min, the synaptosome-rich interface was removed and recentrifuged to remove any remaining Ficoll.

RNA Interference—Stealth™ small interfering RNA (siRNA) duplex oligoribonucleotides against PC12 cell TrkA (GenBank™ number NM_021589) and the p75 neurotrophin receptor ($p75^{NTR}$) (GenBank™ number NM_012610) were synthesized by Invitrogen. The siRNA sequences used were as follows: rTrkA-siRNA (position 1370) sense (5′-GCCCUCCUCCUAGUGCUCAACAAAU-3′) and antisense (5′-AUUUGUUGAGCACUAGGAGGAGGGC-3′); rTrkA-siRNA-control sense (5′-GCCCUCCGAUCUCGUCAACAUCAAU-3′) and antisense (5′-AUUGAUGUUGACGAGAUCGGAGGGC-3′); rp75-siRNA (position 1212) sense (5′-CAGCCUGAACAUAUAGACUCCUUUA-3′) and antisense (5′-UAAAGGAGUCUUAUAUGUUCAGGCUG-3′); rp75-siRNA-control sense (5′-CAGGUAAACAUAUAGUCCCUCCUUA-3′) and antisense (5′-UAAGGAGGGACUAUAUGUUUACCUG-3′). The control siRNA had a random sequence. siRNA oligonucleotides were transfected into PC12 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

RESULTS

Toxicity of Aβ Assembly Formed from Arctic-type Aβ—We treated primary neurons with seed-free wild- or Arctic-type Aβ, which had been preincubated for 2 h in the absence or presence of GM1 ganglioside (10 or 20% molar ratio in the lipids composing liposomes). Unexpectedly, extensive neuronal death was observed in the culture treated with Arctic-type Aβ, which had been preincubated for 2 h in the presence of GM1 ganglioside at a 10% molar ratio in liposomes (Fig. 1*A*). The extent of neuronal death under this condition was greater than that under any other conditions examined in this study (Fig. 1, *A* and *B*).

To quantitatively characterize the toxic Aβ assembly, we examined its toxicity against NGF-treated PC12 cells (PC12N cells). We found that PC12N cells are also sensitive to the toxic Aβ assembly formed from Arctic-type Aβ (Fig. 1*C*). We performed an LDH release assay of cultures of PC12N cells under various conditions. The level of LDH released from the PC12N cells, which were treated with the toxic Aβ assembly, increased depending on Aβ dose (Fig. 1*D*), GM1 ganglioside dose (Fig. 1*E*), and the duration of the exposure of the cells to the toxic Aβ assembly (Fig. 1*F*). In regard to the time course of Aβ preincubation with GM1 ganglioside, the level of released LDH increased with peak value at 2 h and then decreased in conjunction with an increase in the ThT fluorescence intensity of the incubation mixtures (Fig. 1*G*).

The Toxic Aβ Assembly Is Soluble—Importantly, the toxicity of the Aβ incubated in the presence of GM1 ganglioside was observed exclusively in the supernatant obtained by ultracentrifuging the incubation mixture (Fig. 2*A*), suggesting that the toxic Aβ assembly is soluble. To examine the possibility that a TAβ is formed in the presence GM1 ganglioside, we performed dot blotting using an oligomer-specific antibody (anti-Oligo) (23). TAβ in the incubation mixtures was readily recognized by anti-Oligo (Fig. 2*B*). The specificity of TAβ recognition by anti-Oligo was confirmed by the finding that TAβ toxicity was significantly neutralized by coincubating the mixtures with anti-Oligo in the cultures of PC12N cells and primary neurons (Fig. 2*C*). However, coincubation with a monoclonal antibody (4396C), which inhibits Aβ fibrillogenesis through binding to GM1 ganglioside-bound Aβ as a seed (40), failed to inhibit the induction of TAβ toxicity (Fig. 2*D*).

TAβ Formation from Wild-type Aβ—We then examined whether TAβ is also formed from wild-type Aβ (Aβ40). We first investigated how TAβ is formed from wild-type Aβ in the presence of liposomes containing GM1 ganglioside. Interestingly, TAβ is favorably formed from wild-type Aβ in the presence of GM1 ganglioside at a 15% molar ratio in liposomes (Fig. 3*A*). TAβ toxicity was not significant in the nanomolar range of Aβ (Fig. 3*B*).

Biophysical and Structural Features of TAβ—To determine the biophysical and structural features of TAβ, we performed SDS-PAGE of the incubation mixtures containing TAβ. However, no high molecular weight bands corresponding to possible Aβ assemblies were detected. Bands were observed only after cross-linking pretreatment with glutaraldehyde (Fig. 4*A*), consistent with previous findings showing that soluble Aβ assemblies are probably degraded by denaturing gel electrophoresis (6) unless they are cross-linked (44, 45). A morphological analysis of TAβ by electron microscopy failed to detect any definite structure under conditions in which protofibrils, which had been prepared as previously reported (30), were readily detectable (Fig. 4*B*). In contrast, spherical particles with diameters of 10–20 nm, along with rod-shaped structures, were observed by AFM in the supernatant obtained by ultracentrifuging the incubation mixtures containing TAβ (Fig. 4*C*). We then determined the molecular mass of TAβ by size exclusion chromatography, which was followed by dot blotting using anti-Oligo. The immunoreactivity was recovered as a single peak with relative molecular masses of 200–300 kDa (Fig. 4*D*). The recovery of TAβ immunoreactivity in the same fraction was also observed in the incubation mixture containing wild-type Aβ (Aβ40) and GM1 ganglioside at a 15% molar ratio in liposomes (Fig. 4*D*). Furthermore, the collected peak showed a significant toxicity against PC12N cells (Fig. 4*E*).

TAβ Formation in the Presence of Natural Neuronal Membranes—Next, we tested whether TAβ can be formed in the presence of natural neuronal membranes. We incubated Arctic-type Aβ in the presence of synaptosomes prepared from brains of mice from three different age groups. The degree of TAβ formation was significantly higher in the incubation mixture containing synaptosomes prepared from the hippocampus of aged (2-year-old) mouse brains than in any other incubation mixtures, including those containing synaptosomes from the hippocampus or the whole brain minus the hippocampus from younger (1-month-old and 1-year-old) mouse brains (Fig. 5*A*). To determine the possibility that an alteration in the lipid composition of neuronal membranes, particularly GM1 ganglioside, underlies the acceleration of TAβ formation, we determined the levels of GM1 ganglioside, cholesterol, and phospholipids in synaptosomes prepared from hippocampi of young (1-month-old) and aged (2-year-old) mouse brains. Notably, the GM1 ganglioside level significantly increased, whereas cholesterol level significantly decreased with age (Fig. 5*B*).

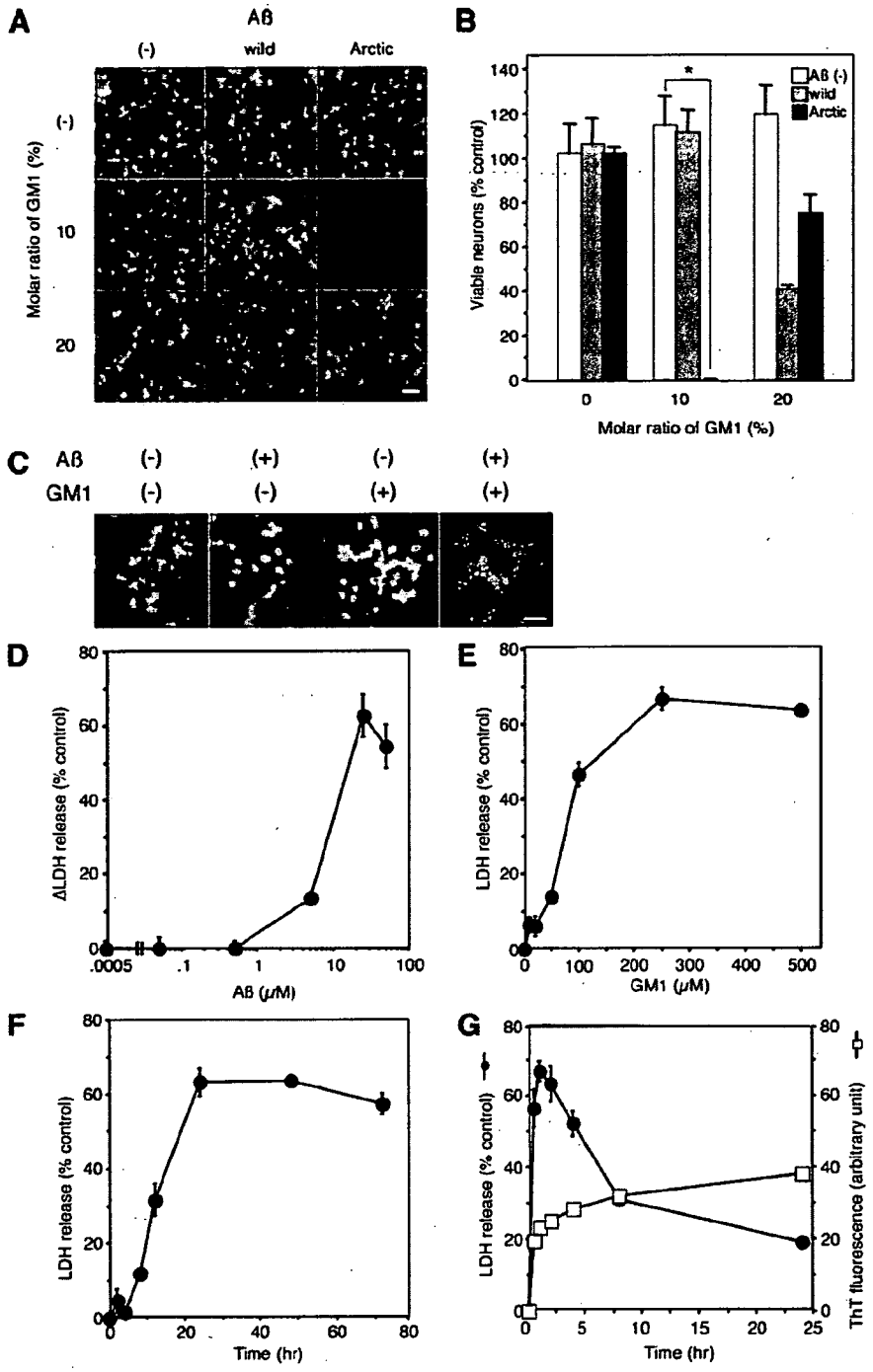

FIGURE 1. **Toxicity of Aβ assembly formed in the presence of GM1 ganglioside against primary neurons and PC12N cells.** *A*, primary cortical neurons cultured for 48 h in serum-free N2-supplemented medium were treated at 37 °C for 48 h with incubation mixtures containing seed-free wild-type Aβ (Aβ40) or Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of GM1 ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM; the molar ratio of GM1 ganglioside in liposomes varied as indicated. Neurons were stained with calcein AM (Invitrogen)/ethidium homodimer, showing green staining for viable cells and *red* staining for dead cells. *Bar*, 50 μm. *B*, the number of viable neurons in the culture shown in *A* was determined. Each *column* indicates the average of three percentages ± S.D. relative to that of control cultures in which neither Aβ nor GM1 ganglioside was added. *, $p < 0.0001$ (one-way analysis of variance combined with Scheffe's test). *C*, representative images of NGF-treated PC12 (PC12N) cells treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of GM1 ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM, and the molar ratio of GM1 ganglioside in liposomes was 10%. *Bar*, 50 μm. *D* and *E*, dose-response curves for the level of LDH released from cells treated with incubation mixtures containing Aβ, which had been preincubated as described in *C*. The concentrations of Aβ and GM1 ganglioside varied as indicated. The LDH value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. *D*, the points indicate LDH levels in the incubation mixtures containing GM1 ganglioside minus those lacking GM1 gangliosides, which were negligible below 25 μM Aβ. *F* and *G*, time course curves for level of LDH released from the cells treated with incubation mixtures containing Aβ, which had been preincubated as described in *A*. The durations of cell treatment (*F*) and Aβ preincubation in the presence of GM1 ganglioside (*G*) varied as indicated. ThT fluorescence intensities in the incubation mixtures are also shown in *G*. *D–G*, each point indicates the average of four values ± S.D.

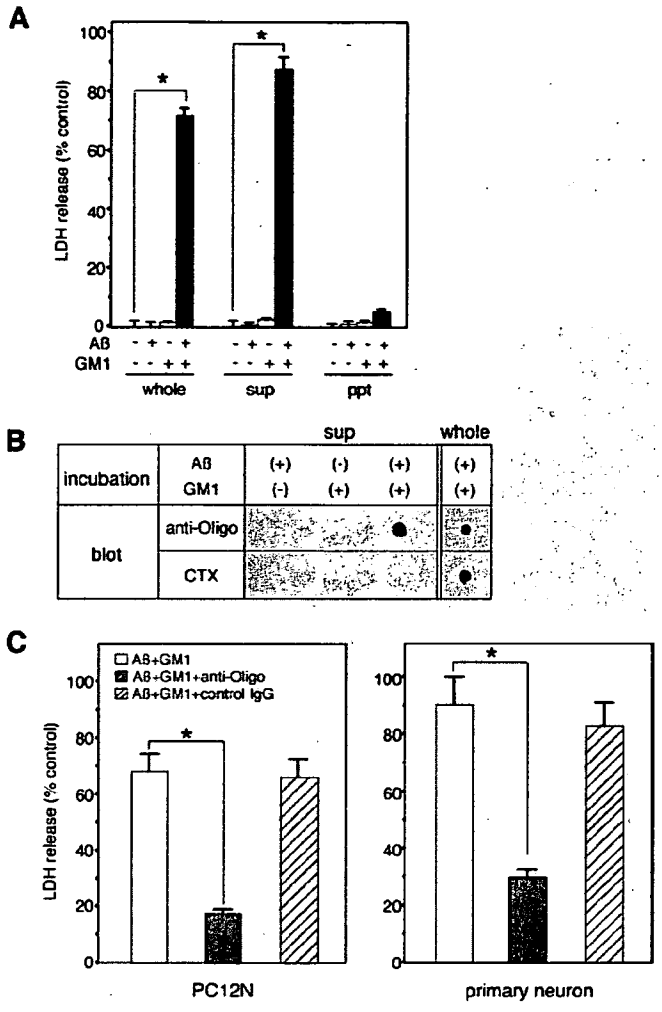

FIGURE 2. **Recognition of toxic Aβ assembly by oligomer-specific antibody.** *A*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with supernatant (*sup*) or precipitate (*ppt*) obtained by ultracentrifuging (540,000 × *g*, 15 min) incubation mixtures (*whole*) containing Arctic-type Aβ (*Aβ40*) at final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). Each value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$. *B*, dot blot analysis of supernatant (*sup*) obtained by ultracentrifuging incubation mixtures (*whole*) containing Arctic-type Aβ alone, GM1 ganglioside alone, or Arctic-type Aβ plus GM1 ganglioside. The blots were reacted with anti-Oligo (BIOSOURCE Inc., Camarillo, CA) or cholera toxin subunit B-horseradish peroxidase conjugate (Sigma) (*CTX*). *C*, the level of LDH released from PC12N cells and primary neurons treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the presence of GM1 ganglioside and anti-Oligo. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$. *D*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with Arctic-type Aβ, which had been preincubated in the presence of GM1 ganglioside and 4396C. Each column indicates the average of three values ± S.D. *n.s.*, not significant.

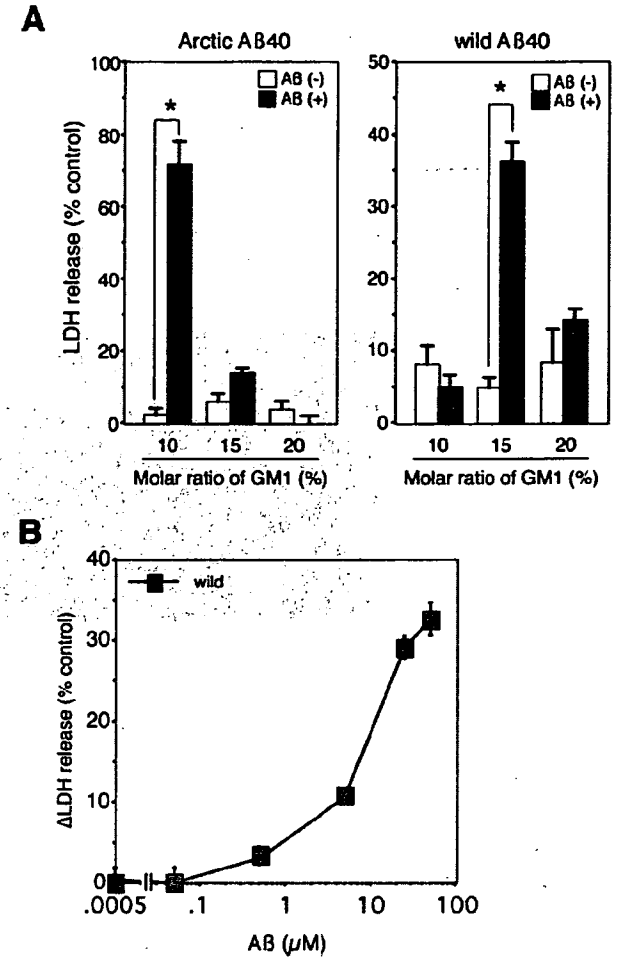

FIGURE 3. **TAβ formation from wild-type Aβ.** *A*, the level of LDH released from NGF-treated PC12 (PC12N) cells treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40), wild-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM for 2 h at 37 °C in the presence of GM1-ganglioside-containing liposomes. The GM1 ganglioside concentration in the incubation mixtures was 500 μM, and the molar ratio of GM1 ganglioside in liposomes varied as indicated. Each value indicates the percentage level of LDH released following treatment with incubation mixtures relative to the level of LDH released following treatment with Triton X-100. Each column indicates the average of three values ± S.D. *, $p < 0.0001$. *B*, the level of LDH released from PC12N cells treated at 37 °C for 48 h with incubation mixtures containing wild-type Aβ at various concentrations, which had been preincubated in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 15%). Each point indicates the LDH level in the incubation mixtures containing GM1 ganglioside minus that of the incubation mixtures lacking GM1 gangliosides, which was negligible below 25 μM for wild-type Aβ.

Putative Mechanism Underlying TAβ-induced Neuronal Death—To characterize cell death induced by TAβ, we performed nuclear staining with a membrane-permeable dye, Hoechst 33258. PC12N cells, which were treated with incubation mixtures containing TAβ for 12 h, showed characteristics of apoptotic changes, including retracted neurites, shrunken

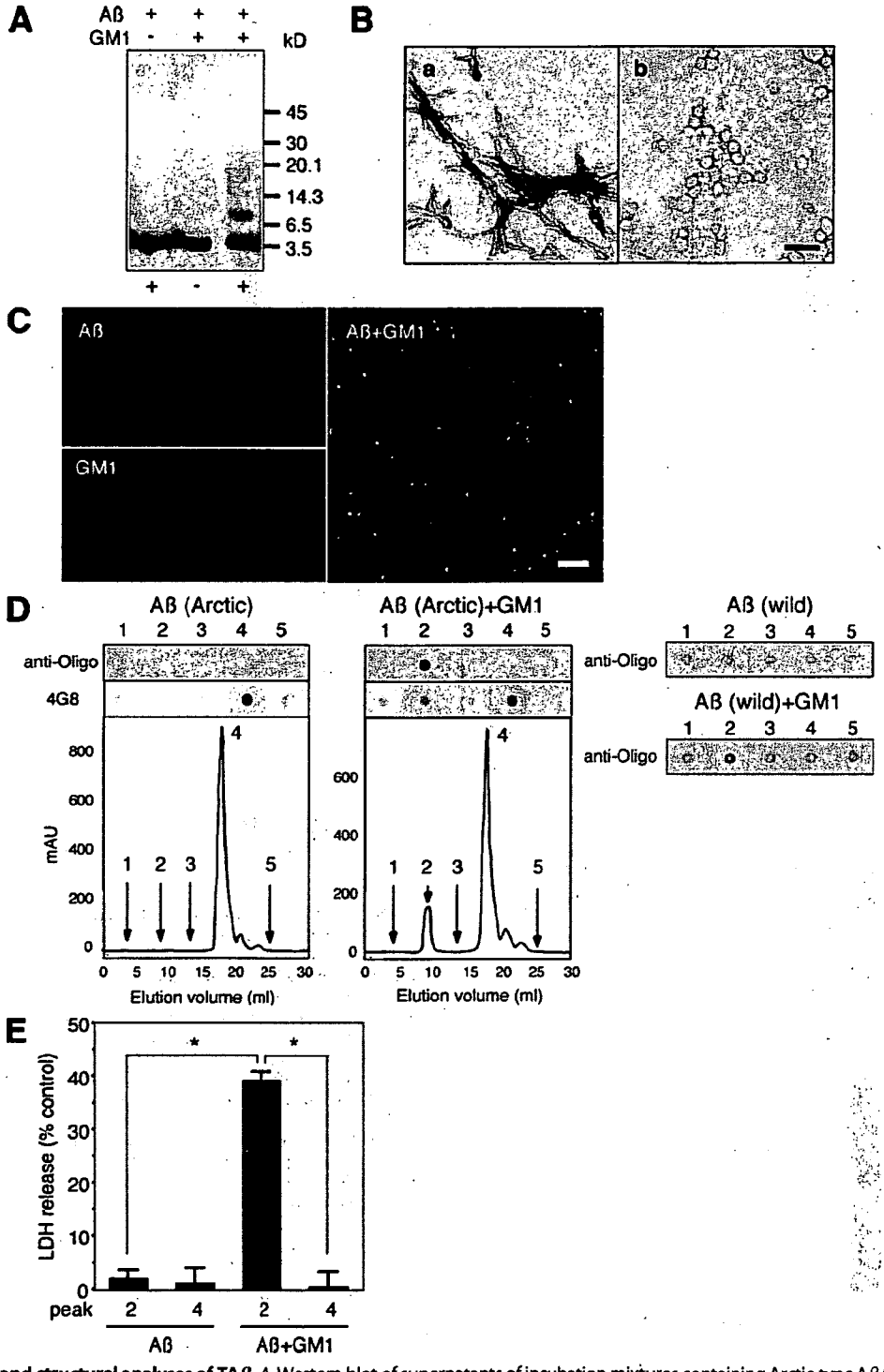

FIGURE 4. **Biophysical and structural analyses of TAβ.** *A*, Western blot of supernatants of incubation mixtures containing Arctic-type Aβ (Aβ40), which had been incubated at 50 μM and 37 °C for 24 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). Ten nanograms of Aβ in the incubation mixtures was subjected to SDS-PAGE (4–20% gradient gel) with (+) or without (−) cross-linking pretreatment using glutaraldehyde. The blot was reacted with 4G8. *B*, electron micrographs of incubation mixture containing Arctic-type Aβ preincubated to allow protofibril formation (*a*) or of incubation mixture containing TAβ formed from Arctic-type Aβ (*b*). Typical protofibril structures were observed in *a*; however, no definite structures aside from liposomes were observed in *b*. *Bar*, 100 nm. *C*, AFM image of fraction containing TAβ formed from Arctic-type Aβ. The supernatant obtained by ultracentrifuging (540,000 × *g*, 3 h) the incubation mixture containing TAβ was subjected to AFM. Spherical particles along with rod-shaped structures were observed. No definite structures were observed in the supernatants of incubation mixtures containing Arctic-type Aβ alone or GM1 ganglioside alone. The amplitude range is 0.1 V. *Bar*, 200 nm. *D*, size exclusion chromatography of incubation mixtures containing Aβ, which had been preincubated in the absence or presence of GM1 ganglioside, on a Superose 12 column. Elution samples from 35 fractions were dot-blotted on nitrocellulose membranes. The blot was reacted with anti-Oligo or 4G8. The immunoreactivity with anti-Oligo was recovered as a single peak with an apparent molecular mass of 200–300 kDa. Five representative fractions are shown. *Peaks 2* and *4* correspond to fractions containing TAβ and monomeric Aβ, respectively. *mAU*, milli-absorbance unit. *E*, toxicities of peaks (*2* and *4*) collected from incubation mixtures containing Arctic-type Aβ (shown in *D*) against PC12N cells. Each *column* indicates the average of three values ± S.D.*, $p < 0.0001$.

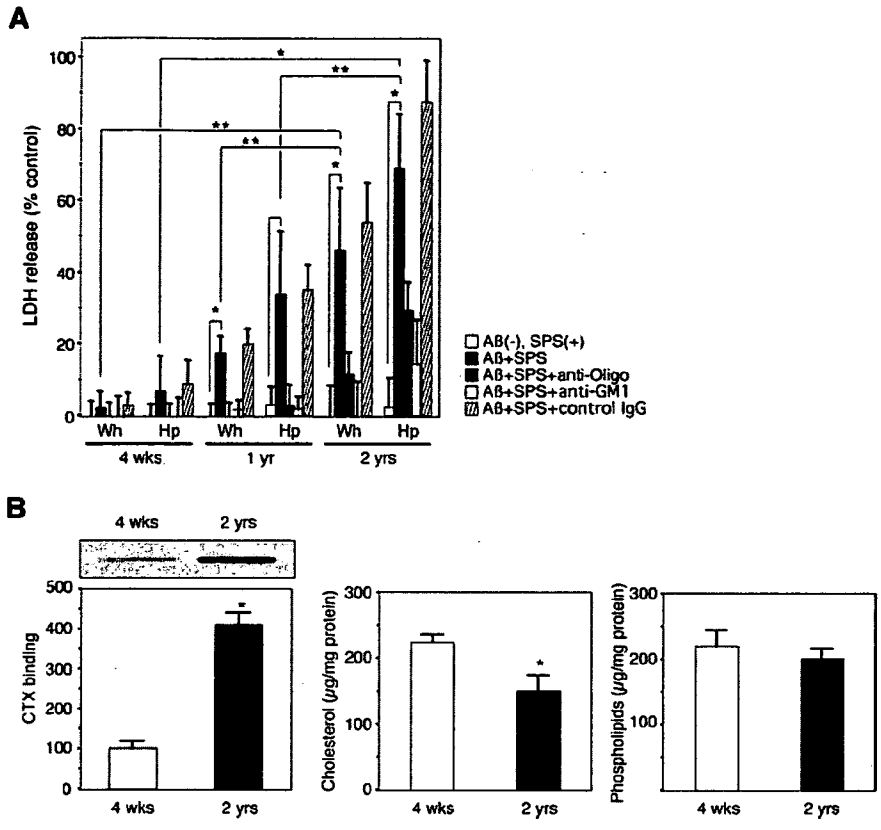

FIGURE 5. **TAβ formation from Arctic-type Aβ incubated in the presence of synaptosomes.** *A*, TAβ formation was assessed by LDH release assay of PC12N cell cultures treated at 37 °C for 48 h with incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of synaptosomes (*SPS*) prepared from brains of mice of three different age groups with or without anti-Oligo or an antibody specific to GM1 ganglioside (Calbiochem). *Wh*, whole brain minus hippocampus; *Hp*, hippocampus. Each *column* indicates the average of four values ± S.D. *, $p < 0.0001$; **, $p < 0.005$. *B*, lipid composition of synaptosomes prepared from young (1-month-old) and aged (2-year-old) mouse brains. GM1 ganglioside levels were determined by densitoscanning the blot following incubation with cholera toxin. Levels of cholesterol and phospholipids were determined using Determiner L (Kyowa, Tokyo, Japan) and phospholipids C (Wako, Osaka, Japan), respectively. Each *column* indicates the average of four values ± S.D. *, $p < 0.0001$.

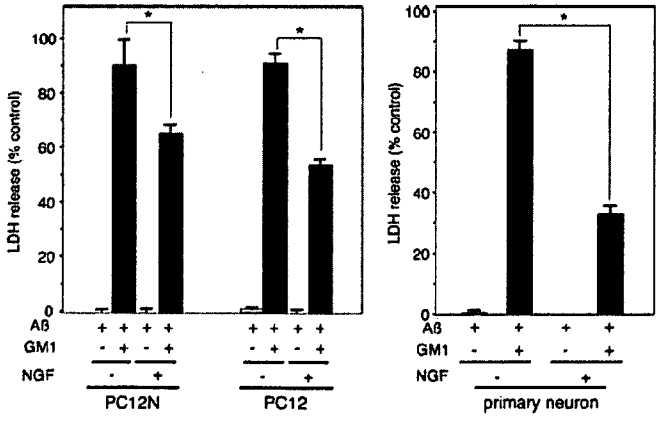

FIGURE 6. **Suppression of TAβ toxicity by the addition of exogenous NGF.** NGF-treated PC12 (PC12N), native PC12 cells, and primary neurons were treated with the incubation mixture containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%) and exogenous NGF (100 ng/ml). TAβ toxicity was assessed by an LDH release assay in these cultures. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$.

cell bodies, and the condensation and fragmentation of nuclei in conjunction with an increase in the level of LDH released from TAβ-treated PC12N cells (data not shown). To determine if TAβ toxicity is mediated by NGF receptors, we first treated PC12N cells, native PC12 cells, and primary neurons with TAβ in the presence of exogenous NGF. In these cultures, cell death was markedly prevented (Fig. 6). We then knocked down the NGF receptors, including TrkA and $p75^{NTR}$, of PC12 cells, SY5Y cells, and primary neurons using specific siRNAs. The knockdown of $p75^{NTR}$ or TrkA markedly suppressed the cell death induced by TAβ in these cultures (Fig. 7).

DISCUSSION

Here, we show that a highly toxic soluble Aβ assembly (TAβ) can be formed more rapidly and to a greater extent from Arctic-type Aβ than from wild-type Aβ. Notably, TAβ formation requires GM1 ganglioside at certain densities. TAβ is probably formed via a pathway different from one that leads to amyloid fibril formation. Biophysical and structural analyses by AFM and size exclusion chromatography revealed that TAβ is spherical with diameters of 10–20 nm and molecular masses of 200–300 kDa. The most striking feature of TAβ is its unique toxicity. Our results suggest that TAβ induces the NGF receptor-mediated apoptosis of cultured cells.

Accumulating evidence suggests that soluble Aβ assemblies are formed as intermediates en route to amyloid fibril formation. This scenario is mainly supported by the formation of soluble Aβ assemblies early during the incubation period *in vitro*, which is frequently followed by the appearance of mature fibrils (5, 6, 8, 13). Indeed, certain inhibitors of Aβ fibrillogenesis are potent for blocking the generation of Aβ oligomers (46). In this study, TAβ was preferably formed in the presence of GM1 ganglioside at lower densities than those required for amyloid fibril formation (36). Furthermore, a monoclonal antibody specific to a seed for amyloid fibril formation (40) failed to inhibit TAβ formation. These results suggest that TAβ is formed via a pathway different from a straightforward pathway leading to amyloid fibril formation, as was previously suggested in the formation of other soluble Aβ assemblies (11, 12).

In this study, monomeric Arctic-type Aβ was converted to TAβ more rapidly and to a greater extent than wild-type Aβ. The propensity of Arctic-type Aβ to form toxic nonamyloid Aβ assemblies has recently attracted interest (13, 30, 34); however,

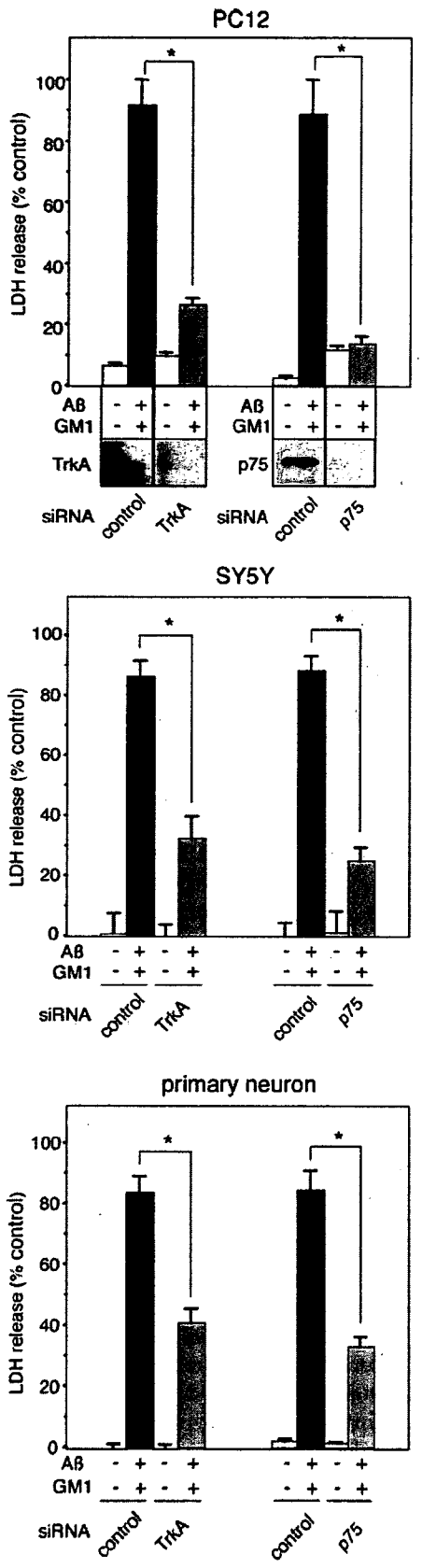

FIGURE 7. **TAβ toxicity mediated by NGF receptors.** PC12 cells, SY5Y cells, and primary neurons, which had been treated with siRNAs against TrkA or $p75^{NTR}$, were exposed to incubation mixtures containing Arctic-type Aβ (Aβ40) at a final concentration of 25 μM, which had been preincubated at 50 μM and 37 °C for 2 h in the absence or presence of 500 μM GM1 ganglioside (the molar ratio of GM1 ganglioside in liposomes was 10%). TAβ toxicity, which was assessed by LDH release assay, was markedly suppressed by the knockdown of TrkA or $p75^{NTR}$. Decreases in TrkA and $p75^{NTR}$ expression levels were confirmed by Western blotting of cell lysates using anti-TrkA and anti-$p75^{NTR}$ antibodies, respectively. Each *column* indicates the average of three values ± S.D. *, $p < 0.0001$.

it remains to be clarified how the assembly of Arctic-type Aβ is accelerated compared with that of wild-type Aβ. We previously found that Aβ fibrillogenesis from Arctic-type Aβ is also enhanced in the presence of SDS as well as GM1 ganglioside (36). Thus, taken together with the results of this study, it is likely that the negatively charged membrane surface is a preferred environment for Arctic-type Aβ to form soluble and insoluble assemblies. A previous study suggested that the lateral distribution of GM1 ganglioside affects the spatial arrangements of the oligosaccharide chain of a molecule (47). Thus, the conformation of GM1 ganglioside may be modulated at certain densities, providing a favorable microenvironment for TAβ formation.

Results of this study imply that GM1 ganglioside potently accelerates the formation of not only amyloid fibrils but also the soluble Aβ assembly. It has recently been reported that Aβ oligomerization is induced in the presence of lipid rafts isolated from brain tissues and cultured cells in a ganglioside-dependent manner (48). Although further studies are necessary, it may be assumed that GM1 ganglioside-rich membrane microdomains, such as lipid rafts, provide a favorable environment that facilitates the formation of soluble Aβ assemblies, including Aβ oligomers and dimers (49).

In this study, the incubation of Arctic-type Aβ with synaptosomes prepared from aged mouse brains markedly induced TAβ formation. Furthermore, the level of GM1 ganglioside significantly increased, whereas that of cholesterol significantly decreased with age. Our observation of an age-dependent alteration in lipid composition of neuronal membranes is in agreement with the result of a recent study of cerebral cortices of AD brains (38). Taking this together with our recent observation that the level of GM1 ganglioside in synaptosomes increases not only with age but also with the expression of apolipoprotein E4 (37), it is possible that TAβ can be formed in the brain in association with the risk factors for AD development.

It was previously reported that Aβ-derived diffusible ligands potently alter NGF-mediated signaling in cultured cells (11). Moreover, many previous studies suggested that Aβ toxicities emerge through the association with $p75^{NTR}$ (50–56) (for a review, see Refs. 57–59). In particular, it is noteworthy that Aβ toxicity mediated by $p75^{NTR}$ depends on a death domain (60) in the cytoplasmic part of $p75^{NTR}$ molecules (56). Evidence indicates the dual function of $p75^{NTR}$: one for survival and the other for death (61) (for a review, see Refs. 57 and 58). Furthermore, a previous study revealed that heteromeric TrkA-$p75^{NTR}$ complexes have different functions from homo-oligomeric TrkA or $p75^{NTR}$ alone (62). Notably, the knockdown of either TrkA or $p75^{NTR}$ is sufficient for suppressing TAβ toxicity. Thus, it may be assumed that the function of heteromeric TrkA-$p75^{NTR}$ complexes is

perturbed by TAβ binding to $p75^{NTR}$ or TrkA, leading to apoptosis through the activation of the death domain of $p75^{NTR}$ (for a review, see Ref. 58). However, it should be noted that conflicting evidence also exists; the expression of $p75^{NTR}$ protects against the toxicity of soluble Aβ assembly or extracellular Aβ (63, 64). These opposite conclusions imply that the signaling pathways of $p75^{NTR}$ are complicated and that the functions of $p75^{NTR}$ vary depending on cell type and context (for a review, see Ref. 57).

To date, various soluble Aβ assemblies with diverse structural features have been detected in a broad range of *in vitro* and *in vivo* studies, which employed different techniques in preparing or isolating such assemblies. As previously reported (11, 65), Aβ assembles into multiple alternative structures. Thus, at this point, it is difficult to determine whether TAβ is identical to or distinct from previously identified soluble Aβ assemblies. However, on the basis of its biophysical features, including its SDS disaggregatability and unsuccessful detection on a carbon-coated grid by EM, TAβ probably differs from previously reported Aβ assemblies, particularly protofibrils, because most protofibrils appear to adsorb equally onto carbon-coated grids (65); moreover, no TAβ is detected by EM under conditions in which protofibrils are readily detected. One interesting soluble Aβ assembly is Aβ*56 (25). Aβ*56 may be a candidate Aβ assembly responsible for plaque-independent cognitive decline in AD; however, its biophysical features, including molecular mass and marked stability in SDS-PAGE, make it distinct from TAβ.

Finally, this study indicates a novel pathological implication of soluble Aβ assemblies. It is well documented that early and severe neuronal loss in the cholinergic basal forebrain in AD is probably responsible for cognitive decline in AD patients. Previous studies suggested that cholinergic phenotype alone is unlikely to be a sufficient condition for inducing neuronal death in AD. Certain cholinergic neurons, such as those in the pontomesencephalon, are unaffected in AD (66). Notably, cholinergic neurons in the pontomesencephalon are free of NGF receptors, whereas those in the basal forebrain, which are early and severely affected in AD, have NGF receptors (67). Taken together, our results suggest that soluble Aβ assemblies, such as TAβ, are responsible for the loss of NGF-dependent neurons in the cholinergic basal forebrain in AD. A future challenge is the production of a monoclonal neutralizing antibody against TAβ toxicity, which would provide promising therapeutic strategies, as suggested by *in vitro* and *in vivo* studies that selectively targeted Aβ oligomers (68, 69).

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Novel action of apolipoprotein E (ApoE): ApoE isoform specifically inhibits lipid-particle-mediated cholesterol release from neurons

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Abstract

Background: Since the majority of apolipoprotein E (apoE) existing in the cerebrospinal fluid is associated with high-density lipoprotein (HDL), one should focus on the role of the apoE-HDL complex rather than on that of free apoE in cholesterol metabolism in the central nervous system. However, the apoE-isoform-specific effect of apoE-HDL on cholesterol transport remains unclarified.

Results: Here we show that apoE3-HDL induced a marked cholesterol release from neurons, while apoE4-HDL induced little. To elucidate the mechanism underlying this phenomenon, we used a complex of lipid emulsion (EM) with recombinant apoE3 or apoE4 (apoE-EM) at various apoE concentrations. When a small number of apoE molecules were associated with EM, apoE3- and apoE4-EM, induced a marked cholesterol release to a level similar to that induced by EM alone. However, when apoE at given concentrations was incubated with EM, apoE3-EM induced a marked cholesterol release, while apoE4-EM induced little. Under these conditions, a greater number of apoE4 molecules were associated with EM than apoE3 molecules. When an increasing number of apoE molecules were associated with EM, both apoE3-EM and apoE4-EM induced little cholesterol release. Preincubation with β-mercaptoethanol increased the number of apoE3 molecules associated with EM similar to that of apoE4 molecules, indicating that the presence (apoE3) or absence (apoE4) of intermolecular disulfide bond formation is responsible for the association of a greater number of apoE4 molecules to EM than apoE3 molecules.

Conclusion: These results suggest that although apoE and a lipid particle are lipid acceptors, when apoE and a lipid particle form a complex, apoE on the particle surface inhibits the lipid particle-mediated cholesterol release from cells in an apoE-concentration-dependent manner.

Background

It has been shown that the prevalence of Alzheimer's disease (AD) is associated with the polymorphisms of genes related to cholesterol metabolism, including *apolipoprotein E (apoE)*[1], *ATP-binding cassette transporter A1 (ABCA1)* [2], and *CYP46*, the gene encoding cholesterol 24-hydroxylase [3,4]. However, before discussing the association of altered cholesterol metabolism with AD pathogenesis, one should delineate mutual interaction between cholesterol metabolism in the circulation and that in the central nervous system across the blood-brain barrier, and also determine how cholesterol is transported within the central nervous system and how altered cholesterol metabolism induces AD pathologies. In the central nervous system, apoE is one of the major lipid acceptors [5,6] and interacts with ABCA1 [7] to remove cholesterol from cells and generate HDL particles [8] in an apoE-isoform-specific manner [9-11]. This isoform-specific action of free apoE to remove cholesterol and to generate HDL would be a possible cause for the altered cholesterol metabolism in an AD brain. On the other hand, it was shown that the majority of apoE existing in cerebrospinal fluid (CSF) and culture media is associated with HDL and the free form of apoE is at a very low level in the CSF [5,6] and culture media [10,12]. Thus, to determine the apoE-isoform-specific cholesterol transport in the central nervous system, one should focus on the role of the apoE-HDL complex rather than on that of free apoE.

Many studies have shown that HDL stimulates cholesterol release from cultured cells [13-15]. It is believed that this removal of cellular cholesterol induced by HDL involves at least two different mechanisms working cooperatively. One involves the biochemical pathway mediated by apolipoproteins [16,17]. The other involves the physicochemical pathway for the bidirectional movement of cholesterol mediated by aqueous diffusion mechanism [18,19]. However, how these two acceptors contribute and modulate the cholesterol release remains to be clarified. Our recent finding that the apoE-isoform-specific ability to generate HDL is associated with an apoE-isoform-specific ratio of apoE molecules per HDL particle [10] led us to examine the effect of apoE3- and apoE4-containing HDLs or lipid emulsions (EMs) at different apoE ratios on cholesterol release from neurons. Here we show that apoE3-HDL induces a strong cholesterol release, while apoE4-HDL induces a very weak release, and that this isoform-specific effect of apoE associated with lipid particle (HDL or EM) is due to the finding that (1) apoE4 has a higher affinity to lipid particles and thus a greater number of apoE4 molecules bind to lipid particles than apoE3, and (2) with increasing number of apoE molecules covering the surface of lipid particle, both apoE3 and apoE4 inhibit the lipid-particle-mediated cholesterol release. These results suggest that both apoE and a lipid particle are strong lipid acceptors; however, when apoE forms a complex with a lipid particle, apoE on the particle surface inhibits the lipid-particle-mediated cholesterol release by covering its surface.

Results

ApoE-isoform-specific lipid release mediated by apoE3- and apoE4-containing HDL

Human apoE3- and apoE4-containing HDL (apoE3-HDL and apoE4-HDL, respectively) were obtained from the conditioned media of each culture as described in the "Experimental Procedures". As many previous studies demonstrated, apoE3-HDL promoted cholesterol and phosphatidylcholine (PC) release from neurons in an HDL-dose-dependent manner (Fig. 1A). In contrast, surprisingly, the amounts of cholesterol and PC released from cultured neurons in the presence of apoE4-HDL remained very low at any HDL-cholesterol concentrations examined (Fig. 1A). Because our previous study demonstrated that apoE4-HDL contains apoE molecules twofold those in apoE3-HDL per particle [10], we determined the amount of apoE molecules in each HDL fraction added and plotted against the amount of cholesterol and PC released at various apoE concentrations. As shown in Fig. 1B, even when a comparable or a greater amount of apoE molecules was included in the apoE4-HDL than that in the apoE3-HDL, it did not promote lipid release, either (Fig. 1B). Analysis of the time dependence of lipid release mediated by apoE-HDL showed that lipid release mediated by apoE-HDL reached the peak 60 min following the addition of apoE3-HDL and apoE4-HDL (Fig. 2).

Effect of apoE-emulsion complex on cholesterol release from cultured neurons

To elucidate the mechanism underlying the apoE-isoform-specific effect on cholesterol release mediated by apoE-HDL, we used a complex consisting of lipid emulsion (EM) and recombinant human apoE3 or apoE4, because one cannot modulate the number of apoE molecules associated with HDL, but one can modulate apoE number associated with EM. Using this system, we can investigate the effect of apoE on EM-mediated lipid efflux. EM is generated using phosphatidylcholine and triolein and its diameter was determined as described in the Experimental Procedures, and the apoE-EM complexes were then re-isolated before use for the various assays performed. When EM was added to the neuronal cultures in which cholesterol was labeled with ^{14}C-acetate, cholesterol and PC were released in a time- and an EM-dose-dependent manner (Fig. 3), showing that EM serves as a lipid acceptor to release cholesterol. Time-dependent kinetics in terms of lipid release showed that the level of lipids released into the conditioned media saturated 30 min following the commencement of treatment. We determined the amounts of cholesterol and PC released

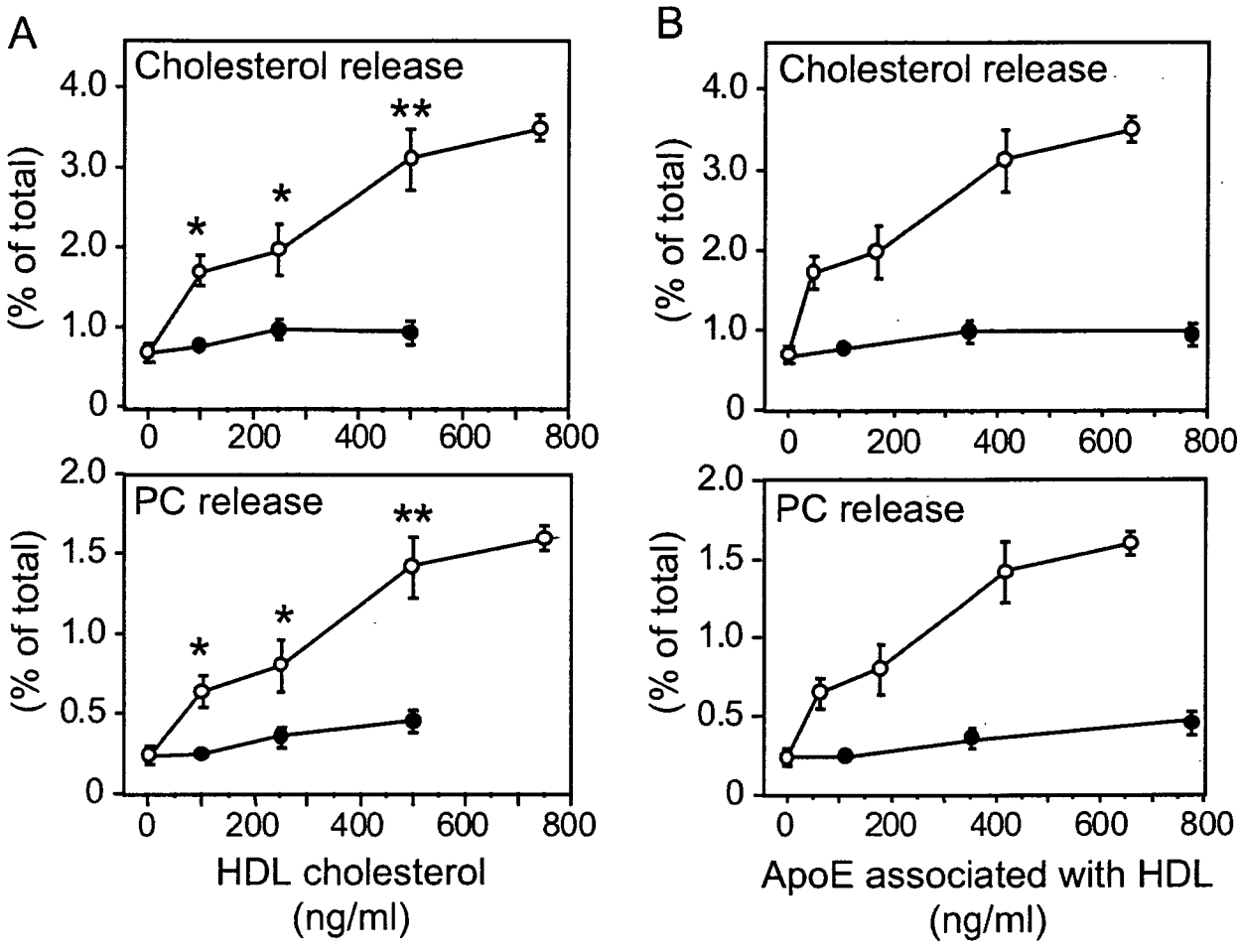

Figure 1

Characterization of cholesterol and PC release from cultured neurons in the presence of apoE3-HDL or apoE4-HDL. Neurons labeled with 37 Bq/ml [^{14}C] acetate were cultured for 3 days at 37°C. These neurons were washed in DMEM three times and incubated in DMEM for 1 h in the presence of apoE3-HDL or apoE4-HDL at various ApoE or HDL cholesterol concentrations, and the amounts of released [^{14}C]-labeled cholesterol and PC were determined. **A)** The amounts of cholesterol and PC released, as induced by apoE3-HDL (○), were significantly greater than those of cholesterol and PC released, as induced by apoE4-HDL (●). **B)** The amounts of cholesterol and PC are plotted against the concentrations of apoE associated with HDL. Data are means ± S.E. of four samples. *$p < 0.05$ and **$p < 0.001$ vs apoE4-HDL at various cholesterol concentrations. Six independent experiments show similar results.

using EM particles at a PC concentration of 5 μg/ml 60 min following the commencement of treatment in the following experiments performed.

ApoE-dose-dependent inhibition of EM-mediated lipid release

We examined the dose-dependent effect of apoE associated with EM on apoE-EM-mediated lipid release. EM alone and the apoE3- and apoE4-EM complexes induced lipid release from neurons; however, with increasing concentration of apoE incubated with EM, the levels of lipid released induced by both apoE3- and apoE4-EM complexes decreased (Fig. 4). However, when the apoE-EM complexes were generated by the incubation of apoE at concentrations of 0.1 and 1 μg/ml with EM (at a PC concentration of 50 μg/ml) and were applied at a PC concentration of 5 μg/ml, apoE3-EM strongly induced cholesterol release, whereas apoE4-EM induced little. The weight ratio of PC per apoE calculated in each treatment is shown in Table 1. The ratio of ratio of PC per apoE at apoE concentrations of 0.1,1,10, and 30 μg/ml was greater in the apoE3-EM complex than in the apoE4-EM complex,

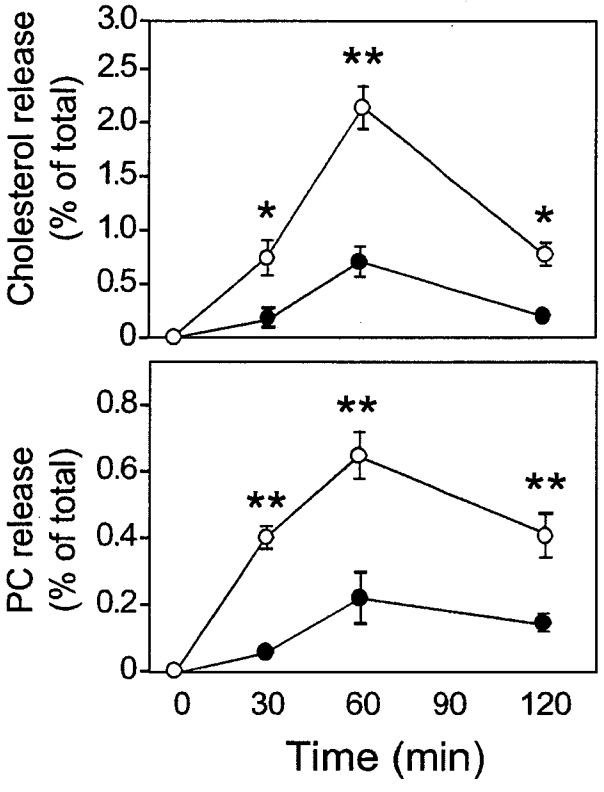

Figure 2
Time-dependent release of cholesterol and PC from cultured neurons in the presence of apoE3-HDL or apoE4-HDL. The kinetics of cholesterol and PC release in the presence of apoE3- or apoE4-HDL at a cholesterol concentration of 500 ng/ml were determined. Both apoE3- (○) and apoE4-HDL (●) induced cholesterol and PC release in a time-dependent manner. Data are means ± S.E. of four samples. *p < 0.001 and **p < 0.0001 vs apoE4-HDL. Six independent experiments showed similar results.

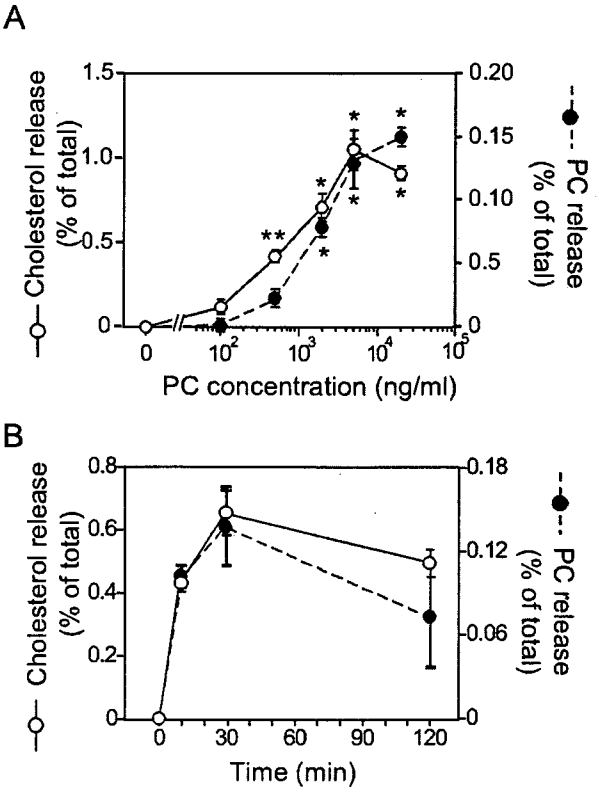

Figure 3
EM mediated cholesterol and PC release from cultured neurons. Neurons labeled with 37 Bq/ml [^{14}C]acetate were cultured for 3 days at 37°C. These neurons were washed in DMEM three times and further incubated in DMEM for 1 h **(a)** or various times **(b)** in the presence of EM at various PC concentrations. The conditioned media were then collected and the amounts of [^{14}C]-labeled cholesterol and PC were determined. **(a)** EM-induced cholesterol (○) and PC (●) release from cultured neurons occurs in an EM-PC-dose-dependent manner. **(b)** The time-dependent release of cholesterol (○) and PC (●) from cultured neurons in the presence of EM at PC concentration of 5 μg/ml is shown. Data are means ± S.E. of four samples. *p < 0.0001 and **p < 0.001 vs PC at 0 ng/ml and time at 0 h. Four independent experiments showed similar results.

indicating that apoE4 has higher binding affinity to EM than apoE3. Interestingly, when the ratios (PC/apoE) were (a) 496 ± 97 (for apoE3) and 269 ± 85 (for apoE4), and (b) 23 ± 6 (for apoE3) and 16 ± 6 (for apoE4), which were obtained by incubating EM with apoE at concentrations of (a) 0.1 and (b) 1.0 μg/ml, respectively (Table 1), the apoE-isoform-specific cholesterol release was observed (Fig. 4) as was the case for apoE-HDL (Fig. 1), that is, apoE3-EM and apoE3-HDL induced cholesterol release from neurons, whereas apoE4-EM and apoE4-HDL induced little release.

We next examined whether the formation of the apoE-EM complex is required for apoE to attenuate the EM-mediated lipid release. When apoE3 and apoE4 at 10 μg/ml were preincubated with 5 μg/ml EM, lipid release in the presence of apoE3-EM or apoE4-EM was significantly reduced compared with that in the presence of EM. On the other hand when they were added into the culture medium without preincubation, they did not inhibit lipid release mediated by EM (Fig. 5).

Dimerization of apoE3 via disulfide bonds alters binding affinity of apoE3 to be similar to that of apoE4

Because apoE3 differs from apoE4 by one amino acid at residue 152 having cysteine instead of arginine, we exam-

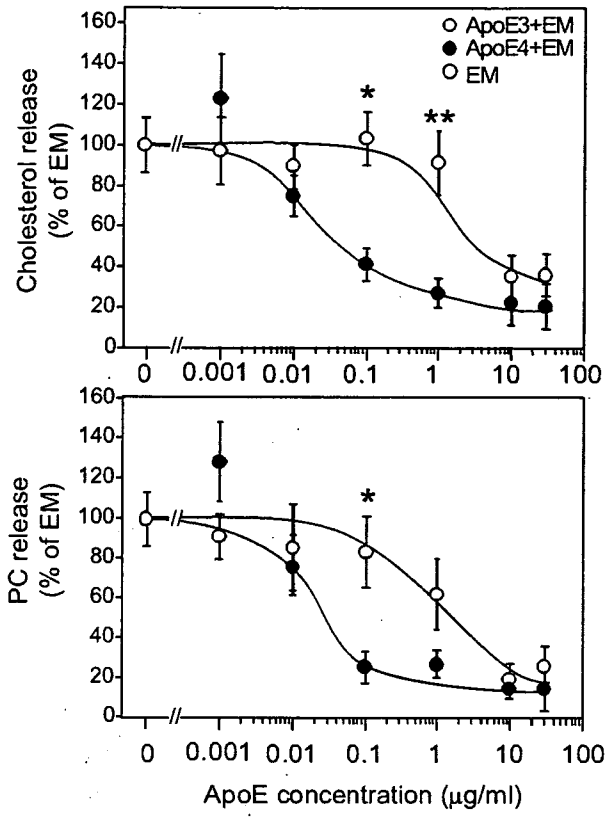

Figure 4
Effect of apoE associated with EM on EM-mediated cholesterol and PC release. Cultured neurons were incubated with serum-free N2 medium containing 37 Bq/ml [^{14}C]acetate for 3 days at 37°C. These neurons were washed in DMEM three times and further incubated in DMEM for 1 h in the presence of the apoE-EM complex at various apoE concentrations. The conditioned media were then collected and the amounts of [^{14}C]-labeled cholesterol and PC released were determined. The amounts of cholesterol and PC released into the conditioned media in the presence of apoE3-EM (○) or apoE4-EM (●) are shown. Data are means ± S.E. of four samples. *$p < 0.01$ and **$p < 0.005$ vs apoE4-EM. Six independent experiments showed similar results.

ined whether the dimerization of apoE3 molecules via disulfide bonds is responsible for the apoE-isoform-specific binding affinity to lipids. In the presence of 5% β-mercaptoethanol, the binding affinity of apoE3 increased to a level similar to that of apoE4 (Fig. 6). The next question is whether EM associated with apoE3 preincubated with β-mercaptoethanol, loses its ability to release cholesterol. However, this experiment was difficult to perform due to the toxic effect of β-mercaptoethanol on neurons.

Discussion

Previous studies showed that HDL induces cholesterol release from various cell types [15,18,20]. The present study also showed that apoE3-HDL induced a strong cholesterol release, whereas apoE4-HDL induced a weak cholesterol release from neurons. As a mechanism underlying this apoE-isoform specificity, we showed a novel action of apoE, that is, although apoE is a lipid acceptor, when apoE is associated with lipid particles such as HDL and EM, apoE inhibits lipid-particle-mediated cholesterol release in an apoE-dose-dependent manner. We also found that more apoE4 molecules are associated with HDL or EM than apoE3. This may explain why apoE4 associated with HDL or EM inhibits HDL- or EM-mediated cholesterol release, whereas apoE3 does not. We also found that the dimerization of apoE3 by disulfide bonds causes the apoE-isoform dependence of the apoE binding affinity to lipids.

Table 1: Binding of apoE3 and apoE4 molecules to emulsion particles

apoE concentration (μg/ml)		0.1	1	10	30
Weight ratio of PC per apoE	apoE3	496 ± 97	23 ± 6	7 ± 2	8 ± 3
	apoE4	269 ± 85	16 ± 6	6 ± 1	7 ± 1

Lipid emulsions were prepared by the method described previously [36] using a high-pressure emulsifier. The emulsion used had a particle size of 34.7 ± 5.2 nm and a weight ratio of TO/PC was 1.63 ± 0.07. For the generation of apoE-EM complex, apoE at various concentrations, from 0.1 to 30 μg/ml, was incubated with EM at PC concentration of 5.0 μg/ml in a 5-ml solution for 1 hr at room temperature. The apoE-EM complex was isolated and assayed by the untracentrifugation method as previously reported [24, 37]. The weight ratio of apoE and PC were determined as described in the Experimental Procedures. Data are means ± S.E. of four samples.

Cellular cholesterol release is mediated by two distinct mechanisms. One is the reaction of lipid-free apolipoproteins, such as apoE or apoAI, and any other molecules containing a certain amount of amphipathic α-helix with a cellular surface protein, ABCA1, resulting in the removal of cellular lipids and the subsequent generation of HDL-like particles [16,21]. The other one is a nonspecific physicochemical interaction causing cholesterol diffusion and its exchange between plasma membrane and lipid particles mediated by EM and liposomes. The cellular cholesterol release mediated by HDL likely involves both mechanisms, because HDL in the central nervous system contains at least two potential acceptors, apoE and lipids. One may reasonably raise questions as to which apoE isoform and to what extent lipids contribute to the HDL-mediated cholesterol release. Previous studies showed that apolipoprotein AI (apoAI) dissociated from HDL cooperatively induces cholesterol release [21,22] and that cholesterol release mediated by HDL is, in part, mediated by apoAI dissociated from HDL in an ABCA1-dependent manner [23]. However, our previous study showed that the level of free apoE in conditioned media and physiological fluid is underdetectable and almost all of the apoE is recovered from the HDL fraction [10].

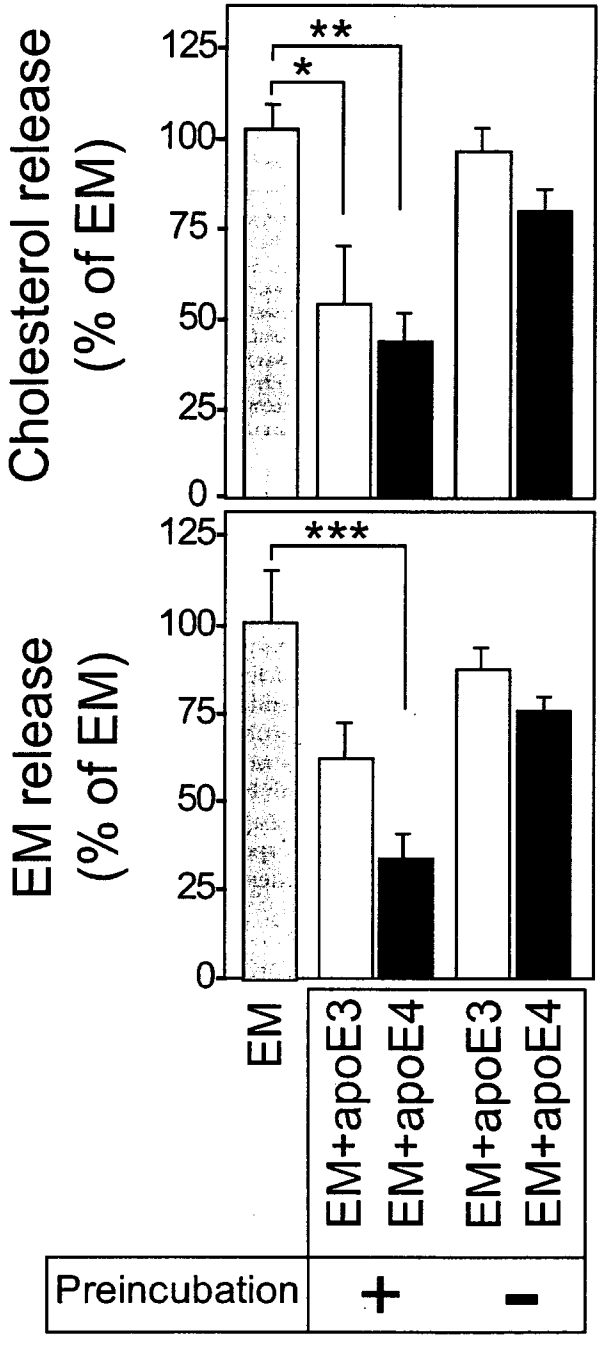

Figure 5
ApoE inhibits EM-mediated lipid release when apoE is associated with EM particles. ApoE-EM complexes were prepared as described in the Experimental Procedures. Neurons were prepared as described in Figure 4 until treatment. Neurons were treated with EM (5 μg/ml); the apoE3-EM complex (10 μg/ml, and 5 μg/ml, respectively) and the apoE4-EM complex (10 μg/ml, and 5 μg/ml, respectively), which was preincubated and isolated by centrifugation; apoE3 (10 μg/ml) and EM (5 μg/ml) or apoE4 (10 μg/ml) and EM (5 μg/ml), which were added separately. Data are means ± S.E. of four samples. *$p < 0.05$, **$p < 0.002$, and ***$p < 0.02$ between the values indicated. Three independent experiments showed similar results.

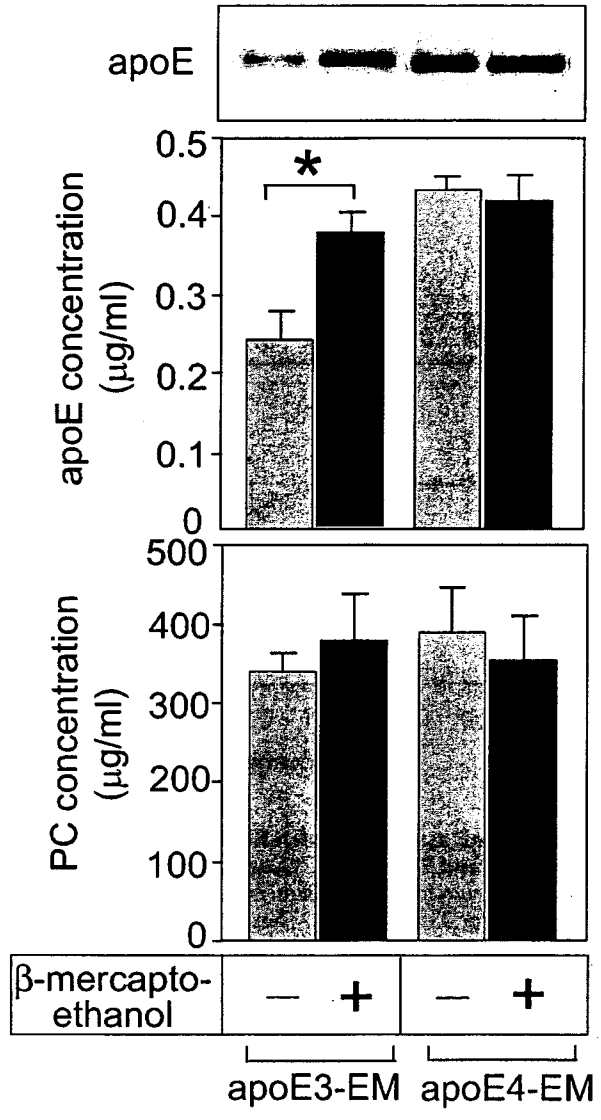

Figure 6
Dimerization of apoE3 by disulfide bonds causes the difference in apoE isoform-specific binding affinity. EM and apoE3 (or apoE4) were incubated in the presence or absence of 5% β-mercaptoethanol for 1 h and EM-PC concentrations and the number of apoE molecules in the apoE-EM complex was determined as described in the Experimental Procedures. Data are means ± S.E. of three samples. *$p < 0.001$. Three independent experiments showed similar results.

Moreover, as we previously reported [10], apoE4-HDL contains number of apoE molecules per particle twofold that of apoE3; however, apoE4-HDL induced a very-weak cholesterol release, whereas apoE3-HDL induced a strong cholesterol release (Figs. 1A and 1B). These results suggest that HDL harboring apoE4 on its surface does not induce cholesterol release. That is, apoE4 on the HDL surface inhibits cholesterol release mediated by HDL. This notion is confirmed by our experiments performed to determine the effect of apoE on EM-induced cholesterol release using the apoE-EM complex at various apoE concentrations on the EM surface, that is, with increasing number of apoE molecules associated with EM, not only apoE4-EM, but also apoE3-EM lose their ability to induce cholesterol release, suggesting that apoE associated with EM strongly inhibits EM-mediated cholesterol release. Interestingly, the apoE-EM complex at certain apoE concentrations and apoE/EM particle ratios induces cholesterol release in an apoE-isoform-specific manner; apoE-EM3 induces cholesterol release, but apoE4-EM does not (Fig. 4), as observed in the cases of apoE3-HDL and apoE4-HDL (Fig. 1). As shown previously [24], our data confirm that more apoE4 molecules are associated with HDL or EM than apoE3 (Table 1). These results lead us to propose a novel hypothesis that apoE molecules covering the surface of lipid particles inhibit the physicochemical interaction occurring between lipids and cell membrane. There are greater numbers of apoE4 molecules associated with EM and HDL, which in turn inhibit the EM- and HDL-mediated physicochemical exchange of cholesterol, whereas in the case of apoE3, the smaller number of apoE3 molecules on the EM or HDL surface allows cholesterol influx to EM or HDL surface from the cell membrane (Fig. 7b).

One may raise the issue that apoE-HDL/EM internalization via several apoE receptors expressed on neurons and recycled-apoE-mediated cholesterol efflux may explain apoE-isoform-dependent cholesterol efflux induced by apoE-HDL/EM. The recycle of apoE4 has been shown to be significantly decreased, resulting in a decreased level of cholesterol efflux [25]. Thus, it is possible that the poor recycling of internalized apoE4 is responsible for the reduced ability of apoE4-containing particles to release cholesterol from neurons. However, the observation that a greater amount of apoE3 associated with EM induces a smaller amount of cholesterol efflux compared with EM without apoE3 (Fig. 4) indicates that this is not the case.

It has been shown that HDL in the central nervous system contains apoE and that neurons express apoE receptors [26]. Thus, different from HDL in systemic circulation, HDL in the central nervous system is internalized into cells via apoE receptors to supply cholesterol to neurons (Fig. 7a). One may question, which is the net cholesterol transport, release from or supply to cells, in the presence of apoE-HDL and apoE-EM? It has been shown that HDL serves as the net cholesterol supplier to neurons. Previous studies showed that HDL promotes synaptogenesis [27], synaptic plasticity [28], and elongation of axons [29], and strongly suppresses cholesterol synthesis (unpublished data), indicating that HDL-cholesterol is taken up by neurons and used for axonal elongation and synaptogenesis.

If HDL, as a whole, functions as a net cholesterol supplier to neurons, what is the biological significance of apoE-HDL-mediated cholesterol release from neurons? Because physicochemical interaction causes a bidirectional cholesterol exchange (nonspecific cholesterol diffusion) between HDL cholesterol and cholesterol in the plasma membrane, it is reasonable to assume that cholesterol exchange may contribute to the maintenance of a fresh supply of cholesterol in the plasma membrane by replacing accumulated oxidized cholesterol in the membrane. Thus, the isoform-specific apoE-HDL action on cholesterol exchange suggests that the apoE3-HDL complex has a greater ability to maintain a fresh supply of cholesterol in the plasma membrane (Fig. 7b). The lower ability of apoE4-HDL may result in the accumulation of oxysterols in the plasma membrane, leading to altered membrane functions e.g., signal transduction, enzyme activities, and ion channel properties.

The last issue to be addressed is the cause of the apoE-isoform dependence of the preferential association of apoE with HDL and EM particles. Previous studies showed that apoE3 forms a disulfide-linked homodimer in plasma [30] and in culture media [10]. Thus, we determined whether this dimerization of apoE3 is responsible for the lesser number of apoE3 molecules associated with EM. The treatment of apoE3 with β-mercaptoethanol significantly increased the number of apoE3 molecules associated with EM (Fig. 6), indicating that when apoE3 remains as a monomer, more apoE3 monomers can associate with EM than apoE3 dimers. The next question is whether EM associated with apoE3, which is treated with β-mercaptoethanol, loses its ability to induce strong cholesterol release. However, this experiment is difficult to perform due to toxic effect of β-mercaptoethanol on neurons. Regarding the effect of apoE polymorphism on lipid interaction, it has been suggested that domain interaction mediated by a salt bridge between Arg-61 in the N-terminus and Glu-255 in the C-terminus, leading to a compact structure, results in the preferential binding of apoE4 to very low density lipoproteins [11,31,32]. The apoE4 domain interaction has been observed in vivo in Arg-61 knock-in mice [33]. Taken all together, it is possible that in addition to structural differences within an apoE molecule such as domain interaction, dimerization also has an effect on the apoE structure (which may also affect domain interaction), leading to the enhancement of the

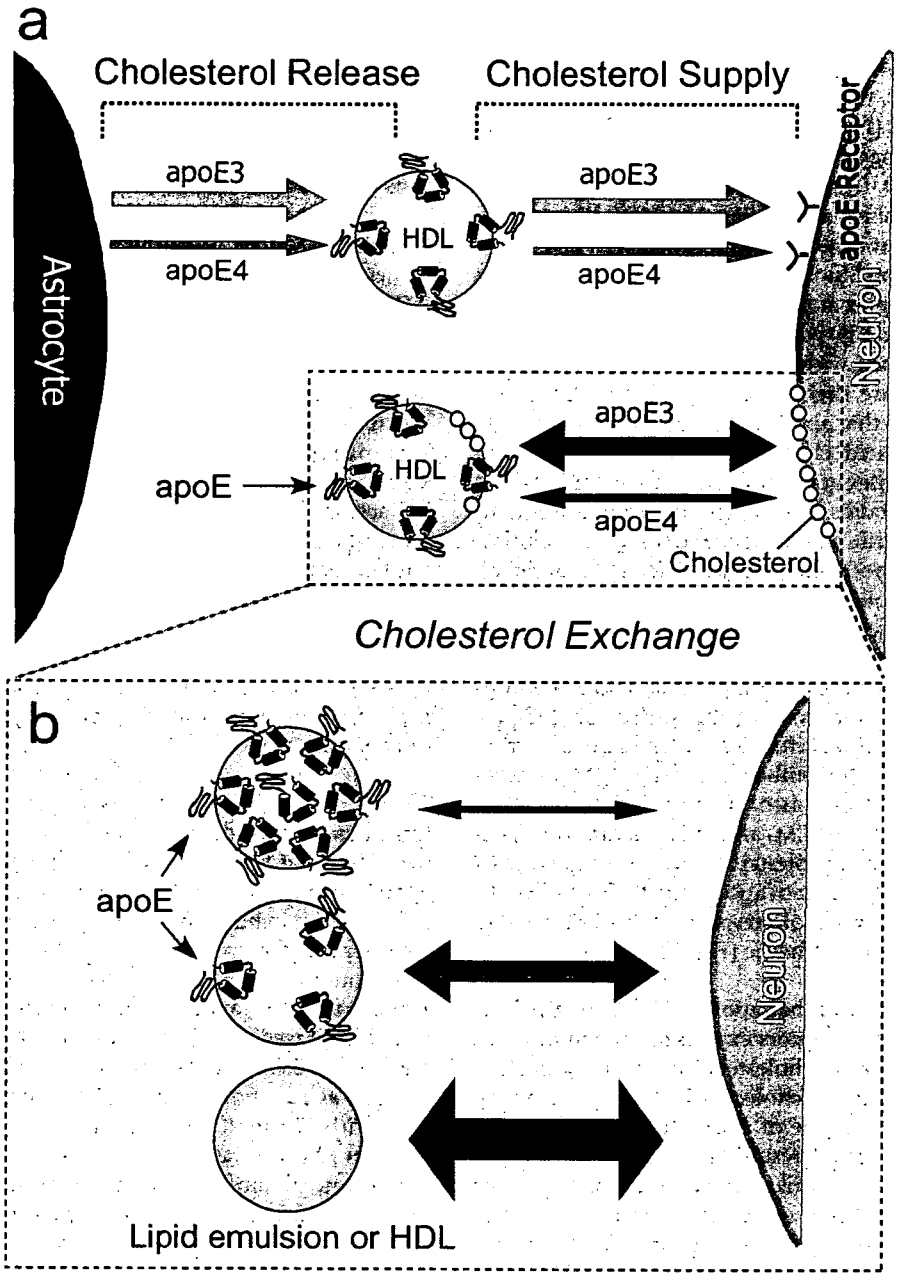

Figure 7

Schema showing the apoE-isoform-specific effect of apoE-HDL and apoE-EM complex on HDL- and EM-mediated cholesterol release. Lipid-free apoE3 released from astrocytes generates a greater number of HDL particles than apoE4 with a similar number of apoE molecules [10], indicating that apoE3-expressing astrocytes can supply more cholesterol as HDL to neurons via apoE receptors **(a)**. Lipid particles such HDL and EM have another role in cholesterol metabolism, that is, physicochemical and nonspecific cholesterol exchange between lipid particles and the cell membrane. Our present results show that with increasing number of apoE molecules on the particle surface, apoE inhibits the particle-mediated cholesterol exchange **(b)**. Because a greater number of apoE4 molecules bind to lipid particles (HDL and EM) than apoE3 molecules, apoE4 inhibits cholesterol exchange (release) by covering the lipid surface, whereas a smaller number of apoE3 molecules on lipid particle surface allows apoE3-HDL- or apoE3-EM-mediated cholesterol release **(b)**.

apoE-isoform-specific effect on the interaction between apoE and lipids.

Conclusion

Here we have shown that although apoE and a lipid particle such as EM are lipid acceptors, when apoE and a lipid particle form a complex, apoE inhibits the lipid particle-mediated cholesterol release from cells in an apoE-dose-dependent manner, probably owing to the occupation of the surface of the lipid particle, thereby inhibiting lipid diffusion and exchange. The observation that a greater number of apoE4 molecules are associated with EM than apoE3 may explain the apoE-isoform-dependent lipid release induced by the apoE-lipid complex. Because lipid particles such HDL and EM induce physicochemical and nonspecific cholesterol exchange between lipid particles and the cell membrane, the lower ability of the apoE4-lipid complex to release lipids may result in the lower lipid replacement and accumulation of oxysterols in the plasma membrane, leading to altered membrane functions, e.g., signal transduction, enzyme activities, and ion channel properties.

Methods

Animals

The animal care and the experiments using animals were carried out in accordance with institutional guidelines. Mice expressing human apoE4 in place of mouse apoE were generated by the gene-targeting technique taking advantage of homologous recombination in embryonic stem cells (knock-in) as previously described [34]. ApoE3 knock-in mice were generated in the same manner except that the transgene carried apoE3 cDNA in place of apoE4 cDNA. Postnatal day 2 mice that possess the homozygous epsilon 3 (3/3) or epsilon 4 (4/4) allele, and correctly expressing human apoE3 or apoE4 proteins, respectively, were used in this study.

Cell culture

Highly astrocyte-rich cultures were prepared according to a previously described method [10]. In brief, the brains of postnatal day 2 mice were removed under anesthesia. The cerebral cortical fragments were incubated in 0.25% trypsin and 20 mg/ml DNase I in phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4,1.5 mM KH_2PO_4O, 137 mM NaCl and 2.7 mM KC1, pH 7.4) at 37°C for 20 min. The fragments were then dissociated into single cells by pipetting. The dissociated cells were seeded in 75-cm^2 dishes at a cell density of 1 × 10^7 in Dalbecco's modified essential medium (DMEM) containing 10% FBS. After 10 days of incubation in vitro, astrocytes in the monolayer were trypsinized (0.1%) and reseeded onto six-well dishes and maintained in DMEM containing 10% FBS until use. Neuron-rich cultures were prepared from rat cerebral cortices as previously described [35]. Dissociated cells were suspended in the feeding medium and plated onto poly-D-lysine-coated twelve-well plates at a cell density of 2 × $10^5/cm^2$. The feeding medium consisted of DMEM nutrient mixture (DMEM/F12; 50%: 50%) and N2 supplements. More than 99% of the cultured cells were identified as neurons by immunocytochemical analysis using a monoclonal antibody against microtubule-associated protein 2, a neuron-specific marker, on day 3 of culture.

Preparation of HDL released into conditioned media of astrocytes expressing apoE3 or apoE4

Astrocytes in 75-cm^2 dishes were washed in DMEM three times and incubated in 12 ml of DMEM for 5 days at 37°C. After incubation, the astrocyte culture medium was collected, centrifuged at 1, 600 × g for 15 min in a 50-ml plastic tube to exclude cell debris, and adjusted to a discontinuous sucrose gradient, which was prepared in a 14 × 89 mm ultracentrifuge tube (Ultraclear, Beckman, Palo Alto, CA) from the bottom to the top, with 1.5 ml of sucrose at a density of 1.30 g/ml, 3 ml at 1.20 g/ml, 4.5 ml at 1.10 g/ml, and 3 ml at 1.006 g/ml medium. The sample in the sucrose gradient was then centrifuged in an SW41-Ti swing rotor (Beckman, Palo Alto, CA) at 16°C for 48 h at 160,000 × gav. Following density gradient centrifugation, twelve 1.0-ml fractions were collected with a mircopipette from the top gradient. The final fraction was stirred to resuspend the pellet. The density of each fraction was determined using a density meter, DMA35N (Anton Paar, Graz, Austria).

Preparation of lipid emulsions (EM) and apoE-EM complex

Egg yolk phosphatidylcholine (PC) was kindly provided by Asahi Kasei (Tokyo, Japan). Triolein (TO) was purchased from Sigma (St. Louis, MO). Lipid emulsions were prepared by the method described previously [36] using a high-pressure emulsifier (Nanomizer System YSNM-2000AR; Yoshida Kikai Co., Nagoya, Japan). The mixture of TO and PC at a weight ratio of 1:1 was suspended in 50% glycerol in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA and 0.01% NaN_3, and subsequently emulsified under 140 MPa of pressure at 60°C. Glycerol was exhaustively removed by dialysis against PBS overnight. The contaminating vesicles and larger particles were removed by ultracentrifugation. The weight-averaged particle size of emulsions was 34.7 ± 5.2 nm determined from dynamic light scattering measurements (Photal LPA-3000/3100; Otsuka Electronic Co., Osaka, Japan). The concentrations of TO and PC were determined using enzymatic assay kits purchased from Wako Pure Chemicals (Osaka, Japan). After ultracentrifugation, the weight ratio of TO/PC was 1.63 ± 0.07 (mean

± S.D., n = 5). For the generation of the apoE-EM complex, apoE at various concentrations, from 0.001 to 30 μg/ml, was incubated with EM at a PC concentration of 50 μg/ml in a 5 ml solution for 1 h at room temperature. The apoE-EM complex was isolated and assayed by the ultracentrifugation method as previously reported [24,37].

Lipid analysis

The extraction of lipids and the subsequent determination of the amounts of cholesterol and phospholipids in the HDL fraction were carried out according to previously described methods [9]. Aliquots (1.0 ml) of conditioned culture media were transferred to clean glass tubes containing 5.0 ml of chloroform: methanol (2:1 v/v). The organic phases were removed, evaporated under N_2 gas, followed by redissolution in 50 μl of isoprapanol for lipid assay. The amount of total cholesterol was determined using a cholesterol determination kit, LTCII (Kyowa Medex, Tokyo, Japan). The amount of phospholipids was determined using a phospholipid determination kit, PLB (Wako, Osaka, Japan). For the determination of PC level in the apoE+EM complex in the presence of 5% β-mercaptoethanol, PC was extracted with chloroform: methanol (2:1 v/v) as described above. The organic phases were removed, evaporated under N_2 gas. The samples were redissolution in 20 μl of chloroform: methanol (2:1 v/v) and 5 μl of each solution was spotted on a chromarod-SIII quartz rod and analyzed by thin-layer chromatography/flameionization detector (Iatroscan MK-5 ; Iatron Lab., Inc., Tokyo, Japan).

Determination of amount of cholesterol and phosphatidylcholine released from neurons labeled with [^{14}C]acetate

Neurons cultured for 2 days were labeled with 37 Bq/ml [^{14}C] acetate (DuPont NEN) for another 2 days. Two days later, these neurons were washed three times with 1.5 ml of DMEM and incubated in DMEM containing reagents such as apoE3-HDL, apoE4-HDL, apoE3-EM, or apoE4-EM at various concentrations. Aliquots of 1.0 ml each of the conditioned culture media were transferred to clean glass tubes containing 5.0 ml of hexane:isopropanol (3:2 v/v). For the extraction of intracellular lipids, dried cells were incubated in hexane:isopropanol (3:2 v/v) for 1 h at room temperature. The solvent from each sample was evaporated and the organic phases were redissolved in 20 μl (for the condition medium) and 200 μl (for the cells) of chloroform, and 10 μl of each sample was spotted on activated silica gel high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany); the lipids were separated by sequential one-dimensional chromatography using chloroform: methanol: acetic acid: water (25:15: 4: 2, v/v/v/v), followed by another run in hexane: diethylether: acetic acid (80: 30: 1). [^{14}C]-Cholesterol and [^{14}C]-phosphatidylcholine were used as standards. The chromatography plates were exposed to radiosensitive films and each lipid was visualized and quantified with BAS2500 (Fuji Film, Tokyo, Japan).

Immunoblot analysis

Samples of each fraction were dissolved in the sample buffer consisting of 100 mM Tris-HCl (pH 7.4), 10% glycerol, 4% SDS, 10% mercaptoethanol and 0.01% bromophenol blue, and analyzed by 4–20% gradient Trids/tricine SDS-PAGE as previously reported [38]. The separated proteins were transferred onto Immobilon membranes with a semidry electrophoretic transfer apparatus (Nihon Eido, Tokyo, Japan) using a transfer buffer (0.1 M Tris, 0.192 M glycine and 20% methanol). Blots were probed for overnight at 4°C with a goat anti-apoE polyclonal antibody, AB947 (1: 2,000; Chemicon, Temecula, CA). Bands were detected using an ECL kit (Amersham Pharmacia Biotech, UK). For the determination of the concentration of apoE released into the culture medium, signals corresponding to apoE of each sample in the immunoblot membrane were quantified by densitometry using NIH image software, at varying concentrations of synthetic apoE protein (Wako, Tokyo, Japan) as standards. Standard signals were demonstrated to be linear in the range of apoE protein amounts from 0 to 2 μg per lane. ApoE concentrations in the HDL fraction and apoE-EM complex fraction within this range were used for analysis. For immunoblot analysis using anti-ABCA1 antibody, the cultured neurons in a 6-well plate were washed in cold PBS and harvested in 500 μl of 50 mM Tric-HCl (Ph 7.4) solution containing 2 mM EGTA. The cell lysates were sonicated and centrifuged at 700 rpm for 10 min at 4°C. The supernatant of each sample was centrifuged at 14,000 rpm for 20 min at 4°C, and the pellet fractions were resuspended in DW containing 0.45 M Urea, 0.1% TritonX-100, and 0.05% Dithiothretol and used for immunoblot analysis as described above.

Statistical analysis

StatView computer software (Windows) was used for statistical analysis. Statistical significance of differences between samples was evaluated by multiple pairwise comparison among the sets of data using ANOVA and the Bonferoni t-test.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

JSG carried out major part of the experiments. SM and TH prepared microemulsion. MK and SCF generated and provided ApoE3- and ApoE4-knock-in mice. KY contributed to interpret the results and make critical intellectual comments. MM participated in its design and coordination

and was involved in the interpretation of the results and in drafting the manuscript.

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