Differential Effects of Apolipoprotein E Isoforms on Metal-Induced Aggregation of Aβ Using Physiological Concentrations[†]

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ABSTRACT: The ϵ4 allele of apolipoprotein E (*APOE*) has been found to be a risk factor for late-onset Alzheimer's disease (AD). While the pathogenic mechanism of *APOE* in AD is not yet clear, APOE isoforms appear to differentially influence the aggregation of Aβ, the principal component of Alzheimer-associated β-amyloid deposits. To date, no data are available for the propensity of Aβ to aggregate in the presence of APOE under conditions where these components are at physiological concentrations (in cerebrospinal fluid, APOE and Aβ are $\approx$100 nM and $\approx$5 nM, respectively). We employed a novel *in vitro* filtration assay for detecting zinc(II)- and copper(II)-induced aggregation of Aβ in solutions containing concentrations of the peptide that are similar to those reported for human cerebrospinal fluid. The potential for resolubilization with EDTA and the relative densities of zinc- and copper-induced Aβ aggregates were also compared. Zinc-induced Aβ aggregates were found to be denser and less easily resolubilized than copper-induced precipitates. Metal-induced aggregation of Aβ was studied in the presence of purified apolipoprotein E2, apolipoprotein E3, and apolipoprotein E4 under conditions that approximate the physiological concentrations and ratios of these proteins. In the presence of all three APOE isoforms, zinc-induced aggregation of Aβ was attenuated, while precipitation with copper was enhanced. Consistent with the increased risk for AD associated with the ϵ4 allele of *APOE*, metal-induced aggregation of Aβ was highest for both zinc and copper in the presence of apolipoprotein E4. Our data are consistent with a role for APOE as an *in vivo* molecular chaperone for Aβ.

The pathogenesis of Alzheimer's disease (AD)[1] involves the abnormal accumulation and deposition of β-amyloid in cerebral blood vessels and in the brain parenchyma as senile plaques (*1*). Brain β-amyloid deposits are insoluble aggregates of the 4 kDa Aβ protein and consist of twisted fibrils of this peptide in a β-pleated sheet conformation (*2*). The Aβ peptide is released by catabolism of a much larger transmembrane protein, the amyloid β-protein precursor (APP) (*3*, *4*). Aβ appears to be a normal product of cellular metabolism and is found as a soluble component of human cerebrospinal fluid (CSF) and plasma (*5*−*7*). Aβ contains between 39 and 43 amino acids, but the predominant species found in brain (*8*, *9*), CSF (*10*), and plasma (*11*) are Aβ_{40} (40 residues) and Aβ_{42} (42 residues). Aβ is reported to be at low (nanomolar) concentrations in CSF and plasma with Aβ_{42} one-fifth to one-tenth as abundant as Aβ_{40} (*11*, *12*).

Aβ has been reported to be toxic to cultured neuronal cells with neurotoxicity shown to be dependent on aggregation state (*13*−*16*). Soluble monomeric species of Aβ are relatively nontoxic as compared to fibrillar Aβ. The transition from monomeric to fibrillar Aβ is accompanied by a change in secondary structure of the protein from random coil and α-helical to β-sheet (*15*, *17*−*20*). In simple aqueous buffers, a significant proportion of a monomeric micromolar solution of Aβ will aggregate into fibrils if left to 'age' (stand for days or weeks) (*19*). Nucleation is the rate-limiting step in spontaneous β-protein amyloid fibril formation (*21*). We and others have previously demonstrated *in vitro* that rapid aggregation of Aβ can be induced by the addition of physiological concentrations of zinc (*22*−*24*) and copper (*25*). Zinc has also been shown to induce aggregation of endogenous Aβ in canine CSF (*26*). Fibril formation by 'aging' requires micromolar concentrations of Aβ. In contrast, zinc and copper induce aggregation at Aβ concentrations reported for biological fluids (low nanomolar range). Zinc-induced Aβ aggregates also stain with Congo Red and exhibit positive birefringence under polarized light, a hallmark of AD amyloid (*23*).

Several studies have reported that zinc (*27*−*29*) and copper (*30*) homeostasis is altered in AD. Recently it has been demonstrated that the normally high concentrations of copper, zinc, and iron in brain parenchyma are further elevated in AD patients (*31*). In addition, a recent study reports that

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[1] Abbreviations: AD, Alzheimer's disease; APP, amyloid β-protein precursor; APOE, apolipoprotein E; α_2M, α_2-macroglobulin; mAb, monoclonal antibody; CSF, human cerebrospinal fluid.

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copper and zinc are also significantly elevated in the serum of AD patients that carry an ϵ4 allele for apolipoprotein E (APOE) (*32*).

Genetic studies have shown that inheritance of the ϵ4 allele of APOE is a risk factor for AD (*33*, *34*) particularly in cases with onset between 61 and 70 years (*35*). APOE normally mediates the clearance of plasma lipoproteins (*36*) and in humans has three major allelic variants, ϵ2 (8%), ϵ3 (77%), and ϵ4 (15%) (*37*) encoding for the protein isoforms APOE2, APOE3, and APOE4, respectively. In addition to data from family-based association studies for AD, a role for APOE in amyloidosis is supported by the observation that the ϵ4 allele is also associated with an increased risk for cerebral amyloid angiopathy (*38*) and inclusion body myositis (*39*), two other Aβ-amyloid diseases. It has also been demonstrated that in AD brains, APOE is concentrated in amyloid deposits and that Aβ deposition increases with ϵ4 dosage (*40*−*46*). The mechanism by which this increased risk is conferred remains unclear. APOE has been shown to bind with Aβ *in vitro* (*33*, *47*−*57*) in CSF (*58*) and in brain (*45*, *59*) and may have a role as an *in vivo* molecular chaperone for the peptide (*40*). Consistent with this model, APOE can also modify Aβ aggregation *in vitro*, although the direction of the effect remains controversial. Investigators have reported both an acceleration (*48*, *60*−*63*) and an inhibition (*21*, *64*, *65*) of fibril formation in the presence of APOE relative to Aβ alone. In a recent study, APOE knockout mice ($APOE^-$) were crossed with human mutant APP transgenic mice ($PDAPP^+$) that normally develop cerebral amyloid deposits. While amyloid deposition was reduced in $APOE^-/PDAPP^+$ mice, soluble Aβ levels remained unaffected (*66*). The finding of this *in vivo* study is also concordant with a direct role for APOE in facilitating amyloidogenic Aβ/Aβ interactions.

APOE/Aβ aggregation studies performed to date have employed micro- or millimolar concentrations of Aβ (3 or 4 orders of magnitude above physiological), 100−1000-fold in excess of APOE levels (*21*, *48*, *60*−*65*). Importantly, in biological fluids, APOE is in excess compared to Aβ (in CSF, the APOE:Aβ ratio is between 20:1 and 40:1). To date, no data are available for the propensity of Aβ to aggregate in the presence of APOE under conditions where these components approach physiological concentrations. Observations on the effect of APOE on fibril formation can be profoundly affected by experimental concentrations of both Aβ and APOE (*64*). We have developed a novel *in vitro* assay for detecting zinc(II)- and copper(II)-induced aggregation in solutions containing Aβ at concentrations that approach physiological (low nanomolar). Using this assay, we find that attenuation or promotion of Aβ aggregation by native APOE is dependent on the metal used to induce precipitation. Consistent with this finding, comparisons of the properties of zinc- and copper-induced Aβ aggregates suggested that the two precipitates have distinct properties. Notably, different isoforms of APOE were found to differentially modify the propensity of Aβ to aggregate in the presence of metals following the order: APOE4 > APOE3 $\approx$ APOE2. Overall, our findings are consistent with a role for APOE as an *in vivo* chaperone for the Aβ peptide.

EXPERIMENTAL PROCEDURES

Materials. Electrophoretic molecular weight markers were purchased from Amersham (Buckinghamshire, U.K.). Electrophoresis reagents were from Bio-Rad (Richmond, CA), precast acrylamide Tricine gels were from Novex (San Diego, CA), and poly(vinylidene difluoride) (PVDF) membrane was from Millipore Corp. (Bedford, MA). Bicinchoninic acid (BCA) protein assay reagent and Stable Peroxide Substrate Buffer containing 1 mg/mL 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB/SPS buffer) and the ELIFA apparatus were from Pierce (Rockford, IL). Zn(II)-coated 96-well plates were purchased from Xenopore Corp. (Saddle Brook, NJ). Sheets of cellulose acetate filtration membrane (0.22 μm) used with the ELIFA were from MSI (Westboro, MA) and cellulose acetate spin filters from Costar (Cambridge, MA). The monoclonal antibody (mAb) 6E10 recognizes an epitope near the amino terminus of Aβ and was purchased from Senetek (Maryland Heights, MI). Nycodenz was from Nyocomed Pharma As (Oslo, Norway). MAb MAB1062 was purchased from Chemicon International (Temecula, CA).

Human $A\beta_{40}$ peptide was synthesized, purified, and characterized by HPLC analysis, amino acid analysis, and mass spectroscopy by W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). APOE isoforms were purified without organic solvents under nondenaturing conditions from the conditioned media of cells stably transfected with human APOE2, APOE3, or APOE4 according to the method of Miyata and Smith (1996) (*67*).

Preparation of Aβ Solutions and Experimental Buffers. Prior to use, all buffers and stock solutions of metal ions were filtered through a 0.22 μm filter to remove particulate matter. Aβ solutions were prepared and incubated in presiliconized polypropylene 1.7 mL microcentrifuge tubes from National Scientific Supply Co. (San Rafael, CA). Nonspecific loss of Aβ to the walls of theses tubes was <15% (data not shown). Stock Aβ solutions were prepared by the addition of HPLC-grade water to the powdered peptide to a final concentration of between 800 and 1000 μg/mL. Undissolved peptide was solubilized by light-sonication of stock solutions in an ultrasonic water bath for 3 min. Following sonication, the stock Aβ solution was centrifuged through a cellulose acetate membrane spin filter (0.1 μm) and the filtrate assayed by BCA. Working concentrations of Aβ were prepared by serial dilution of the filtrate in TBS. Stock Aβ solutions were prepared fresh on the day of experimentation.

Aβ Incubations and Filtration Assay. Preassay incubations of Aβ were carried out for 30 min at 37 °C in 1.7 mL siliconized tubes. Incubations were performed in TBS containing 50 μg/mL BSA as a nonspecific blocking agent. For incubations under acidic conditions, 100 mM MES, pH 5.5, was added to the TBS buffer. For assay, samples were transferred to the wells of an ELIFA apparatus (200 μL/well) containing a clamped 0.22 μm pore cellulose acetate filtration membrane. Samples were filtered by vacuum at $\approx$100 μL/min per well. Wells were then incubated for 15 min with 200 μL of 0.1% glutaraldehyde in TBS to fix retained Aβ aggregate to the filter membrane. The filtration membrane was removed from the ELIFA apparatus and washed (×5) with TBS buffer. Subsequent immunodetection of Aβ fixed on the filtration membranes was the same as described for PVDF Western blots.

Immunoblotting and Electrophoresis. Samples were subjected to electrophoresis on SDS−PAGE (10−20% Tricine gels) and blotted to PVDF membrane. Membranes from

SDS−PAGE or the filtration assay were blocked for 1 h with BSA (3%) prior to incubation with mAb 6E10 (1:3000) for 3 h. Following a 2 h incubation with anti-mouse IgG coupled to horseradish peroxidase (1:10 000), blots were developed for exposure to enhanced chemiluminescence (ECL) film. Quantitation of the Aβ signal on the ECL film was determined by transmittance analysis as described by Bush et al. (1992) (*68*). All incubations were carried out at room temperature.

Aβ/APOE Binding Assay. The wells of a Zn(II)-coated 96-well plate (Xenopore Corp.) were preincubated with TBS containing 0, 0.5, 1, 2, or 4 ng of Aβ. The wells were then blocked by the addition of 200 μL/well of TBS containing 1% BSA. Wells were incubated with 100 nM APOE3 in blocking buffer. MAb MAB1062 in blocking buffer (1:200) was added to each well, followed by a further incubation with anti-mouse IgG coupled to horseradish peroxidase (1:10 000). Bound antibodies were detected by incubation for 60 min with TMB/SPS buffer, addition of 2 M sulfuric acid (5 μL), and measurement of absorbance at 450 nm. All incubations were for 2 h at room temperature in a total volume of 50 μL unless otherwise indicated. Between incubations the plate was washed ×5 with TBS (200 μL/well per wash).

Nycodenz Cushion Centrifugation of Aβ Aggregates. The sample (100 μL) was carefully layered on top of a Nycodenz cushion (1 mL) prepared in siliconized 1.7 mL tubes. Except for the presence of Nycodenz in the bottom layer, buffer conditions and components remained constant for the sample and Nycodenz cushion. The tube was centrifuged at 16000*g* for 10 min in a fixed-angle rotor. Following removal from the Microfuge, the tube was place at −170 °C for 1 h. The bottom tip of the tube was then removed with clipping shears (bottom 7 mm containing pelleted material and ≈100 μL of frozen Nycodenz solution). After thawing, SDS sample buffer (×4) containing 10 mM EDTA was added (25 μL) to the severed tube bottom and vigorously mixed. An aliquot (20 μL) was then subjected to electrophoresis on SDS−PAGE and Western-blotted with the anti-Aβ mAb 6E10.

RESULTS

A novel assay was developed for detecting Aβ aggregation in simple solutions. The assay employed a 0.22 μm pore size cellulose acetate membrane clamped in a 96-well format ELIFA apparatus. Samples for assay were transferred to the ELIFA wells and drawn through the filter membrane by the application of vacuum. The retained Aβ aggregate was fixed to the membrane by incubation with glutaraldehyde and then detected immunochemically using an anti-Aβ monoclonal antibody (mAb 6E10) and ECL Western blotting techniques. Aβ signal was quantified by transmittance analysis of ECL film according to the method of Bush et al. (1992) (*68*).

Initial experiments were directed at characterizing the Aβ aggregates using the filtration assay. Increasing concentrations of $Aβ_{40}$ incubated in TBS alone or with 25 μM $ZnCl_2$, $CuCl_2$, or EDTA were assayed for amount of aggregate. Signal was observed to increase linearly with increasing peptide concentrations for both copper- and zinc-induced aggregation. Under the conditions used, zinc and copper induced the aggregation of Aβ in solutions containing peptide concentrations in the low nanomolar range (Figure 1). Signal strength was greatly reduced in the absence of metals and further reduced by the addition of EDTA. In additional experiments, the Aβ signal was compared in samples before and after filtration. Collected filtrates and corresponding samples put aside before filtration were drawn through a PVDF membrane clamped in the ELIFA apparatus. The PVDF membrane is a strong protein binding material that will immobilize both soluble and aggregated Aβ. Comparison of the relative amounts of mAb 6E10 immunoreactive material captured by the PVDF membrane shows that the

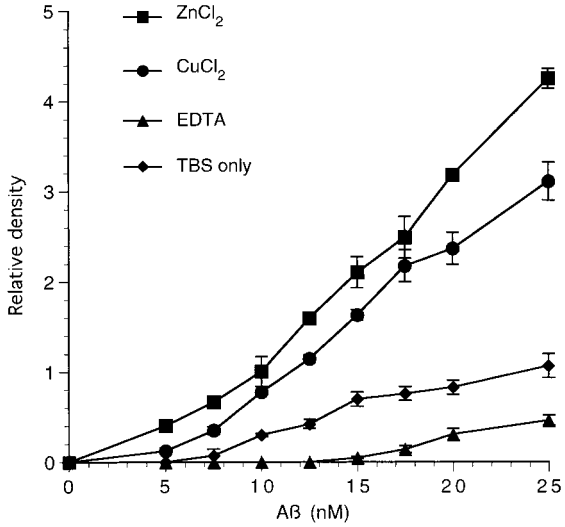

FIGURE 1: Sensitivity of Aβ aggregation filtration assay. Increasing concentrations of $Aβ_{40}$ (200 μL volume) were incubated (30 min) in TBS alone or with 25 μM $ZnCl_2$, $CuCl_2$, or EDTA. Samples were then filtered through a cellulose acetate membrane (0.22 μm) clamped in a 96-well format ELIFA apparatus. Following fixing with glutaraldehyde, the membrane was probed with anti-Aβ/APP mAb 6E10 and developed for exposure to ECL film. The figure shows the relative signal strength as determined by transmittance analysis of the ECL film. All samples were assayed in triplicate, and average values are shown ± standard error.

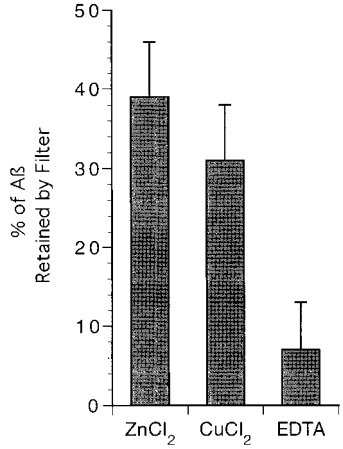

FIGURE 2: Aβ signal before and after filtration through cellulose acetate membrane. $Aβ_{40}$ (50 nM) was incubated (30 min) in TBS with 25 μM $ZnCl_2$, $CuCl_2$, or EDTA. Half of each sample was filtered through a cellulose acetate membrane (0.22 μm) clamped in an ELIFA apparatus and the filtrate collected. Unfiltered and filtered samples were then drawn through a second PVDF membrane clamped in the ELIFA apparatus. The PVDF membrane was probed with mAb 6E10 and developed for exposure to ECL film. Signal strengths were determined by transmittance analysis of the ECL films. The figure shows Aβ retained by the filter as a percentage of signal in unfiltered material for each sample. All samples were assayed in triplicate, and average values are shown ± standard error.

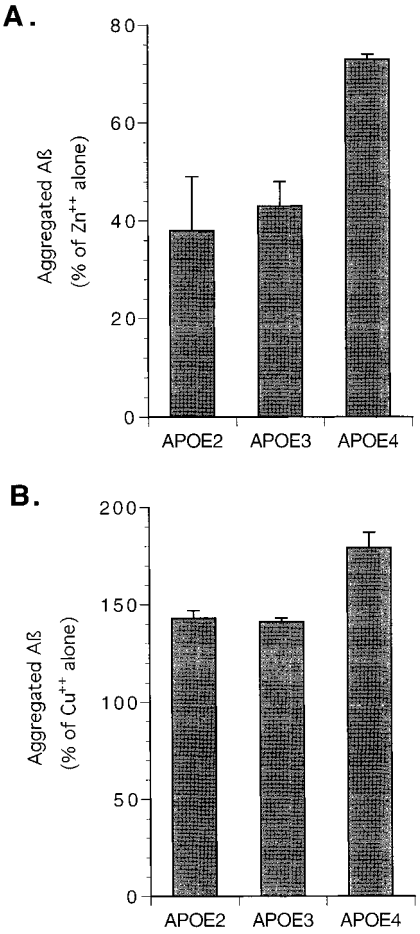

FIGURE 3: Metal-induced Aβ aggregation in the presence of APOE2, APOE3, or APOE4. Samples containing 8 nM Aβ_{40} were preincubated for 30 min at 37 °C in TBS containing 2.5 μM BSA and in the presence of 150 nM APOE2, APOE3, or APOE4. Twenty-five micromolar $ZnCl_2$ (panel A) or $CuCl_2$ (panel B) was added, and the samples were incubated a second time. Incubants were then assayed for precipitated peptide using the Aβ aggregation filtration assay. Aggregation in the presence of the APOE forms is shown as a percentage of precipitation with metal alone. All samples were assayed in triplicate, and averages are shown ± standard error.

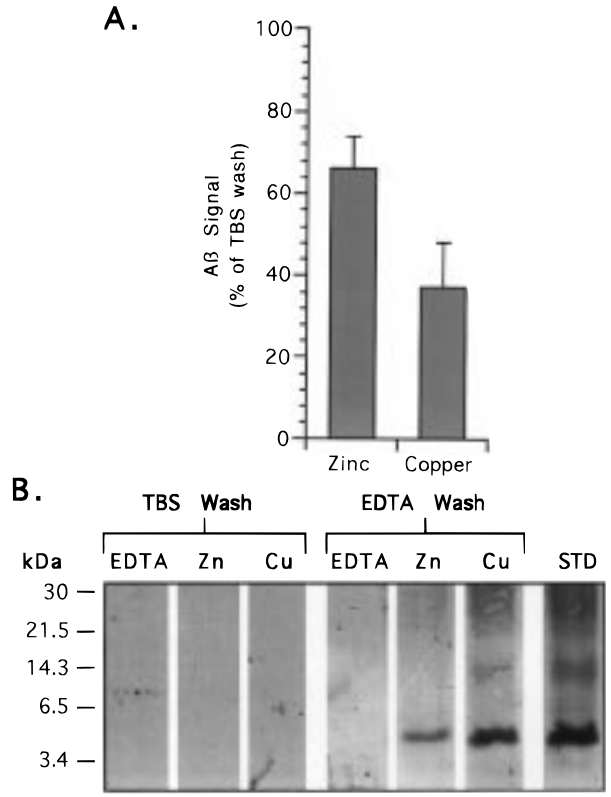

FIGURE 4: Resistance of zinc- and copper-induced Aβ aggregates to resolubilization by EDTA. Aβ_{40} (50 nM) was preincubated in TBS with 25 μM $ZnCl_2$ or $CuCl_2$ and then filtered through a cellulose acetate membrane (0.22 μm) clamped in an ELIFA apparatus (200 μL/well). Wells were washed (200 μL/well) with TBS without or containing EDTA (2 μM), and the filtrate was collected. The membrane was fixed, probed with the anti-Aβ/APP mAb 6E10, and developed for exposure to ECL film. Relative signal on membranes was determined by transmittance analysis of the ECL film. Panel A shows relative Aβ signal on the filtration membrane following washing with EDTA. All samples were assayed in triplicate, and values are expressed as a percentage of the Aβ signal after washing with TBS alone (±standard error). For panel B, the experiment was repeated using 200 nM Aβ and with collection of the sample filtrates. The filtrates and a Aβ standard were lyophilized and then Western-blotted and probed with mAb 6E10.

amount of Aβ removed by filtration following incubation with zinc or copper was 39% and 31%, respectively (Figure 2). The average amount of Aβ (from 50 and 100 nM Aβ_{40} solutions) retained by filtration after incubation with EDTA was 7%, indicating low nonspecific binding to cellulose acetate filtration membranes. These results are consistent with the metal-induced aggregation of Aβ that we previously reported for solutions containing micromolar concentrations of the peptide (*23*, *24*).

The filtration assay was next used to investigate metal-induced Aβ aggregation in the presence of APOE. Experiments used concentrations of both Aβ and APOE that approach those reported for CSF. Aβ_{40} (8 nM) was preincubated with 150 nM APOE2, APOE3, or APOE4. Aggregation of Aβ was induced by the addition of 25 μM zinc, copper, or EDTA. Figure 3 shows the results for Aβ aggregation in the presence of the APOE isoforms relative to metal alone. All three APOE isoforms attenuated zinc-induced Aβ aggregation but enhanced the peptide's propensity to aggregate in the presence of copper. Consistent with the increased risk for AD associated with the ϵ4 allele, Aβ aggregation was most robust for both metals in the presence of APOE4. It should also be noted that metal chelation by APOE is unlikely to significantly affect free zinc or copper concentrations in our experiments since both metals were in excess by 2 orders of magnitude.

Results from the experiments assessing the effects of APOE isoforms on Aβ precipitation suggest that zinc and copper may induce peptide aggregation via different mechanisms. To explore this possibility, we carried out experiments to compare the physiochemical properties of zinc- and copper-induced Aβ aggregates. In the first experiment, the aggregates were compared for the ability to be resolubilized by EDTA. Zinc- or copper-induced Aβ aggregates (50 or 200 nM Aβ_{40} incubated for 30 min with 25 μM zinc or copper) were first captured by filtration through a 0.22 μm filter membrane clamped in an ELIFA apparatus. The aggregates were then washed with 200 μL of TBS with or without 2 μM EDTA, and the filtrates collected. The membrane was removed, fixed, and probed with mAb 6E10. Collected filtrates from the 200 nM Aβ samples were lyophilized, electrophoresed, and Western-blotted with mAb 6E10. The Western blot included an Aβ standard prepared

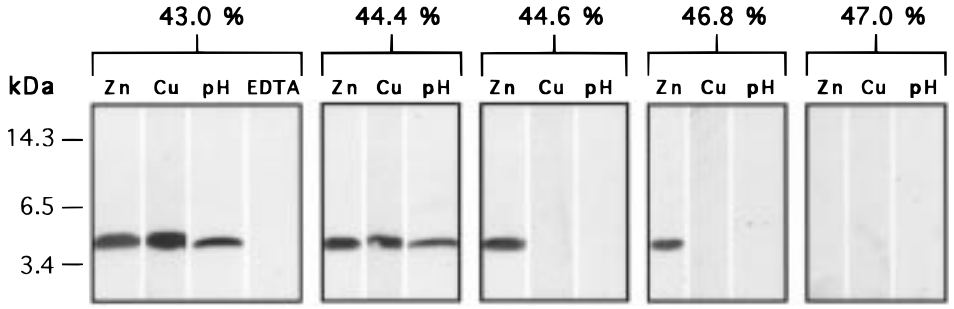

FIGURE 5: Sedimentation analysis of copper-, zinc-, and pH-induced Aβ aggregates using Nycodenz cushion centrifugation. $A\beta_{40}$ (2 μM) was preincubated in TBS with 25 μM $ZnCl_2$, $CuCl_2$, or EDTA or 100 mM MES buffer, pH 5.5. Following incubation, 100 μL aliquots of the Aβ samples were layered on top of 43, 44.4, 44.6, 46.8, or 47% solutions of Nycodenz (1 mL). The tubes were centrifuged, and pelleted material was resuspended in SDS sample buffer containing EDTA (10 mM). The resuspended pellets were Western-blotted and probed with anti-Aβ/APP mAb 6E10.

from powdered $A\beta_{40}$. While approximately two-thirds of the copper-induced Aβ aggregates were resolubilized by EDTA, only one-third of the peptide precipitated by zinc was dissolved by the metal chelator (Figure 4A). MAb 6E10 Western blots of resolubilized Aβ from both zinc- and copper-induced aggregates are shown in Figure 4B. It should be noted that while only monomeric $A\beta_{40}$ was detected following a 30 min incubation with copper, apparent dimeric and trimeric species are observed on SDS−PAGE immunoblots following longer incubation times (>12 h) (data not shown).

The second experiment compared the density of zinc- and copper-induced Aβ aggregates using Nycodenz cushion centrifugation. Acidic conditions have been reported to induce precipitation of Aβ from solutions containing micromolar concentrations of the peptide. As a positive control, pH-induced aggregation was also examined. $A\beta_{40}$ (2 μM) was preincubated in TBS with 25 μM zinc, copper, or EDTA, or in buffer adjusted to pH 5.5 by the addition of 100 mM MES. Aliquots of the incubated material were then layered on top of 43.0, 44.4, 44.6, 46.8, or 47.0% solutions of Nycodenz. Samples were centrifuged, and the pelleted material was Western-blotted using mAb 6E10 (Figure 5). Copper- and pH-induced Aβ aggregates did not pellet at Nycodenz concentrations greater than 44.4%. However, the zinc-induced Aβ aggregates could be pelleted through Nycodenz concentrations of up to 46.6%. Collectively, these data are consistent with copper and zinc inducing structurally distinct aggregates of Aβ and help to explain the differential effects of APOE on aggregation in the presence of copper versus zinc.

Binding between Aβ and APOE was investigated next by employing a solid-phase ELISA. The concentrations of Aβ and APOE used in this experiment approach those found physiologically. A Zn(II)-coated 96-well microtiter plate was first used to immobilized increasing amounts of $A\beta_{40}$ (0, 0.5, 1, 2, or 4 ng per well). The plate wells were then incubated with APOE3 (100 nM). Bound APOE3 was detected with mAb MAB1062 (anti-APOE antibody). Signals in wells containing immobilized Aβ were elevated relative to those that received no preincubation with Aβ peptide (Figure 6). Furthermore, signal strength for bound APOE increased with the amount of immobilized Aβ. These observations suggest that Aβ and APOE bind at physiologically relevant concentrations. At present, the sensitivity of this assay is insufficient for reliable comparison of Aβ binding between APOE isoforms.

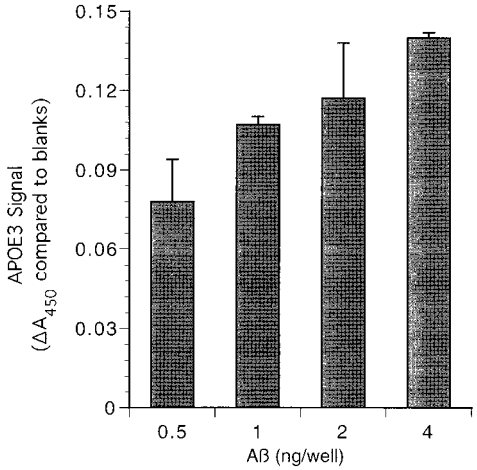

FIGURE 6: Capture of APOE3 by immobilized Aβ. The wells of a Zn(II)-coated 96-well microtiter plate were preincubated with 0, 0.5, 1, 2, or 4 ng of $A\beta_{40}$ in TBS (50 μL/well). Unbound sites on the well surfaces were then blocked by incubation with TBS containing 1% BSA. The wells were incubated with blocking buffer containing 100 nM APOE3. Anti-APOE mAb MAB1062 was added to the wells followed by anti-mouse horseradish peroxidase conjugate. The plate was developed with TMB/SPS buffer and absorbance measured at 450 nm. The figure shows the increase in absorbance compared to blanks (wells not preincubated with Aβ). All assays were performed at room temperature in triplicate, and values are shown ± standard error.

DISCUSSION

The role of APOE in AD pathology remains unresolved. However, observations that APOE binds Aβ (*33*, *40*) have lead to the hypothesis that APOE acts as a chaperone for Aβ. In this hypothesis, APOE isoform-specific differences between APOE/Aβ complexes are proposed to lead directly to differential Aβ/Aβ interactions with regard to amylodogenicity (*40*). Consistent with this hypothesis, several studies have reported that APOE isoforms differentially affect *in vitro* fibril formation (*21*, *48*, *60*−*65*). However, previous aggregation experiments have used nonphysiological Aβ concentrations 2 or more orders of magnitude in excess of APOE levels. We have developed a novel high-sensitivity assay for monitoring aggregation of nanogram amounts of Aβ. Using this assay, we have been able to test aggregation under conditions where APOE levels are 1 order of magnitude in excess of Aβ, thereby simulating physiological concentrations of these proteins.

Characterization studies showed the novel filtration assay to have high precision, with signal increasing linearly with Aβ levels. Analysis of assay filtrates showed 30−40% of Aβ normally aggregates in the presence of 25 μM zinc or copper. The proportion of Aβ that aggregates with a given amount of metal also remained constant over the peptide concentration range we tested. Together with findings from similar previous studies (*25*) that employed micromolar concentrations of peptide, our data suggest that for a given amount of metal, the ratio of aggregate to soluble species is relatively insensitive to total Aβ concentration over several orders of magnitude.

Aβ binds within the N-terminal 191 amino acids of APOE (*21*) with a 1:1 stoichiometry (*58*, *62*). Dissociation constants of 10 nM (*53*) and 20 nM (*51*) have been reported for the interaction. Our experiments using a novel solid-phase ELISA confirmed that Aβ and APOE bind at physiologically relevant concentrations. APOE was also observed to affect Aβ aggregation in the presence of metals with inhibition of zinc-induced and enhancement of copper-induced precipitation. The binding of APOE has previously been reported to induce a shift in Aβ conformation toward a β-sheet structure (*63*). Acidic conditions that induce a β-sheet conformation in Aβ (*69*) also favor copper-induced aggregation of the peptide (*25*). In contrast, zinc-induced Aβ aggregation is enhanced by α-helical-promoting solvents (*70*). Our data may be explained by a model in which APOE binding induces a β-sheet confirmation in the peptide that can attenuate zinc-induced aggregation and potentiate copper-induced precipitation. APOE may also modulate metal-induced Aβ aggregation by changing the affinity of the peptide for zinc or copper.

Our findings reveal a differential effect of the three APOE isoforms on Aβ aggregation. Aβ aggregation was most robust in the presence of APOE4 for both zinc- and copper-induced precipitation. This finding is consistent with the increased risk for AD and greater β-amyloid burden associated with the ϵ4 allele, and is also concordant with previous reports of the effects of APOE on aggregation of 'aged' Aβ (*21*, *48*, *60*−*65*). The differential effect of APOE2, APOE3, and APOE4 on fibrillogenesis has been attributed to APOE isoforms having distinct Aβ binding avidity. Data from several studies appear to be consistent with this model. In these studies, incubation of micromolar Aβ with APOE2, APOE3, or APOE4 gave different yields of SDS-stable APOE/Aβ complexes. Yields of APOE4/Aβ complex have been reported to be either greater (*48*, *55*) or reduced (*49*, *52*, *54*, *56*, *57*, *71*) relative to APOE3/Aβ and APOE2/Aβ complexes. However, these previous studies used the formation of SDS-resistant APOE/Aβ complexes as a measure of binding affinity between APOE and Aβ, which may not be the most accurate measure of differential binding between APOE isoforms and Aβ. A recent study has shown that APOE4 has less antioxidant activity than APOE3 or APOE2 (*67*). The formation of SDS-stable APOE/Aβ complexes has also been shown to be dependent on oxidative modification (*49*, *55*). Thus, the differential formation of SDS-stable APOE/Aβ complexes with APOE isoforms may be due to differences in the resistance of variants to oxidative cross-linking and not to discrete binding affinities. Such a model is consistent with the equivalent dissociation constants observed for Aβ and APOE2, APOE3, and APOE4 (*51*) and the similar yields of APOE2/Aβ, APOE3/Aβ, and APOE4/Aβ complexes reported for nondenaturing incubation conditions (*50*). Synthetic Aβ at micromolar concentrations can generate free radicals (*72*) and induce lipid peroxidation of synaptosomes (*73*). Our laboratory has also recently found that Aβ has strong oxidative activity in the presence of copper and iron (personal communication, Atwood and Huang). The concentrations of Aβ used in these previous experiments are likely to generate oxidative conditions that promote cross-linking reactions.

As previously discussed, modulation of metal-induced Aβ aggregation by APOE may be caused by changes in Aβ's affinity for copper and zinc. It is possible that the differential effects of APOE2, APOE3, and APOE4 may also be caused by isoform-specific metal interactions of APOE/Aβ complexes rather than preferential binding between Aβ and APOE variants.

The concentrations of copper, zinc, and iron in brain parenchyma (70 μM, 350 μM, and 340 μM, respectively) have been reported to be elevated in AD patients, reaching concentrations of 300 μM, 800 μM, and 700 μM, respectively (*31*). These metal ions are further concentrated within the core and periphery of β-amyloid plaques. A recent study has reported that copper is also significantly elevated in the serum of AD patients that carry an ϵ4 allele for APOE (*32*). Our data show that copper-induced Aβ aggregation is potentiated by APOE4. It is possible that elevated copper levels lead to greater metal-induced Aβ deposition in APOE4 cases than for ϵ2 or ϵ3 carriers. Thus, a combination of APOE4 and elevated copper levels may increase Aβ deposition. In any case, these recent finding are consistent with a role for metal-induced Aβ aggregation in AD patients with an ϵ4 allele for APOE.

It is unclear how the structural differences between APOE isoforms may influence the interactions of APOE/Aβ complexes with metals. APOE has been reported to bind copper and zinc as well as iron (both ferrous and ferric) (*67*). Although the metal binding region of APOE has yet to be determined, the four-helix bundle of the N-terminus may allow a tetrahedral coordination of metal ions (*67*). Residues 12−28 of Aβ have been identified as a possible APOE binding region (*51*). This portion of Aβ also includes the zinc binding domain (*23*) and is important for β-sheet formation (*17*, *74*). If APOE does mediate metal-induced aggregation by modulating Aβ/metal binding, then possible mechanisms for isoform-specific effects include differences in the ease with which free metals access Aβ bound in APOE/Aβ complexes, differences in bound Aβ's proximity/availability to zinc and copper complexed to APOE, or differences in the conformational changes in Aβ induced by APOE binding. One important structural difference between APOE variants that may prove important for isoform-specific effects is that, unlike APOE2 and APOE3, APOE4 does not form disulfide dimers (*57*).

In our experiments, zinc(II) or copper(II) was used to induce $A\beta_{40}$ aggregation. Previous *in vitro* studies of fibrillogenesis induced Aβ aggregation by 'aging' the peptide. To 'age' Aβ, high concentrations (micro- to millimolar range) of the peptide are incubated over days or weeks. Some protocols also include a 'seeding' step where small amounts of preformed Aβ fibrils are added to act as nuclei for further β-amyloid formation. In contrast to this method, inducing Aβ to aggregate by the addition of zinc or copper has the

advantage of being very rapid (15 min or less) and reproducible, and a relatively large proportion of Aβ leaves the bulk phase (≈30%) using only low nanomolar concentrations of the peptide. Submicromolar concentrations of metals are sufficent to induce Aβ aggregation (*25*), and our most recent studies suggest that contaminating metals in laboratory buffers may play a major role in fibril formation during Aβ 'aging', possibly by initiating nucleation. We have found that the inclusion of specific copper chelators to incubation buffers significantly extends Aβ solubility in aqueous solutions (Atwood and Huang, private communication). This suggests that the 'aging' of Aβ solutions used to induce fibril formation in many previous Aβ aggregation studies may have been metal-dependent.

APOE for this study was purified under nondenaturing conditions from the culture media of APOE stably transfected cells. Purification procedures that denature and delipidate APOE can dramatically change both Aβ binding and the protein's effect on Aβ aggregation (*56*). At least some of the contrariety in findings between previous studies appears to be due to differences in APOE isoform preparation methods. APOE binding has also been reported to be sensitive to the conformational state of Aβ (*51*, *63*). The conformation of Aβ in solution is dependent on multiple factors including concentration (*15*, *17*), pH (*25*, *75*), and salinity (*70*, *76*). Conditions are most likely to approach the situation *in vivo* when, as in this study, physiological concentrations of native APOE and Aβ are employed.

To characterize zinc- and copper-induced aggregation, we compared the physiochemical properties of the metal Aβ aggregates. Zinc-induced aggregates differed from copper-induced precipitates in relative density and resolubilization by EDTA. The structurally distinct nature of zinc- and copper-induced aggregates is consistent with different aggregation mechanisms. Our findings show that, in addition to the conformational states of APOE and Aβ, the mechanism used to initiate Aβ aggregation is also a factor that can have an important effect on experimental observations.

APOE appears to be involved in mediating the *in vivo* accumulation (versus clearance) of Aβ. Mutations in several other proteins associated with this pathway have now been linked to Alzheimer's disease, including the following: α_2-macroglobulin (*77*) (α_2M), another Aβ binding protein (*78*, *79*) involved in Aβ clearance and degradation (*80*); APP (*81*, *82*), the precursor to Aβ; and the low-density lipoprotein receptor related protein (*83*, *84*), a cell surface receptor for APOE (*85*), α_2M (*86*, *87*), and APP (*88*). These findings strongly support the Aβ chaperone model in which APOE/Aβ binding plays a central role in the rate of amyloidosis. A possible, albeit still speculative, conjecture is that APOE modulation of zinc- or/and copper-induced Aβ aggregation has an important role in this function. However, confirmation of this hypothesis must await further testing of the significance of metal-induced Aβ aggregation *in vivo*.

In summary, we employed a novel *in vitro* filtration assay to investigate metal-induced aggregation of Aβ in the presence of APOE2, APOE3, and APOE4 at physiologically relevant concentrations. In the presence of all three APOE isoforms, zinc-induced aggregation of Aβ was attenuated, while precipitation with copper was enhanced. Consistent with the increased risk of AD associated with the ϵ4 allele, metal-induced aggregation of Aβ was highest for both zinc and copper in the presence of APOE4. Our data are consistent with a role for APOE as a molecular chaperone for Aβ, even when physiological concentrations and ratios of these molecules are employed.

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