritance of the apolipoprotein E (apoE) ϵ4 allele is a prevailing risk factor for sporadic and familial Alzheimer's disease (AD). ApoE isoforms bind directly to Alzheimer's amyloid β (Aβ) peptides both *in vitro* and *in vivo*. Recent studies suggest that association of apoE with lipids may modulate its interaction with Aβ. We examined the binding of lipid-associated and delipidated apoE3 and apoE4 isoforms to Aβ utilizing a solid-phase binding assay and estimated the dissociation constants for the interaction of various apoE and Aβ species. Using native apoE isoforms from stably transfected RAW 264 and human embryonic kidney 293 cells, apoE3 had greater affinity than apoE4 for both Aβ1-40 and Aβ1-42. Delipidation of apoE decreased its affinity for Aβ peptides by 5–10-fold and abolished the isoform-specificity. Conversely, incorporation of apoE isoforms produced by baculovirus-infected Sf9 cells into reconstituted human high-density-lipoprotein lipoparticles restored the affinity values for Aβ peptides and resulted in preferential binding of apoE3. The data demonstrate that native lipid-associated apoE3 binds to Aβ peptides with 2–3-fold higher affinity than lipid-associated apoE4. Since the isoforms' binding efficiency correlate inversely with the risk of developing late-onset AD, the results suggest a possible involvement of apoE3 in the clearance or routing out of Aβ from the central nervous system as one of the mechanisms underlying the pathology of the disease.

Key words: Aβ, apoE isoform, dissociation constant, HDL, lipid.

INTRODUCTION

Amyloid β (Aβ) peptide is the major constituent of the fibrils deposited in senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) and related disorders. The Aβ peptide extracted from senile plaques is mainly 42–43 amino acids long (Aβ42/43), whereas the vascular amyloid consists mainly of shorter Aβ species (39–40 amino acids, Aβ39/40). Although both the short and long species self-aggregate and form fibrils *in vitro*, biochemical and immunohistochemical studies have shown that Aβ42/43 has a higher propensity for aggregation and fibrillogenesis (reviewed in [1]). Soluble Aβ, predominantly 40 amino acids long, is also normally present in plasma, cerebrospinal fluid [2–4], urine [5] and brain parenchyma [6,7]. An increased amount of soluble Aβ in AD and Down's syndrome brains precedes the appearance of Aβ deposits [8], suggesting that soluble Aβ species may be the immediate precursors of deposited Aβ fibrils. Alternatively, a particular species of soluble Aβ may be responsible for the neurotoxic effect observed *in vitro*, as well as for the neuronal loss *in vivo* not spatially correlated with fibrillar deposits [9–11].

Apolipoprotein E (apoE) co-localizes with cerebral amyloid deposits in patients with AD [12–15]. Its three major isoforms in humans (E2, E3 and E4) are products of three alleles at a single gene locus [16]. Genetic studies have shown that inheritance of apoE4 is a significant risk factor for AD [17,18], decreasing the age of onset and duration of disease [18,19]. In addition, the extent of Aβ deposition in AD patients correlates with the doses of the *APOE4* gene [20,21]. On the contrary, the ϵ2 allele may confer lower risk for the disease than ϵ3 and ϵ4. The precise mechanisms by which the apoE isoforms are involved in the development of AD still remain unclear.

The three apoE isoforms bind Aβ peptides *in vitro*, leading to the formation of complexes [18,22,23], although it is controversial whether there are differences in the binding interaction of the different isoforms with Aβ peptides. Initially, it was shown that purified (non-lipidated) apoE4 binds Aβ to form an SDS-stable complex more rapidly than apoE3 [22], although the efficiency of binding of the two apoE isoforms seemed comparable after prolonged incubations. Subsequent studies utilizing recombinant particle-associated apoE (native apoE) from human plasma and conditioned media of transfected eukaryotic cells, showed a preferential binding of apoE3 to Aβ, suggesting that the lipid association of apoE could play a relevant role in the isoform-specific interaction, probably by inducing a conformational modification in the apolipoprotein molecule [24–28]. All these studies detected the formation of complexes by SDS/PAGE followed by Western-blot analysis. This protocol provides limited information since it only detects SDS-stable apoE–Aβ complexes and does not result in quantitative estimation of the binding data. In the present report the binding of particle-associated and purified delipidated apoE3 and apoE4 to both Aβ40 and Aβ42 species was assessed by solid-phase binding assays that allow the calculation of the corresponding dissociation constants based on saturation profiles. Our results demonstrate that the affinity of native apoE3 for Aβ peptides is 2–3-fold higher than that of apoE4 and that native apoE molecules that contain lipid-associated moieties express 5–10-fold higher Aβ-binding affinity than the delipidated isoforms.

Abbreviations used: Aβ, amyloid β; AD, Alzheimer's disease; apoE, apolipoprotein E; BS3, bis(sulphosuccinimidyl) suberate; HDL, high-density lipoprotein; rHDL, reconstituted HDL; HEK, human embryonic kidney.

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EXPERIMENTAL

Synthetic peptides and proteins

Peptides DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV (Aβ40) and DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA (Aβ42), identical to residues 672–711 and 672–713 of Aβ-precursor protein 770 respectively, were synthesized at the W. M. Keck Facility at Yale University (New Haven, CT, U.S.A.) using *N*-t-butyloxycarbonyl chemistry and purified by HPLC. Aliquots of the final products were lyophilized and stored at −20 °C until use. For preparation of aggregated peptides, 50 μg of either Aβ40 or Aβ42 were dissolved in 100 μl of PBS (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl) and incubated at 37 °C for 72 h. apoE3 and apoE4 produced in Sf9 insect cells by the baculovirus expression system were purchased from PanVera (Madison, WI, U.S.A.). In all cases, protein purity was corroborated by SDS/PAGE and N-terminal sequence analysis.

apoE expressed by eukaryotic cell lines

Human apoE3 or apoE4 were expressed individually in RAW 264 mouse macrophage cells (A. T. C. C. TIB71) stably transfected with genomic DNA encoding the human apoE isoforms, and in human embryonic kidney (HEK) 293 cells (A.T.C.C. CRL1573) stably transfected with cDNA encoding the human apoE isoforms, and harvested in serum-free conditioned medium for each cell line as described in [24,25,29,30]. Concentrations of secreted apoE were determined by capture-ELISA (ApoTek ApoE; PerImmune, Rockville, MD, U.S.A.) after incubating the harvested conditioned media with 0.05 % β-octyl glucopyranoside for 1 h. Aliquots of conditioned media containing apoE3 or apoE4 were stored at 4 °C and used within 2 weeks of harvesting.

Purification of apoE from RAW-264-cell conditioned media

Polyclonal AB947 anti-apoE antibody (1 ml; Chemicon, Temecula, CA, U.S.A.) was coupled to 2 ml of CNBr-activated Sepharose 4B according to the manufacturer's instructions. Conditioned media containing apoE3 or apoE4 was loaded on to the AB947-affinity matrix. Bound apoE was eluted with 0.2 M acetic acid, pH 2.2, and immediately neutralized. The elution profile was monitored at 280 nm and the pertinent fractions were pooled and dialysed against 0.02 M Tris/HCl, pH 8.5, containing 0.1 M NaCl.

Isolation of apoE-containing particles from HEK-293-cell conditioned media

Aliquots of serum-free conditioned media from HEK-293 cells stably transfected with human apoE3 or apoE4 cDNA, in which the apolipoproteins constituted approx. 50 % of the total protein content, were concentrated 50-fold with Centricon-10 (Amicon; Millipore, Bedford, MA, U.S.A.) as described previously [24]. Particles that contained apoE3 or apoE4 were isolated from the corresponding concentrated conditioned media by FPLC using tandem Superose 6 HR10/30 columns (Pharmacia, Piscataway, NJ, U.S.A.) equilibrated in 0.02 M sodium phosphate, pH 7.4, containing 0.05 M NaCl, 0.03 % EDTA and 0.02 % sodium azide as described in [31].

Delipidation of apoE isoforms purified from conditioned media of eukaryotic cell lines and from baculovirus-transfected Sf9 cells

apoE isoforms from RAW-264 and HEK-293 cells, purified as described above, as well as apoE produced in Sf9 insect cells by the baculovirus expression system (PanVera), were delipidated in aqueous state using diethyl ether and ethanol as described in [32]. Briefly, the lipoprotein-containing samples were extracted with an equal volume of a 3:2 (v/v) diethyl ether/ethanol mixture, followed by four subsequent extractions of the aqueous phase with a 3:1 (v/v) diethyl ether/ethanol solution. After the final extraction, the remaining solvent was evaporated under a N_2 stream and the apoE concentration determined as described above.

Incorporation of apoE into reconstituted high-density lipoprotein (rHDL) particles

Extraction of total lipids from the human HDL fraction

Human HDL fractions were isolated by preparative gradient ultracentrifugation of plasma obtained from normal healthy subjects, ages 25–40, after a 10–12-h fast as described previously [33]. The HDL fractions were dialysed extensively at 4 °C against PBS containing 1 mM EDTA, and the total lipid fractions (HDL-lipid) were extracted with a mixture of chloroform and methanol (1:2, v/v) and centrifuged at 1700 ***g*** for 5 min. The supernatant was combined with chloroform and 0.88 % KCl (1:1, v/v) and centrifuged at 1700 ***g*** for 5 min. The bottom layer that contained the extracted lipids was collected, dried under a N_2 atmosphere, dissolved in chloroform and stored at −70 °C until use. The amount of total cholesterol, total triacylglycerols and phospholipids in the HDL and HDL-lipid fractions were determined enzymically with Sigma (St. Louis, MO, U.S.A.) diagnostic kits.

Preparation of reconstituted apoE-containing HDL particles

rHDL particles containing apoE were prepared as described in [34] using recombinant apoE expressed in baculovirus-infected Sf9 cells and the HDL-lipids extracted from human plasma HDL lipoparticles. In a typical experiment, the HDL-lipids (500 μg) were placed in a glass tube, dried under N_2 atmosphere, resuspended in 0.01 M Tris/HCl buffer, pH 8, containing 0.15 M NaCl (TBS) and 0.001 M EDTA. After the addition of 280 μg of sodium cholate, the suspension was incubated at 4 °C for 12 h. Subsequently, 500 μg of either Sf9 apoE3 or apoE4 was added to the reaction, incubated at 4 °C for another 12 h, and dialysed extensively at 4 °C against PBS containing 0.01 % EDTA. The fractions containing apoE incorporated into lipoparticles (apoE-rHDL) were separated from lipid-free apoE by gel-filtration chromatography using a Superose 12 column (Pharmacia) equilibrated in 0.02 M phosphate buffer, pH 7.4, containing 0.05 M NaCl, 0.03 % EDTA and 0.02 % sodium azide, at a flow rate of 0.8 ml/min. Collected fractions were analysed by native PAGE using 4–20 % Tris/glycine gels and Western-blot analysis employing a monoclonal anti-apoE antibody (3D12; BioDesign, Kennebunk, ME, U.S.A.). The fractions containing apoE-rHDL were pooled for solid-phase binding studies and the concentration of apoE in the lipoparticles was determined using the ApoTek ApoE system as described above.

Electron microscopy analysis of rHDL particles

rHDL particles containing either apoE3 or apoE4 were examined by transmission electron microscopy. Droplets (10 μl) of each of the 5-fold-concentrated apoE-rHDL fractions were placed on a Formvar carbon-coated grid and stained with 2 % sodium phosphotungstate as described in [35,36]. The shape and diameter of the lipoparticles were evaluated from enlarged prints of photomicrographs and the mean diameter was calculated from 100 particles.

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Chemical cross-linking of rHDL particles

apoE molecules reconstituted into HDL particles were cross-linked using bis(sulphosuccinimidyl) suberate (BS3) as described in [36,37]. Briefly, BS3 was added to the apoE-rHDL fraction at a concentration of 0.002 M in PBS, incubated at room temperature for 4 h, and the reaction stopped by the addition of 0.03 M Tris/HCl buffer, pH 7.4. After desalting with Microcon10 (Amicon, Millipore) and lyophilization, the cross-linked samples were separated by Tris/Tricine PAGE (10 % polyacrylamide), transferred on to an Immobilon-P membrane (Millipore) and reacted with monoclonal 3D12 anti-apoE antibody followed by horseradish-peroxidase-conjugated anti-mouse IgG. The Western blot was developed by chemiluminescence using the Super-Signal kit (Pierce, Rockford, IL, U.S.A.).

Solid-phase binding assays

The binding of apoE to Aβ species was studied by ELISA using immobilized freshly prepared (non-aggregated) or 72-h-aggregated Aβ40 and Aβ42 as described in [38] and apoE3 or apoE4 isoforms with different degrees of lipidation. Polystyrene microtitre plates (Immulon 2; Dynex Technology, Chantilly, VA, U.S.A.) were coated for 2 h at 37 °C with either fresh or aggregated Aβ40 and Aβ42 (400 ng in 100 μl of 0.1 M $NaHCO_3$, pH 9.6, per well). Under these conditions, 10 ng of fresh Aβ40, 9.6 ng of fresh Aβ42, 10.2 ng of aggregated Aβ40 and 10.9 ng of aggregated Aβ42 (representing 2.5, 2.4, 2.6 and 2.7 % of the peptide offered, respectively) were coated to the microtitre wells, as determined by a modification of the Quantigold assay (Diversified Biotech, Boston, MA, U.S.A.) for protein quantification. After blocking with Superblock (Pierce), increasing concentrations of apoE (0–150 nM in TBS; 100 μl per well) were added to the Aβ-coated wells and incubated for 3 h at 37 °C. Bound apoE was detected with monoclonal anti-apoE antibody (3D12, 1:1000) followed by alkaline-phosphatase-conjugated goat F(ab′)2 anti-mouse IgG (1:3000; BioSource International, Camarillo, CA, U.S.A.). The reaction was developed for 30 min with *p*-nitrophenyl phosphate in diethanolamine buffer (Bio-Rad, Hercules, CA, U.S.A.), and quantified at 405 nm on a Microplate Reader (Cambridge Technology, Watertown, MA, U.S.A.). For Scatchard analysis, bound apoE values were expressed in fmol with the aid of a calibration curve in which known concentrations of apoE coated to microtitre wells (as determined by Quantigold assay) were reacted with 3D12, followed by alkaline-phosphatase-conjugated F(ab′)2 anti-mouse IgG under conditions identical with those described above. Under the experimental conditions employed, an excess of apoE was reacted with solid-phase Aβ; therefore, only a small fraction of added ligand bound to the immobilized peptide and the concentration of free ligand was considered equivalent to the total apoE added. Non-linear regression analysis, Scatchard plots, estimation of dissociation constants and comparison of protein-binding data for statistical significance by calculation of the *F* ratios were assessed using GraphPad Prism software (GraphPad, San Diego, CA, U.S.A.).

RESULTS

apoE3 and apoE4 isoforms produced by baculovirus-infected Sf9 cells were incorporated into rHDL particles employing the lipids extracted from human plasma HDL fractions. The resulting lipoparticles were characterized in terms of molecular mass, particle size, number of apoE molecules per particle, and lipid content. As indicated in Figure 1, both apoE3- and apoE4-rHDL were eluted from the gel-filtration matrix at a retention time consistent with a molecular mass of approx. 230000 Da, which corresponds to the mass of HDL particles. apoE molecules not incorporated into the particles were recovered mostly in a second peak at a retention time corresponding to 135000–140000 Da and a minor peak at 35000 Da that consisted of apoE monomers and which represented approx. 10 % of the total apoE. Delipidated apoE preparations subjected to gel-filtration chromatography, under identical conditions, were eluted as a major peak at 135000–140000 Da, corresponding to the tetrameric form of the molecule, and a minor peak (10–20 %) that corresponded to the 35000 Da monomer (results not shown), in agreement with data reported previously [39] that demonstrated that apoE in aqueous solutions and in the absence of lipids self-associates and forms stable tetramers.

Electron microscopy studies revealed that apoE3- and apoE4-rHDL were spherical in shape and relatively homogeneous in size (Figure 2A), with a diameter that ranged from 7.1 to 17.9 nm for the apoE3-rHDL (mean, 11.8 nm) and from 7.0 to 18.1 nm for the apoE4-rHDL (mean, 11.9 nm). The number of apoE molecules incorporated per particle, assessed by covalent cross-linking followed by Western-blot analysis, was similar for both isoforms. Per particle, 1–4 molecules of apoE were incorporated (Figure 2B). The majority of the lipoparticles in both apoE3-rHDL and apoE4-rHDL preparations contained three molecules of apolipoprotein per particle. The lipid composition of rHDLs was 18.7 % total cholesterol, 9.0 % triacylglycerols and 72.3 % phospholipids, comparable with the plasma HDL fraction.

To study the effect that apoE lipidation exerts on the binding interaction with Aβ peptides the following apoE preparations were employed in the solid-phase assays: (i) apoE molecules

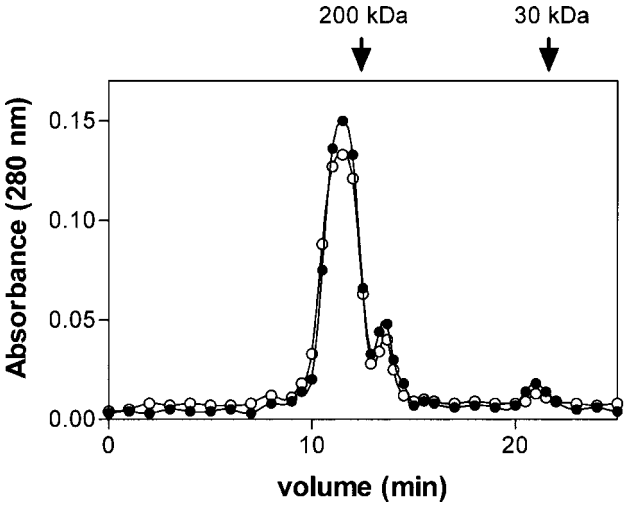

Figure 1 Purification of apoE-containing rHDL particles by gel-filtration chromatography

rHDLs were prepared by incorporating apoE3 and apoE4 produced in the baculovirus expression system into HDL particles employing the lipids extracted from human HDL fractions, as described in the Experimental section. apoE-containing rHDL particles were separated from lipid-free apoE by gel-filtration chromatography using a Superose 12 column (Pharmacia) equilibrated in 20 mM phosphate buffer, pH 7.4, containing 50 mM NaCl and 0.03 % EDTA, at a flow rate of 0.8 ml/min. The corresponding elution profiles for apoE3 (●) and apoE4 (○) were monitored by A_{280}, and the presence of apoE in the eluted fractions was corroborated by Western-blot analysis employing monoclonal anti-apoE antibody 3D12. The elution profile indicates that the apoE molecules incorporated into rHDL particles were eluted at a retention time that ranged from 9 to 12 min, corresponding to a molecular mass of 230 kDa. Lipoprotein-free apoE monomers were eluted at 22 min, a retention time that corresponded to a molecular mass of approx. 35 kDa.

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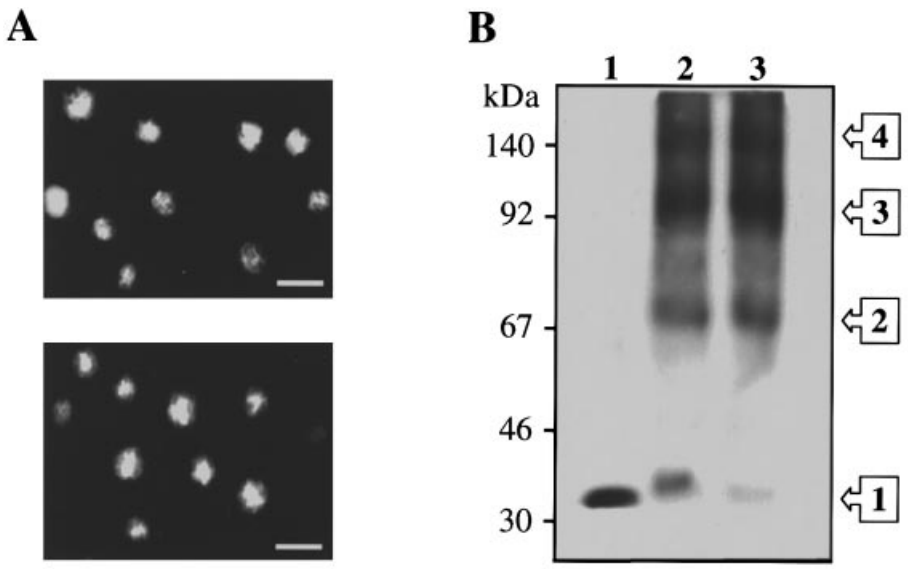

Figure 2 Biochemical characterization of apoE-containing rHDL

(**A**) Electron microscopy: rHDL particles containing apoE3 (upper panel) or apoE4 (lower panel) were placed on a Formvar carbon-coated electron microscopy grid and negatively stained with 2% sodium phosphotungstate. The lipoparticles consisted of spherical particles relatively homogeneous in size with an average diameter of 11.8 nm for apoE3, and of 11.9 nm for apoE4. The scale bar corresponds to 30 nm. (**B**) Covalent cross-linking and Western-blotting analysis of apoE molecules in rHDL particles. apoE3 or apoE4 molecules incorporated into rHDL were cross-linked for 4 h using BS3. After desalting and lyophilization, the cross-linked samples were separated by Tris/Tricine gel electrophoresis, transferred to an Immobilon P membrane and analysed by Western blotting using monoclonal 3D12 anti-apoE antibody. Visualization was carried out by chemiluminescence. Lane 1, delipidated apoE4; lane 2, apoE3 incorporated into rHDL particles; lane 3, apoE4 incorporated into rHDLs. Arrows indicate the positions of the monomers, dimers, trimers and tetramers of apoE incorporated into the lipoparticles.

produced by baculovirus-infected Sf9 insect cells, including native preparations, delipidated apoE isoforms and delipidated apoE molecules reconstituted into lipoparticles with human HDL lipids, and (ii) apoE secreted by stably transfected RAW and HEK cells, both in their native state and after removal of the lipid moieties. In all cases, increasing concentrations of the apoE3 or apoE4 isoforms with or without lipids were allowed to interact with a constant amount of immobilized Aβ peptides (Aβ40 and Aβ42) at physiological pH and temperature. The binding curves were generated by determining the amount of each apoE isoform bound to the Aβ peptides for each concentration of added apoE monomer. A dose–response relationship that reached saturation and fitted a rectangular hyperbola was obtained in each case. The dissociation constants were calculated as the half-maximal concentration of apoE monomer required for saturation, assuming that each monomer behaves as an independent entity and that there is no co-operative or synergistic interaction among the different molecules within the lipoparticle. In support of this assumption it is interesting to note that apoE molecules appear not to self-associate on lipid surfaces [40]. Scatchard plots were used to display the results more graphically and demonstrate the differences in affinity showed by the different apoE preparations. As indicated in Figure 3, removal of the lipid moieties from the different apoE preparations tested resulted in a lower binding activity for both Aβ40 and Aβ42. In addition, the lack of lipid-associated molecules translated into the loss of the isoform specificity for the interaction with Aβ peptides. A higher binding for Aβ peptides was observed for the apoE3 than for the apoE4 isoform when the lipoproteins contained lipid-associated molecules. This isoform specificity was abolished by delipidation that resulted in an overlapping of the binding curves for both apoE3 and apoE4 from stably transfected HEK (Figures 3B and 3E) and RAW cells (Figures 3C and 3F). A similar effect was observed when delipidated apoE molecules derived from Sf9 cells were used for the binding interaction (Figures 3A and 3D). Interestingly, incorporation into rHDL-like lipoparticles restored the binding activity difference between the two isoforms, with higher values obtained for apoE3. In all cases non-specific binding, determined as the binding of increasing concentrations of apoE to blocked microtitre wells that were not coated with Aβ peptides, never exceeded 8% of the specific binding.

Table 1 displays the dissociation constants estimated from the corresponding saturation curves shown in Figure 3. The lipidated apoE isoforms (E3 and E4) showed, in all cases, higher affinity for Aβ peptides than the delipidated molecules ($P < 0.01$). In addition, there was a statistically significant ($P < 0.01$) higher affinity of binding for the apoE3 isoform than for the apoE4 for both Aβ peptides in lipidated apolipoprotein preparations (RAW- and HEK-derived) and in Sf9-derived molecules upon incorporation into rHDL particles. On the contrary, in lipid-depleted isoforms the binding of apoE3 and apoE4 to all Aβ species tested did not differ significantly ($P > 0.05$), confirming that lipid association confers isoform specificity. A quantitative difference in Aβ binding was also observed for nascent apoE secreted by transfected eukaryotic cells in culture compared with apoE produced by the baculovirus expression system, with a higher affinity of binding for the native lipoparticles. The K_d values obtained for Sf9-derived apoE3 for Aβ40 and Aβ42 were 9.3 ± 0.6 nM and 13.3 ± 1.3 nM, respectively, while for apoE4 binding to Aβ40 and Aβ42 the corresponding K_d values were 10.3 ± 0.9 nM and 13.9 ± 1.4 nM. These results are in agreement with previous reports that found that native apoE molecules secreted by BHK-21 transfected cells have higher tendency to form complexes with Aβ than the baculovirus-generated apolipoproteins [28]. It is likely that the differences in Aβ-binding affinity of the apoE isoforms from various sources reflect a dissimilar degree of lipid association. In the case of BHK-21 cells, flotation

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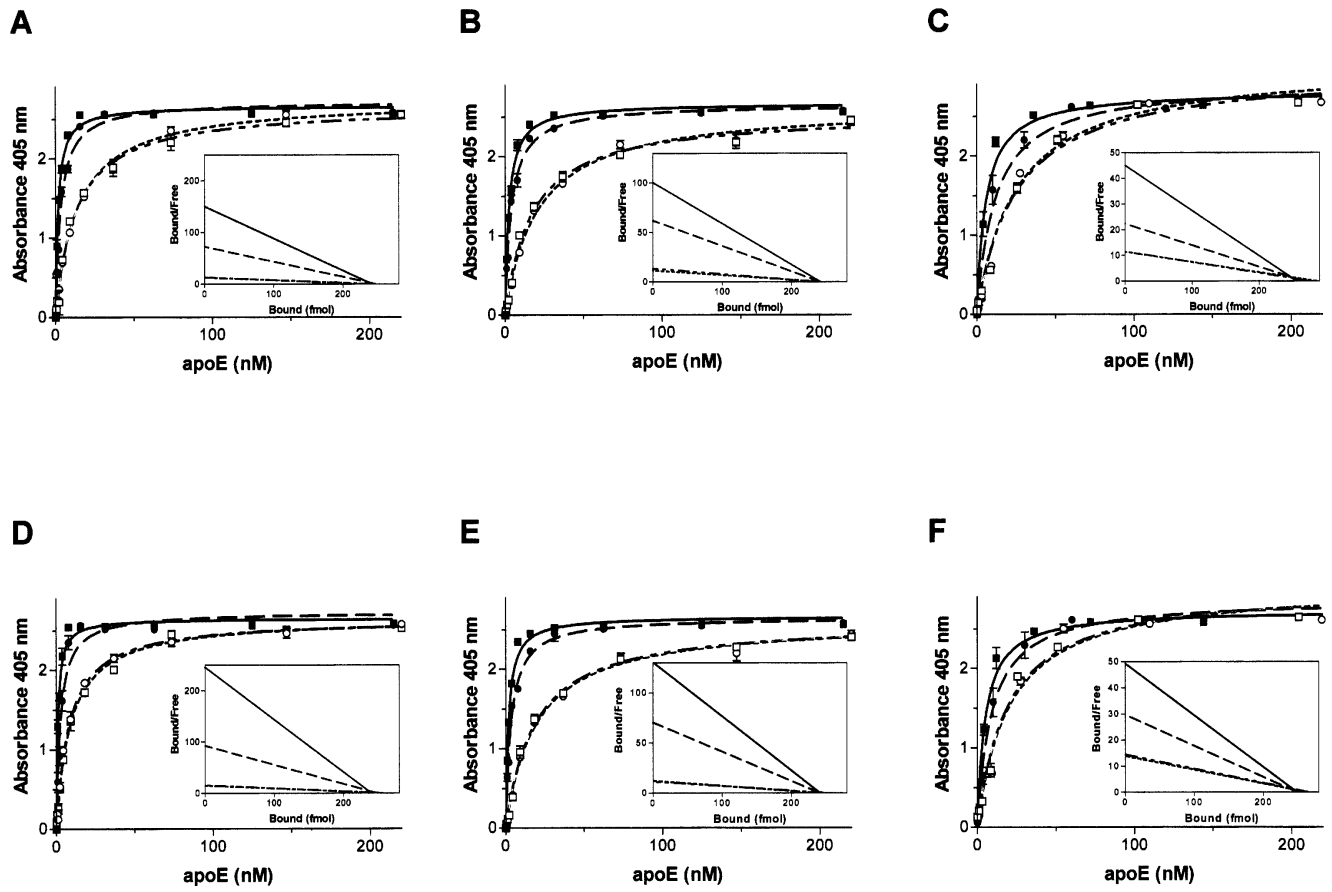

Figure 3 Binding curves for the interactions of apoE3 and apoE4 with immobilized Aβ peptides

Increasing concentrations (0–150 nM) of various apoE3 (■, □) or apoE4 (●, ○) preparations were reacted with microtitre ELISA wells coated with non-aggregated Aβ40 or Aβ42 for 3 h. Bound apoE was detected, in all cases, with monoclonal 3D12 anti-apoE antibody, followed by alkaline-phosphatase-labelled anti-mouse IgG. Insets, Scatchard transformation of the binding data. (**A**, **D**) Binding to Aβ40 and Aβ42, respectively, of Sf9-derived apoE3 and apoE4, both delipidated (□, ○) and upon incorporation into rHDLs (■, ●). (**B**, **E**) Binding to Aβ40 and Aβ42, respectively, of HEK-derived apoE3 and apoE4, both in their native HDL particles (■, ●) and following delipidation (□, ○). (**C**, **F**) Binding to Aβ40 and Aβ42, respectively, of RAW-derived apoE3 and apoE4, both in their native HDL particles (■, ●) and following delipidation (□, ○). In all cases, each point represents the mean ± S.D. of experiments performed in triplicate. ——, Lipidated apoE3; – –, lipidated apoE4; – – –, delipidated apoE3; ----, delipidated apoE4.

Table 1 Effect of lipidation in the interaction of non-aggregated Aβ40 and Aβ42 with apoE3 and apoE4

Values represent the mean dissociation constants (± S.D.; expressed in nM) of triplicate experiments. Statistical significance: lipidated versus non-lipidated, $P < 0.01$; lipidated apoE3 versus lipidated apoE4, $P < 0.01$; non-lipidated apoE3 versus non-lipidated apoE4, $P > 0.05$ (all F tests).

	Dissociation constant (nM)			
	Lipidated		Non-lipidated	
apoE (source and isoform)	Aβ40	Aβ42	Aβ40	Aβ42
Sf9 cells				
apoE3	1.6 ± 0.1	1.0 ± 0.1	22.7 ± 2.7	18.6 ± 2.7
apoE4	3.3 ± 0.3	2.7 ± 0.4	23.3 ± 3.4	22.5 ± 4.1
HEK cells				
apoE3	2.4 ± 0.2	1.8 ± 0.1	17.8 ± 3.1	18.6 ± 3.6
apoE4	3.9 ± 0.4	3.4 ± 0.3	18.2 ± 3.0	19.7 ± 3.2
RAW cells				
apoE3	5.7 ± 0.9	5.0 ± 0.7	23.9 ± 3.1	19.2 ± 2.4
apoE4	11.9 ± 2.5	9.1 ± 1.2	23.2 ± 3.3	20.3 ± 3.2

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analysis demonstrated that 45% of the secreted apoE is associated with HDL-like particles, 40% with lipid-poor particles, and 15% is found in the lipid-free fraction [41]. Removal of the associated lipids from Sf9-, RAW- and HEK-derived apoE molecules resulted in similar affinities for Aβ peptides (Table 1). Upon incorporation into rHDL particles the binding affinity of the Sf9-derived apoE isoforms reached values almost identical with those of the HEK-derived apolipoprotein (e.g. $K_d = 1.6$ nM versus 2.4 nM for the apoE3–Aβ40 and $K_d = 3.3$ nM versus 3.9 nM for the apoE4–Aβ40, respectively). In addition, the Sf9-derived apoE molecules reconstituted into HDL particles showed isoform-specific binding behaviour with a higher binding affinity for the apoE3 isoform. This contrasts with previous reports that baculovirus-produced apoE isoforms incorporated into lipoparticles generated with synthetic lipids (1-palmitoyl-2-oleoyl glycero-3-phosphocholine) showed a diminished efficiency to form complexes with Aβ peptides, although the preferential binding of apoE3 apoE4 was maintained [28].

Binding studies using immobilized aggregated Aβ peptides showed similar results to the non-aggregated species. A preferential binding for the apoE3 isoform derived from RAW cells was observed for both aggregated Aβ40 and Aβ42 (K_d values were 6.5 ± 1.1 nM and 6.7 ± 1 nM, respectively), with an approx. 3-fold higher affinity than the apoE4 isoform ($K_d = 16.5 \pm 2.7$ nM for Aβ40 and $K_d = 18.2 \pm 2.8$ nM for Aβ42). This isoform specificity disappeared following delipidation that resulted in K_d values of 30.4 ± 2.8 nM and 28.3 ± 2.8 nM for the binding to Aβ40 of apoE3 and apoE4, respectively; the corresponding constants for apoE3 and apoE4 binding to Aβ42 were 24.6 ± 3 nM and 27.6 ± 2.9 nM.

DISCUSSION

apoE is a ubiquitous protein synthesized predominantly in liver, brain, spleen, lung, adrenal gland, kidney and muscle. In the brain apoE mRNA is present mainly in astrocytes and microglia [42,43]. During synthesis, apoE is incorporated into lipoprotein particles that are involved in lipid transport and clearance through the binding to the low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) receptors, as well as to low-density lipoprotein receptor-related protein 1, apoE receptor 2 and glycoprotein 330 (also known as megalin or low-density lipoprotein receptor-related protein 2) [44]. In the central nervous system the secretion of apoE by non-neuronal cells increases dramatically following neuronal injury, and it has been suggested that apoE participates in the growth and repair of the nervous system by providing the lipid moieties necessary for membrane synthesis.

The three apoE isoforms have been demonstrated to bind Aβ peptides, resulting in the formation of SDS-stable complexes [18,22,23]. Our results provide for the first time quantitative data clarifying the isoform-specific binding of apoE to Aβ by means of the estimation of the corresponding dissociation constants. In addition, by comparing the affinity constants for the Aβ interaction of apoE from different sources, both lipid-associated and delipidated, these results demonstrate quantitatively that lipidation of apoE molecules modifies their affinity for different species of Aβ. Lipidation not only increased the affinity of both apoE3 and apoE4 for Aβ, but also accentuated the isoform differences.

Purified apoE molecules have been shown to require lipids to maintain their biological activity in most other assay systems, presumably by allowing the molecules to acquire their functional configuration in the hydrophobic lipidic environment. These apoE biological assays include such diverse functions as receptor binding, modulation of neuritic growth and antioxidant properties. It is known that association with lipids is essential for apoE binding to cellular apoE receptors [31]. Purified delipidated apoE is not a functional ligand for the apoE receptor; however, the receptor-binding activity is restored after apoE is reconstituted with phospholipid moieties [45]. Another example of lipid requirement for the expression of the biological activity of apoE is provided by primary dorsal root ganglia cultures. In this system purified apoE3 and apoE4 have no effect on neuritic growth. However, when employed in the presence of VLDLs, both apoE isoforms affect neuritic growth, with apoE3 increasing neuritic extension and decreasing branching and apoE4 decreasing both branching and extension [46]. Finally, another setting in which the lipid association affects the activity of apoE are the antioxidant and anti-cytotoxic effects indicated by apoE isoforms secreted by stably transfected RAW-246 cells that are enhanced by lipid association [30]. In summary, the association with lipid moieties seems to be a prerequisite for the expression of the diverse biological activities of apoE in their full capacity, presumably by contributing to the acquisition of an optimal three-dimensional configuration.

apoE has been related to the pathogenesis of AD. Epidemiological studies have shown that the ϵ4 allele is a risk factor for AD, although the mechanisms underlying this association remain to be defined. Substantial evidence suggests that the binding interactions between apoE and Aβ peptides may be critical in the pathogenesis of the disease by affecting the aggregation properties and/or the clearance of the Aβ peptide and thereby influencing Aβ deposition. Recent studies have provided compelling evidence for a role of apoE in Aβ deposition *in vivo*. In transgenic mice that over-express a mutant amyloid precursor protein and develop age-dependent Aβ deposition, breeding with apoE-knock-out animals (apoE$^{-/-}$) resulted in a significant decrease of Aβ deposition in comparison with animals expressing endogenous apoE [47]. In a strikingly contrasting result, it was found that the expression of human apoE3 and apoE4 isoforms at physiological levels, in amyloid precursor protein-transgenic mice with apoE$^{-/-}$ background, suppress early Aβ deposition [48]. This suggests that human apoEs have different binding affinities for Aβ peptides from the mouse isoforms, resulting in different clearance capabilities. Further studies will provide a better understanding of the Aβ catabolism/deposition pathways and the involvement of the apoE, and other chaperones in the biochemical mechanisms leading to AD. Systematic analysis of the interactions of Aβ peptides with different intra- and extracellular protein ligands, including apoE, as well as clarification of the effects that post-translational modifications exert on the various ligand interactions, may provide new insights into the molecular events leading to the pathogenesis of the disease.

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