Role of Apolipoprotein E in β-Amyloidogenesis

**ISOFORM-SPECIFIC EFFECTS ON PROTOFIBRIL TO FIBRIL CONVERSION OF Aβ IN VITRO AND BRAIN Aβ DEPOSITION IN VIVO***

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Background: ApoE is a genetic risk factor for Alzheimer disease.

Results: As compared with apoE2/3, apoE4 failed to inhibit the conversion of Aβ protofilbrils to fibrils in vitro. Intraparenchymal injection of Aβ protofilbrils complexed with apoE3 attenuated Aβ deposition, whereas apoE4 did not.

Conclusion: ApoE3, not apoE4, impedes β-amyloid formation.

Significance: Interaction between Aβ and apoE is a critical determinant of β-amyloid formation.

Human APOE e4 allele is a strong genetic risk factor of Alzheimer disease. Neuropathological and genetic studies suggested that apolipoprotein E4 (apoE4) protein facilitates deposition of amyloid β peptide (Aβ) in the brain, although the mechanism whereby apoE4 increases amyloid aggregates remains elusive. Here we show that injection of Aβ protofilbrils induced Aβ deposition in the brain of APP transgenic mice, suggesting that Aβ protofilbrils acted as a seed for aggregation and deposition of Aβ in vivo. Injection of Aβ protofilbrils together with apoE3 significantly attenuated Aβ deposition, whereas apoE4 did not have this effect. In vitro assays revealed that the conversion of Aβ protofilbrils to fibrils progressed more slowly upon coincubation with apoE2 or apoE3 compared with that with apoE4. Aβ protofilbrils complexed with apoE4 were less stable than those with apoE2 or apoE3. These data suggest that the suppression effect of apoE2 or apoE3 on the structural conversion of Aβ protofilbrils to fibrils is stronger than those of apoE4, thereby impeding β-amyloid deposition.

Alzheimer disease (AD) is pathologically characterized by massive deposition of amyloid β peptides (Aβ) as senile plaques in brains. The amyloid hypothesis postulates the central role of Aβ aggregation as the major cause of neuronal degeneration in AD (1). A subset of AD is inherited as an autosomal dominant trait. Genetic analysis of familial AD (FAD) cases revealed three genes, Aβ precursor protein (APP), presenilin 1, and presenilin 2, that are causative for FAD (1). Subsequent studies revealed that these mutant genes increase the production of Aβ or accelerate fibrillization of Aβ, thereby leading to AD. However, it has not been clarified whether production or fibrillization of Aβ is up-regulated in the brains of patients with sporadic form of AD, which comprises the major population of AD in the elderly.

A number of non-Aβ proteinaceous components are deposited in senile plaques associated with Aβ (2). These proteins may interact with Aβ and modify its deposition in AD brains. The best characterized of these proteins is apolipoprotein E (apoE). ApoE is a 299-amino acid protein secreted from liver into blood plasma, which mediates lipoprotein metabolism. In the central nervous system, apoE is produced and secreted primarily from astrocytes and microglial cells (3–5). Three major polymorphisms in human apoE gene, i.e. e2, e3, and e4, that alter the coding of residues 112 and 158 (E2: Cys-112/Cys-158, E3: Cys-112/Arg-158, E4: Arg-112/Arg-158) have been recognized, of which e4 is associated with an increased risk for developing AD (6, 7). It has been reported that apoE is codeposited with Aβ and acquires insolubility along with Aβ in AD brains (8, 9). Moreover, the density of senile plaques in APOE e4 homozygous AD patients is higher than those carrying e3/e3 or e3/e4 genotypes (10). These studies indicate that apoE proteins, especially apoE4, may be associated with the pathogenesis of AD through interaction with Aβ. However, the mechanism whereby apoE4 affects Aβ in the pathogenesis of AD has been unknown.

In vitro studies have revealed that formation of Aβ amyloid fibrils is a complex process, comprised of nucleation and elongation phases (11, 12). In the nucleation phase, seeds are formed from monomer Aβ through conformational changes. Following the nucleation phase, Aβ forms fibrils by binding to preformed seeds in the elongation phase. It has been shown that apoE inhibits the nucleation phase of Aβ fibrillization, although this effect is independent of the apoE isoforms (13, 14). Furthermore, it has not been well understood how apoE affects the Aβ seeds in the inhibition of nucleation. Along with the classical fibrillation process of Aβ, a variety of metastable intermediates (e.g. paranuclei, protofibrils, Aβ-derived diffusible ligands)
Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo

have been reported (15–19). Aβ protofibrils, separated in the high molecular weight fraction (>200 kDa) by size exclusion chromatography (SEC), are flexible short fibrils of ~5 nm in diameter and in lengths not exceeding ~150 nm (15). Because an intra-Aβ E22G (Arctic) FAD mutation accelerates the Aβ protofibril formation (20, 21) and the Aβ protofibrils are shown to be potently neurotoxic (22, 23), Aβ protofibrils have been deemed as a causative species leading to neurodegeneration in AD brains. However, it remains unclear how Aβ protofibrils impact the pathogenesis of AD.

In this study, we found that intrabrain injection of Aβ protofibrils promoted Aβ deposition in APP transgenic mice, demonstrating that Aβ protofibrils act as aggregation seeds in brains in vivo. We further showed that injection of mixture of Aβ protofibrils and apoE3 attenuated Aβ deposition compared with Aβ protofibrils alone or with mixture of Aβ protofibrils and apoE4. Based on these data, we hypothesized that apoE affects Aβ fibrillization, especially during the process of conversion from protofibrils to fibrils, in an isoform-dependent manner. To test this hypothesis, we carried out in vitro experiments using SEC analysis and thioflavin T (ThT) binding assay (21) and showed that apoE2 or apoE3 induced the slower conversion of Aβ protofibrils to fibrils than apoE4. We also found that Aβ protofibril-apoE2 or -apoE3 complex was more stable than Aβ protofibril-apoE4 complex. These results support the notion that apoE2 or apoE3 inhibits Aβ fibrillization and attenuates Aβ deposition through suppression of the conversion of Aβ protofibrils to fibrils, whereas apoE4 had a weaker effect.

Experimental Procedures

Peptide and Reagents—Synthetic Aβ(1–42) peptides were purchased from Peptide Institute, Inc.. Peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Kanto Chemical) and filtrated with 2% (v/v) DMSO (Kanto Chemical) at a concentration of 1 mg/ml, dried, resolubilized in PBS containing 100 μg/ml 6 min) and then immunostained with an anti-Aβ mouse monoclonal antibody 82E1 (IBL) by avidin-biotin complex method using diaminobenzidine as chromogen. To biochemically quantitate the insoluble Aβ, the hippocampus of the injected side was extracted by stepwise homogenization by radioimmuno precipitation assay buffer (50 mM of Tris-base, pH 7.4, 150 mM NaCl, 1% of Nonidet P-40, 1% of sodium deoxycholate, 0.1% of SDS) buffer containing Complete protease inhibitor mixture (Roche), 2% SDS, and 70% formic acid.

The levels of Aβ in formic acid fractions (insoluble Aβ) were measured by two-site ELISA. The ratio representing the increase in Aβ deposition was calculated as follows: [A7(Aβ) − A7(PBS)] / [wt(Aβ) − wt(PBS)]/A7(PBS), where A7(Aβ) is insoluble Aβ42 in the hippocampus of A7 mice injected with Aβ; A7(PBS) is insoluble Aβ42 in the hippocampus of A7 mice injected with PBS (i.e. contralateral side of Aβ injection); wt(Aβ) is insoluble Aβ42 in the hippocampus of wt mice injected with Aβ; and wt(PBS) is insoluble Aβ42 in the hippocampus of wt mice injected with PBS (i.e. contralateral side of Aβ injection).

In experiments comparing Aβ protofibril injection without apoE and with apoE3 or apoE4, the ratios of the levels of insoluble Aβ42 in the hippocampus injected with protofibril Aβ and apoE divided by those without apoE were calculated. In experiments comparing the effects of apoE3 and apoE4, the ratios of the levels of insoluble Aβ42 in the hippocampus injected with Aβ protofibrils with apoE4 divided by those with apoE3 were calculated.

In Vitro Assays for the Formation of Aβ Protofibrils and Fibrils—In vitro Aβ fibrillization assays were performed as previously described (21). Briefly, synthetic Aβ(1–42) was solubilized at a concentration of 22 μM without or in the presence of indicated proteins (apoE, α1-microglobulin, or α2M) at a concentration of 220 nM and incubated at 37 °C. Following incubation for the indicated times, 50-μl aliquots were put immediately on ice to prevent further fibril formation and were centrifuged at 17,000 × g for 5 min. After centrifugation, samples before and after centrifugation (total ThT and sup ThT, respectively) were mixed with 500 μl of 3 μM ThT (Tokyo
Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo

Chemical in 0.1 m glycine-NaOH (pH 8.5) to monitor fibril formation. Fluorescence levels were then assayed using a Hitachi F2500 fluorometer (λex = 443 nm and λem = 484 nm). The supernatants were fractionated by SEC on Superdex 75 column (GE Healthcare Bio-Sciences) attached to a series 1100 high performance liquid chromatograph (Hewlett-Packard) at a flow rate of 0.5 ml/min to monitor protofibril formation at room temperature. Aβ protofibrils and LMW Aβ were detected at elution times of ~15 and ~27 min, respectively, by UV absorbance at 214 nm. The start time of Aβ fibrillation was defined as the point when the difference of ThT binding between sample prior to and after centrifugation exceeds 1.0.

Negative Stain Electron Microscopy—Samples were spread on 400-mesh collodion-coated grids, negatively stained with 2% (w/v) phosphotungstic acid (pH 7.0; Wako Pure Chemical), and viewed in an electron microscope (JEOL 1200EXII), as described (21). Immunoblot Analysis—SDS-PAGE was performed as previously described (21). Samples were loaded on 10–20% Tris-Tricine gradient gels (Cosmo Bio), and Aβ bands were detected by a rabbit monoclonal antibody 11-S (Alpha Diagnostic Intl. Inc.) or a goat polyclonal antibody 3H1 (University of Ottawa Heart Institute Research Corporation) or a goat polyclonal antibody BAN50, as described (27). ApoE or with apoE by SEC analysis was incubated at 37 °C for the indicated times. Fluorescence levels were then assayed using a Hitachi F2500 fluorometer (λex = 443 nm and λem = 484 nm). The supernatants were fractionated by SEC on Superdex 75 column (GE Healthcare Bio-Sciences) attached to a series 1100 high performance liquid chromatograph (Hewlett-Packard) at a flow rate of 0.5 ml/min to monitor protofibril formation at room temperature. Aβ protofibrils and LMW Aβ were detected at elution times of ~15 and ~27 min, respectively, by UV absorbance at 214 nm. The start time of Aβ fibrillation was defined as the point when the difference of ThT binding between sample prior to and after centrifugation exceeds 1.0.

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Results

Aβ Protofibrils Exhibit a Seeding Effect on Aβ Deposition in the Brains of APP Transgenic Mice—In this study, we used APP transgenic mice (A7 line), which overexpress human APP with Swedish and Austrian double FAD-linked mutations (KM670/ 671NL + T714I) in neurons under the control of Thy1.2 promoter (25). A7 mice develop progressive Aβ deposition in the cerebral neocortices and hippocampi at the age of ~11–12 months in an age-dependent manner.

It has been reported that intracerebral inoculation of Aβ amyloid induces Aβ deposition in the brains of APP transgenic mice, acting as an aggregation seed for Aβ amyloid (26, 28). To elucidate whether Aβ protofibrils also work as an aggregation seed for Aβ amyloid in vivo, we prepared LMW Aβ, Aβ protofibrils, and Aβ fibrils using synthetic human Aβ (1–42) (21) and injected them individually into the brains of 8-month-old A7 or wt mice (Fig. 1A). We also injected PBS into the contralateral hemisphere as a control. After 4 months, immunohistochemical analyses of the brains of A7 mice showed that Aβ protofibrils and Aβ fibrils, but not LMW Aβ, induced Aβ deposition around the injection sites (Fig. 1B). In sharp contrast, no Aβ deposits were seen in the PBS-injected contralateral hemisphere (Fig. 1B) or in both hemispheres of wt mice (Fig. 1C). The levels of insoluble Aβ in the brains injected with Aβ protofibrils were significantly higher than those with LMW Aβ, and those in mice injected with Aβ fibrils were higher compared with those injected with Aβ protofibrils, as quantified by Aβ specific ELISA (Fig. 1D). These data suggested that not only Aβ fibrils but also Aβ protofibrils harbor a seeding potential on Aβ amyloidogenesis in vivo.

ApoE, but Not ApoE4, Attenuates Aβ Deposition Induced by Aβ Protofibrils—We hypothesized that apoE may affect the seeding effects of Aβ protofibrils in an isoform-dependent manner. To test this, we injected Aβ protofibrils alone into one side of the hippocampus of 12-month-old A7 mice and Aβ protofibrils with apoE3 or apoE4 into the other side (Fig. 2A). We found that Aβ protofibrils with apoE3 induced significantly less abundant Aβ deposition as revealed by immunohistochemistry (Fig. 2B) or as the amount of biochemically extractable insoluble Aβ compared with those injected with Aβ protofibrils alone (Fig. 2C). In contrast, Aβ protofibrils with apoE4 induced Aβ deposition at a similar extent to Aβ protofibrils alone (Fig. 2D). No significant difference in the amount of insoluble Aβ was seen between mice injected with Aβ protofibrils with apoE4 or Aβ protofibrils alone (Fig. 2E). We speculated that these contrasting results were due to the difference between the isoforms of injected apoE. To examine this idea, we injected Aβ protofibrils with apoE3 into one side, and Aβ protofibrils with apoE4 into the other side of the hippocampus of 12-month-old A7 mice and found that Aβ protofibrils with apoE4 induced higher levels of Aβ deposition as detected by immunohistochemistry and as insoluble Aβ by biochemistry compared with Aβ protofibrils with apoE3 (Fig. 2, F and G). These data indicated that apoE3, but not apoE4, attenuated Aβ deposition induced by Aβ protofibrils, suggesting that apoE may affect the seeding effect of Aβ protofibrils in an isoform-dependent manner.

ApoE Suppresses the Fibril Conversion of Aβ in an Isoform-dependent Manner in Vitro—To elucidate the mechanism whereby apoE affects the seeding effects of Aβ protofibrils in an isoform-dependent manner, we investigated the role of apoE in the formation of Aβ protofibrils and fibrils in...
**Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo**

by SEC analysis and ThT binding assay, respectively, as previously reported (21) (Fig. 3A). Briefly, the formation of Aβ protofibrils and LMW Aβ including monomer and low oligomers, were quantitated as areas corresponding to the >100-kDa peak and the ~10-kDa peak, respectively, detected by SEC analysis. Simultaneously, the formation of Aβ fibrils was evaluated as the difference of ThT binding between samples prior to and after centrifugation (total ThT and sup ThT, respectively). We defined the start time of Aβ fibril formation as the point when the difference of ThT binding between samples prior to and after centrifugation exceeds 1.0. When Aβ(1–42) was incubated, Aβ protofibrils were rapidly formed within 3 h, in parallel with the decrease in LMW Aβ, followed by a decrease in Aβ protofibrils; protofibrils disappeared by 14 h, whereas Aβ fibrils started to increase after 4.6 h of incubation, which leveled off at ~14 h (Fig. 3B). Using these assays, we examined the effect of apoE on the formation of Aβ protofibrils and fibrils. In comparison with the incubation of Aβ alone (Fig. 3B), coincubation with apoE2 or apoE3 extended the lifetime of Aβ protofibrils and delayed the start of Aβ fibril formation by 7.6 and 13.9 h, respectively (Fig. 3C). In sharp contrast, coincubation with apoE4 did not extend the lifetime of Aβ protofibrils, and fibril formation started at 5.2 h (Fig. 3C). The time course of Aβ fibrillization in the presence of apoE4 was comparable to those injected with PBS. The mean values ± S.D. are shown. *, p < 0.05. One-way analysis of variance was used.

**FIGURE 1.** *In vivo* seeding effects of Aβ protofibrils and fibrils in the brains of A7 mice. A, schematic representation of the timeline of experiments. A7 mice were injected with Aβ or PBS into the neocortex and hippocampus at 8 months. Then at 4 months after injection, the both hemispheres were immunohistochemically or biochemically analyzed. B and C, Aβ immunostaining of the hippocampi and neocortices of mouse brains injected with PBS, LMW, protofibril, or fibril Aβ. A7 mice (B) or wt (C) mice were injected with PBS, LMW, protofibril, or fibril Aβ, respectively, into the hippocampi and neocortices at 8 months old, and Aβ deposits around the trajectories of injection (arrows) were immunostained by 82E1 at 12 months. Representative images in each group (n = 4) are shown. Scale bar, 100 μm. D, relative levels of insoluble Aβ42 in the hippocampi of A7 mice injected with different Aβ preparations. Hippocampi of A7 mice injected with PBS, LMW, protofibril, or fibril Aβ, respectively, at 8 months old were dissected at 12 months, and the levels of insoluble Aβ42 were measured by two-site ELISA. The bars represent the mean ratios of the levels of insoluble Aβ42 after injection of LMW (n = 4), protofibril (n = 3), or fibril (n = 3) forms of Aβ divided by those injected with PBS. The mean values ± S.D. are shown. *, p < 0.05. One-way analysis of variance was used.
lifetime of Aβ protofibrils (Fig. 3D); notably, the capacity of lipidated apoE4 particles to prolong the lifetime of Aβ protofibrils was smaller than that of lipidated apoE3 (>24 h in apoE3 versus 14.3 h in apoE4; Fig. 3D). To determine whether apoE altered the structure of protofibrils or fibrils of Aβ, we observed the ultrastructure of Aβ protofibrils or fibrils by negative stain electron microscopy. Aβ protofibrils presented with a short and curved fibril-like structure and Aβ fibrils exhibited a long, straight, and unbranching structure as described (15), which were almost identical between samples of Aβ protofibrils and fibrils in the presence or absence of apoE3 (Fig. 3E). These data suggested that apoE2 and apoE3 potentially suppress the conversion of Aβ protofibrils to fibrils, resulting in the prolongation of the lifetime of Aβ protofibrils and delay in the start of Aβ fibril formation. In contrast, apoE4 has a lesser effect on the conversion of Aβ protofibrils to fibrils compared with apoE2 or apoE3, thereby allowing Aβ protofibrils with apoE4 to rapidly form fibrils compared with those with apoE2 or apoE3.

ApoE Forms SDS-stable Complex with Aβ Protofibrils—Because apoE affected the protofibril to fibril conversion of Aβ in an isoform-dependent manner, we next examined the interaction of apoE with Aβ protofibrils, as well as its isoform dependence. To this end, we coincubated Aβ with different isoforms of apoE in vitro and evaluated their interaction by immunoblotting. It has been reported that apoE forms an SDS-stable complex with Aβ (31–33).

FIGURE 2. In vivo effects of apoE on the seeding effects of Aβ protofibrils. A, schematic representation of the timeline of experiments. A7 mice were injected with Aβ protofibrils preincubated with or without apoE into the neocortex and hippocampus at 12 months. At 4 months after injection, both hemispheres were immunohistochemically or biochemically analyzed. B and C, A7 mice were injected with Aβ protofibrils preincubated without apoE on one side of the neocortex and hippocampus and those with apoE3 on the contralateral side. The both hemispheres were immunohistochemically analyzed for Aβ using 82E1 antibody (B; n = 3 in each group) or subjected to biochemical quantification of insoluble Aβ (C; n = 5). Insoluble Aβ levels were quantitated by two-site ELISA, and the ratios of those in the contralateral side (i.e. injected with protofibrils preincubated with apoE3) divided by those in the side injected with Aβ protofibrils alone) were calculated (C). Similarly, those injected with protofibrils preincubated without apoE on one side and with apoE4 on the contralateral side (D; n = 3 in each group, E; n = 5) and those injected with protofibrils preincubated with apoE3 on one side and with apoE4 on the contralateral side (F; n = 3 in each group, G; n = 5) were immunohistochemically and biochemically analyzed. Scale bars, 100 μm. The mean values ± S.D. are shown. *, p < 0.05.
ApoE3, an anti-Aβ antibody (BAN50) revealed ∼40-kDa bands in addition to the Aβ monomer and oligomer bands (Fig. 4, A and B, upper panels). The ∼40-kDa bands were also labeled with an anti-apoE antibody (3H1) (Fig. 4, A and B, lower panels), indicating that the ∼40-kDa bands represented the SDS-stable complex of Aβ and apoE2 or apoE3. Intriguingly, the ∼40-kDa bands appeared in a time-dependent manner: the ∼40-kDa band did not exist at 0 h, which emerged during 3–9 h upon coincubation with apoE2 or 3–14 h upon coincubation with apoE3, concomitantly with the Aβ protofibril formation (Fig. 3C). After 9 h of coincubation with apoE2 or 14 h of coincubation with apoE3, the ∼40-kDa bands disappeared, which coincided with the emergence of Aβ fibrils (Fig. 3C). These findings are consistent with the idea that apoE was precipitated with Aβ fibrils. In contrast, the ∼40-kDa bands were present at 3–6 h of incubation when Aβ was coincubated with apoE4 (Fig. 4C).
Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo

FIGURE 4. Formation of SDS-stable Aβ-apoE complex in vitro. A–C, time course of formation of the SDS-stable Aβ-apoE complex. 22 μM of Aβ(1–42) was incubated for 0, 3, 6, 9, 14, 19, or 24 h with rec apoE2 (A), rec apoE3 (B), or rec apoE4 (C). After centrifugation, SDS-stable Aβ-apoE complex was monitored by immunoblot analyses with an anti-Aβ antibody (BAN50, upper panel) and an anti-apoE antibody (3H1, bottom panel). Arrowheads indicate the SDS-stable Aβ-apoE complex bands. D, formation of SDS-stable Aβ-apoE complex in the Aβ protofibril fraction. Immunoblot analyses of isolated protofibrils without apoE (lanes 1 and 2), with rec apoE3 (lanes 5 and 6), with rec apoE4 (lanes 7–10), and of rec apoE3 alone (lanes 3 and 4) with an anti-Aβ antibody (BAN50, upper panels) and an anti-apoE antibody (bottom panels). Lanes 9 and 10 were loaded with double the amount of samples. Arrowheads indicate the bands corresponding to SDS-stable Aβ-apoE complex migrating at ~40 kDa (n = 3). E, 22 μM of Aβ(1–42) was incubated alone (Aβ) or with rec α1-microglobulin, rec α2M, rec apoE2, rec apoE3, or rec apoE4 for 6 h. After centrifugation at 17,000 × g for 5 min, SDS-stable complex was monitored by immunoblotting analyses using an anti-Aβ antibody (BAN50, upper panel), an anti-apoE antibody (3H1, the second panel from the top), an anti-α2M antibody (the third panel from the top), and an anti-α1-microglobulin antibody (bottom panel). Arrowheads indicate ~40-kDa bands corresponding to the SDS-stable Aβ-apoE complex, and the arrow indicates the SDS-stable Aβ-α2M complex migrating at ~190 kDa. F, quantitative measurement of the band intensities in E. The mean values ± S.D. in three independent experiments are shown as ratios relative to the values of apoE3 as 1.0. *, p < 0.05. One-way analysis of variance was used. G, in vitro Aβ fibrillization assay in the presence of binding proteins. 22 μM of Aβ(1–42) was incubated in the absence (filled circles) or presence of rec α1-microglobulin (the molar ratio of Aβ/α1-microglobulin at 100:1, filled squares), rec α2M (the molar ratio of Aβ/α2M at 100:1, filled triangles), rec apoE2 (the molar ratio of Aβ/apoE2 at 100:1, open circles), rec apoE3 (the molar ratio of Aβ/apoE3 at 100:1, open squares), or rec apoE4 (the molar ratio of Aβ/apoE4 at 100:1, open triangles) for 0, 3, 6, 9, 14, 19, and 24 h, and then ThT fluorescence was measured. The mean values in three independent experiments are shown.

The time course of the emergence of the ~40-kDa bands was coincident with that of Aβ protofibril formation in the presence of apoE4 (Fig. 3C).

Because the formation of SDS-stable Aβ-apoE complex coincided with the protofibril formation of Aβ, we further examined whether apoE was directly bound to Aβ protofibrils by incubating Aβ with or without apoE for 6 h and isolating the Aβ protofibril fraction using SEC. When Aβ was incubated without apoE, Aβ-positive bands corresponding to Aβ monomer and oligomers were detected in the protofibril fraction (Fig. 4D, lane 2), suggesting the dissociation of Aβ protofibrils during SDS-PAGE into monomer or oligomers. When apoE3 was incubated alone, no apoE-positive band was detected in the Aβ protofibril fraction (Fig. 4D, lane 4). Importantly, when Aβ was coincubated with apoE3, Aβ monomer, oligomers, apoE3, and the ~40-kDa SDS-stable Aβ-apoE3 complex were detected in

JUNE 12, 2015 • VOLUME 290 • NUMBER 24
JOURNAL OF BIOLOGICAL CHEMISTRY 15169
the protofibril fraction (Fig. 4D, lane 6), indicating that apoE3 directly interacted with Aβ protofibrils and formed the ~40-kDa SDS-stable complex with Aβ protofibrils. In addition, the ~40-kDa band corresponding to the SDS-stable Aβ-apoE complex was also detected in the Aβ protofibril fraction by coincubation of Aβ with apoE4 (Fig. 4D, lanes 8 and 10). It is notable that the amount of SDS-stable Aβ-apoE complex in the Aβ protofibril fraction was markedly smaller than that of SDS-stable Aβ-apoE or Aβ-apoE3 complex (Fig. 4, D–F), whereas the levels of Aβ in these fractions were similar. These data suggest that apoE formed a SDS-stable complex with Aβ protofibrils in an isoform-dependent manner. To further ascertain the specificity of apoE in the formation of SDS-stable complex with Aβ protofibrils, we coincubated Aβ with α1-microglobulin, α2M, recombinant apoE2, apoE3, or apoE4 and examined the level of SDS-stable complex by immunoblotting (Fig. 4E). We confirmed that apoE2 formed the ~40-kDa SDS-stable Aβ-apoE complex at a similar extent to apoE3, whereas apoE4 formed smaller amount of SDS-stable Aβ-apoE complex compared with apoE3 (Fig. 4, E and F). α1-Microglobulin did not form the SDS-stable complex with Aβ, whereas α2M, which is capable of binding with Aβ, formed Aβ-α2M SDS-stable complex as a ~190-kDa band. Because α2M did not affect the Aβ fibrillation in vitro (Fig. 4G), we presumed that the interaction of α2M with Aβ did not affect the conversion of Aβ from protofibrils to fibrils.

Aβ Interacts with apoE Prior to Protofibril Formation—Since apoE formed the SDS-stable complex with Aβ protofibrils in an isoform-dependent manner, we examined whether the binding affinity of apoE to Aβ differs among apoE isoforms. To examine the interaction between apoE and Aβ in solution, we performed chemical cross-linking assay using the PICUP method (16). We found that all apoE isoforms bound to Aβ at 0 or 6 h with no difference in the levels of the Aβ-apoE complex of ~40 kDa.
(Fig. 5, A and B). To determine whether apoE binds to the pre-formed Aβ protofibrils, we coincubated preformed Aβ protofibrils with apoE. No bands corresponding to SDS-stable Aβ-apoE complex were detected (Fig. 5C), suggesting that apoE initially binds to soluble Aβ and that apoE may form SDS-stable complex with Aβ protofibrils in an isoform-dependent manner during the course of Aβ fibrillation.

**ApoE Forms a Less Stable Complex with Aβ Protofibrils Compared with ApoE2 or ApoE3**—It remained unknown why apoE2 or apoE3 suppressed the conversion of Aβ protofibrils to fibrils more efficiently than apoE4 and formed a more SDS-stable complex with Aβ protofibrils than apoE4. We hypothesized that apoE may have a role in stabilizing Aβ protofibrils in an isoform-dependent manner. To test this, we first evaluated the stability of Aβ protofibrils isolated by SEC. We incubated the SEC-isolated Aβ protofibrils at 37 °C for 24 and 48 h, respectively, centrifuged, and monitored the remaining Aβ protofibrils in the supernatants by ThT binding (Fig. 6A). We found that ~56.2% and ~46.7% of Aβ protofibrils formed in the presence of apoE2 and apoE3, respectively, remained after 48 h of incubation. In contrast, ~9.9% and ~26.0% of Aβ protofibrils formed without apoE or in the presence of apoE4, respectively, persisted after incubation. Simultaneously, we measured the levels of Aβ fibrils, which were formed during the incubation period, by ThT binding assay. The ratios of Aβ fibrils formed with apoE2 and apoE3 were ~21.1% and ~24.7%, respectively, after 48 h of incubation (Fig. 6B), whereas those with apoE4 or without apoE were ~46.0% and ~36.8%, respectively (Fig. 6B). These data strongly suggested that apoE3 had the capacity to stabilize Aβ protofibrils by suppressing the conformational change into fibrils, whereas apoE4 had a weaker effect on the stabilization of Aβ protofibrils.

To further examine the effect of apoE2 or apoE3 on the stability of Aβ protofibrils, we tested the stability of the protofibril Aβ-apoE complex by chemical-induced denaturation. We incubated the SDS-stable Aβ-apoE complex in different concentrations of urea solution (0, 2, 4, 6, or 8 M) and found that complexes of Aβ-apoE2 or Aβ-apoE3 were stable, even in the
Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo

presence of 8 M urea, whereas SDS-stable Aβ-apoE4 complex was dissociated in 4, 6, or 8 M of urea solution (Fig. 6C). We also incubated the SDS-stable Aβ-apoE complex in 0, 5, 10, 15, 20, or 25% of HFIP and found that the Aβ-apoE2 or Aβ-apoE3 complex was more stable than the Aβ-apoE4 complex (Fig. 6, D and E). These data also suggested that apoE4 rendered the Aβ protofibrils less stable compared with apoE2 or apoE3. Taken together, we concluded that apoE2 or apoE3 forms a more stable complex with Aβ protofibrils, resulting in the suppression of structural conversion of Aβ protofibrils to fibrils, whereas apoE4 does not have this effect.

Discussion

In this study, we showed that both Aβ protofibrils and fibrils are capable of inducing Aβ deposition in the brain as an amyloid seed, similarly to Aβ amyloid extracted from the brains of AD patients or APP transgenic mice (26, 28), and that apoE3, but not apoE4, had a suppressive effect on the Aβ deposition induced by Aβ protofibrils. We further showed in vitro Aβ protofibril/fibril formation assays that apoE2 and apoE3 prolonged the lifetime of Aβ protofibrils and suppressed the conversion of Aβ protofibrils to fibrils, whereas apoE4 had a lesser effect on the retention of Aβ protofibrils and failed to inhibit the conversion of Aβ protofibrils to fibrils compared with apoE2 or apoE3. Furthermore, we found that Aβ protofibrils interacted with apoE and formed a SDS-stable complex. Finally, we showed that SDS-stable Aβ-apoE4 complex was less stable than those containing apoE2 or apoE3, occurring in the lower level of SDS-stable Aβ-apoE4 complex compared with those with apoE2 or apoE3. These data suggest that apoE2 or apoE3 plays a role in the retention of Aβ protofibrils, thereby suppressing the conversion of Aβ protofibrils to fibrils, whereas apoE4 is incapable of sustaining the lifetime of Aβ protofibrils in a loss of function manner. This view is consistent with the results that apoE3 suppressed Aβ fibril formation in vitro and attenuated the protofibril-induced Aβ deposition in the brains of APP transgenic mice, whereas apoE4 did not have these effects. It has been reported that the Aβ burden in the brains of patients with AD is increased with ε4 allele in a dose-dependent manner (10, 34). Overexpression of human apoE4 in the absence of endogenous murine apoE increased the deposition of fibrillar Aβ in the hippocampus of mice, whereas that of apoE3 did not have this effect (35, 36). Taken together, these data support the notion that apoE4 promotes the amyloid fibril formation through the lack of suppression of conversion of Aβ protofibrils to fibrils.

We coincubated Aβ and apoE in vitro, separated the protofibril fraction by SEC (~600-kDa ranges; Fig. 3A), and detected a ~40-kDa SDS-stable band by SDS-PAGE that is positive both for Aβ and apoE on immunoblots (Fig. 4, A–D). We speculated that the ~40-kDa band was derived from a subfraction of Aβ protofibrils-apoE complex and examined their amount or stability in detail, although it remains to be determined whether the characteristics of the SDS-stable Aβ-apoE complex represents those of Aβ protofibrils potentially interacting with apoE, because the SDS-stable Aβ-apoE complex comprised a relatively minor fraction of apoE or Aβ (Fig. 4D). Nonetheless, we have shown that the stabilizing activity of apoE4 on Aβ protofibrils was lower than those of apoE2 or apoE3, underscoring the differential effects of apoE isoforms on the stability of protofibrils (Fig. 6A).

It has been reported that the level of Aβ monomer-apoE4 complex was smaller than that of Aβ monomer-apoE3 complex in vitro by immunoblotting (32). Recently, it has also been reported that soluble Aβ-apoE4 complex is less stable than Aβ-apoE2 or Aβ-apoE3 in vitro as measured by Aβ-apoE complex-specific ELISA and that the levels of soluble Aβ-apoE complex are lower in 5xFAD-apoE4TR mice compared with those in 5xFAD-apoE3TR or 5xFAD-apoE2TR mice in vivo (37). In the present study, we show that the level of SDS-stable Aβ-apoE4 protofibril complex in the Aβ protofibril fraction was lower than that of SDS-stable Aβ-apoE3 protofibril complex. These data suggest an isoform-specific effect of apoE on the stability of the Aβ-apoE complex, i.e., Aβ-apoE3 complex being more stable than Aβ-apoE4 complex (38). Furthermore, we show that the stability of Aβ protofibril-apoE4 complex was lower than that of Aβ protofibril-apoE2 or Aβ protofibril-apoE3 complex in vitro, suggesting that apoE isoforms differentially stabilize both protofibrils and monomers of Aβ. However, the mechanism of the isoform-specific stabilization of Aβ protofibrils by apoE remains unclear. The Aβ protofibril-apoE4 complex may have a thermodynamically higher energy state compared with Aβ protofibril-apoE2 or apoE3 complexes; this could enable the faster transition of the conformation of Aβ protofibrils with apoE4 into fibrils compared with apoE2 or apoE3, because of the lower activation barrier. It has been documented that apoE4 forms a compact structure by the salt bridge between Arg-61 and Glu-253, whereas apoE2 and apoE3 have an open structure without the salt bridge (39, 40). Moreover, substitution of Thr for Arg-61 in apoE4, which mimics the open structure of apoE3, formed more SDS-stable Aβ-apoE complex than apoE4 (41). These observations support the notion that the difference in the tertiary structure of apoE affects the isoform-dependent differences in the stability of Aβ protofibril-apoE complexes, which may result in affecting the rate of the conformational changes of Aβ protofibrils to fibrils.

Our in vivo experiments showed that the injection of Aβ protofibrils into the brain of APP transgenic mice induced Aβ deposition. Our results indicate that Aβ protofibrils are capable of acting as an aggregation seed in vivo. This also suggests that the Aβ protofibrils act as “on pathway” intermediates that promote Aβ amyloid formation. Aβ protofibrils have been identified as a toxic, soluble, and fibril-like intermediate in an in vitro Aβ fibrilization assay (22, 23). It has been reported that Aβ protofibrils were detected in the brain and cerebrospinal fluid of APP transgenic mice using an antibody specific for Aβ protofibrils (42) and that passive immunization with the Aβ protofibril-specific antibody significantly reduced the amyloid burden in the brains of APP transgenic mice (43). In addition, it has been reported that E22G (Arctic) FAD mutation that alters an amino acid residue within Aβ amyloid extracted from the brains of AD patients or APP transgenic mice (43). In addition, it has been reported that the Aβ amyloid burden in the brains of AD patients as an aggregation seed.
Roles of ApoE in β-Amyloidogenesis In Vitro and In Vivo

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the Aβ deposition induced by inoculation of Aβ prototibrils into the brains of APP transgenic mice. These results suggest that apoE affects the seeding effect of Aβ prototibrils in an isoform-dependent manner.

In the in vitro prototibril/fibril formation study, the lifetime of Aβ prototibrils coinubated with apoE4 was shorter than that of prototibrils with apoE2 or apoE3. This apparently contradicts with the finding that the FAD-linked E22G (Arctic) mutation increases the level of Aβ prototibrils (20). The discrepancy may partly be explained by the limited amount of Aβ peptides within the reaction tube in the in vitro experimental setting, resulting in the monophasic emergence of Aβ prototibrils and subsequent conversion to fibrils. In human brains in vivo, Aβ is continuously produced and supplied to the brain parenchyma; once Aβ starts to aggregate in the brain, we postulate that the level of prototibrils is maintained at a plateau, at an equilibrium between formation of Aβ prototibrils and structural conversion to fibrils, resulting in an increase in the level of Aβ deposition in the brain, without reducing the level of pathogenic Aβ prototibrils. We have previously reported that H6R (English) and D7N (Tottori) FAD-linked mutations accelerated carriers in AD. It also remains possible that Aβ oligomers more markedly than apoE2 or apoE3 in the brains of AD patients (30, 37). Taken together, it is conceivable that the entire process of Aβ fibrillation is suppressed by apoE2 or apoE3, thereby causing the attenuation of amyloid deposition in the brain, whereas apoE4 does not have these effects, thereby causing the enhanced amyloid deposition phenotype observed in APOE carriers in AD. It also remains possible that Aβ prototibrils complexed with apoE4 have a higher neuronal toxicity compared with those with apoE2 or apoE3.

In present study, we specifically investigated the effects of apoE on the process of Aβ fibrillation and deposition. Recent studies also highlight the roles of apoE in the metabolism and clearance of Aβ in the brain (45). Using an in vivo microdialysis technique, it is reported that apoE-null mice, in which reduced Aβ deposition has been documented (46), had a significantly shorter half-life of soluble Aβ in the brain interstitial fluids (47). These data suggest that the effects of apoE on the metabolism and clearance of Aβ should also be taken into account in the understanding of Aβ economy in brains.

In summary, we show that apoE is involved in the process of Aβ fibrillation and Aβ deposition in an isoform-dependent manner: apoE2 or apoE3 suppresses the conversion of Aβ prototibrils to fibrils and attenuates Aβ deposition, whereas apoE4 is incapable of suppressing the conversion, presumably by the lack of a stabilizing activity. These findings add to our understanding as to why APOE e4 allele is a major risk factor for AD and support the idea that regulating the interaction between Aβ and apoE may be a promising therapeutic target for the inhibition of fibrillation and deposition of Aβ in the brain.
Roles of ApoE in β-Amyloidogenesis in Vitro and in Vivo

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