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Mechanism Underlying Apolipoprotein E (ApoE) Isoform-dependent Lipid Efflux From Neural Cells in Culture

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Abstract

We determined the molecular mechanisms underlying apolipoprotein E (ApoE)-isoform-dependent lipid efflux from neurons and ApoE-deficient astrocytes in culture. The ability of ApoE3 to induce lipid efflux was 2.5- to 3.9-fold greater than ApoE4. To explore the contributions of the amino- and carboxyl-terminal tertiary structure domains of ApoE to cellular lipid efflux, each domain was studied separately. The amino-terminal fragment of ApoE3 (22-kDa-ApoE3) induced lipid efflux greater than 22-kDa-ApoE4, whereas the common carboxyl-terminal fragment of ApoE induced very low levels of lipid efflux. Addition of segments of the carboxyl-terminal domain to 22-kDa-ApoE3 additively induced lipid efflux in a length-dependent manner; in contrast, this effect did not occur with ApoE4. This observation, coupled with the fact that introduction of the E255A mutation (which disrupts domain–domain interaction) into ApoE4 increases lipid efflux, indicates that interaction between the amino- and carboxyl-terminal domains in ApoE4 reduces the ability of this isoform to mediate lipid efflux from neural cells. Dimeric 22-kDa or intact ApoE3 induced higher lipid efflux than monomeric 22-kDa or intact ApoE3, respectively, indicating that dimerization of ApoE3 enhances the ability to release lipids. The adenosine triphosphate-binding cassette protein A1 (ABCA1) is involved in ApoE-induced lipid efflux. In conclusion, there are two major factors, intramolecular domain interaction and intermolecular dimerization, that cause ApoE-isoform-dependent lipid efflux from neural cells in culture.

Keywords

Alzheimer's disease; apolipoprotein E; high-density lipoprotein (HDL); neurons; astrocyte

The lipoprotein found in the central nervous system (CNS) is the high-density lipoprotein (HDL), and apolipoprotein E (ApoE) is one of the major apolipoproteins regulating lipid

transport in CNS (Roheim et al., 1979; Pitas et al., 1987b; Weisgraber et al., 1994). Astrocytes and microglia synthesize and secrete ApoE (Boyles et al., 1985; Nakai et al., 1996), which interacts with adenosine triphosphate (ATP)-binding cassette protein A1 (ABCA1) (Krimbou et al., 2004) to remove cholesterol from cells and generate HDL particles in the cerebrospinal fluid and cultured media (Pitas et al., 1987a; Borghini et al., 1995; LaDu et al., 1998).

ApoE-inducible lipid efflux is ApoE-isoform dependent (Michikawa et al., 2000; Gong et al., 2002; Xu et al., 2004), and ApoE3 generates a similar number of HDL particles to but with a smaller number of ApoE molecules than ApoE4 (Gong et al., 2002). HDL synthesis mediated by ApoE contributes to cholesterol release from the cell membrane. On the other hand, HDL associated with ApoE is taken up by cells via ApoE receptors and the cholesterol in HDL is used for maintaining cholesterol homeostasis in CNS neurons. Thus, this isoform-specific action of ApoE to remove cholesterol and generate HDL may be the cause of altered cholesterol metabolism in an Alzheimer's disease (AD) brain (Demeester et al., 2000; Molander-Melin et al., 2005) and may explain how ApoE4 serves as a strong risk factor for AD development (Corder et al., 1993; Strittmatter et al., 1993). However, the molecular mechanism underlying ApoE-isoform-dependent HDL generation remains to be elucidated.

The ApoE molecule has two distinct domains, namely, the 22-kDa amino-terminal (residues 1–191) and 10-kDa carboxyl-terminal domains (residue 218–299), that unfold independently of each other (Wetterau et al., 1988; Morrow et al., 2000). It has been demonstrated that ApoE4 amino- and carboxyl-domain interaction is responsible for the ApoE-isoform dependent association with lipid particles (Weisgraber, 1990; Dong and Weisgraber, 1996; Saito et al., 2003). The domain interaction in ApoE4 under physiological conditions has also been confirmed at the cellular level (Xu et al., 2004) and in vivo (Raffai et al., 2001; Ramaswamy et al., 2005), suggesting that the presence or absence of the domain interaction can explain ApoE-isoform dependent lipid efflux. In addition, there are ApoE-isoform dependent differences in the structure and stability of the 22-kDa amino-terminal fragment (22-kDa-ApoE), which affect their binding affinities to lipids (Morrow et al., 2000, 2002; Segall et al., 2002; Hatters et al., 2005), suggesting that the 22-kDa-ApoE that lacks the domain interaction may also explain an isoform-dependent lipid efflux.

In this study, we investigated the molecular mechanisms, by which intact ApoE3 has a greater ability to induce cholesterol efflux than intact-ApoE4 by using cultured rat neurons and astrocytes prepared from ApoE knockout mouse brain to exclude the effect of endogenously generated and secreted ApoE. We found that the intramolecular amino- and carboxyl-terminal domain interaction is partially responsible for this ApoE-isoform dependency. To our surprise, 22-kDa-ApoE3 has a greater ability to induce cholesterol efflux than 22-kDa-ApoE4. This is because 22-kDa-ApoE3 forms dimers, whereas 22-kDa-ApoE4 does not. These findings suggest that cholesterol efflux induced by ApoE is regulated by two major factors: the presence or absence of intermolecular dimer formation and the intramolecular domain interaction.

EXPERIMENTAL PROCEDURES

Cell Culture

All experiments were performed in compliance with existing laws and institutional guidelines. Neuron-rich cultures were prepared from rat cerebral cortices as previously described (Michikawa et al., 2001). Cerebral cortices from rat brains were dissected, freed of meninges, and diced into small fragments. Cortical fragments were incubated in 2.5% trypsin and 2 mg/ml DNase I in phosphate-buffered saline (PBS) (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl; pH 7.4) at 37°C for 15 min. The fragments

were then dissociated into single cells by pipetting. The dissociated cells were suspended in a feeding medium and plated onto poly-D-lysine-coated 12-well plates at a cell density of 1×10^6 /ml in Dulbecco modified Eagle's medium nutrient mixture (DMEM/F-12; 50:50%) containing N_2 supplements plus 7.5% bovine albumin fraction V.

Highly astrocyte-rich cultures were prepared according to a previously described method (Michikawa et al., 2001). In brief, brains of postnatal day 2 ApoE knockout mice were removed under anesthesia. The cerebral cortices from the mouse brains were dissected, freed of meninges, and diced into small fragments. Cortical fragments were incubated in 0.25% trypsin and 2 mg/ml DNase I in PBS (8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 137 mM NaCl and 2.7 mM KCl; pH 7.4) at 37°C for 15 min. The fragments were then dissociated into single cells by pipetting. The dissociated cells were seeded in 75-cm² dishes at a cell density of 1×10^7 in DMEM nutrient mixture containing 10% FBS and 1% penicillin/streptomycin solution (Invitrogen Corporation, Carlsbad, CA). After 10 days of incubation in vitro, astrocytes in the monolayer were trypsinized (0.1%) and reseeded onto twelve-well dishes. The astrocyte-rich cultures were maintained in DMEM containing 10% FBS until use.

ApoE Preparation

The full-length human ApoE3 and ApoE4 and their 22- and 10-kDa fragments were expressed and purified as described (Saito et al., 2001). The cDNA for full-length human ApoE3 and ApoE4, the 22-kDa fragments, or the 10-kDa fragment were ligated into a thioredoxin fusion expression vector pET32a and transformed into the *Escherichia coli* strain BL21 star (DE3). The transformed *E. coli* were cultured in LB medium at 37°C, and thioredoxin-ApoE expression was induced with isopropyl- β -D-galactopyranoside for 3 hr. After the bacterial pellet was sonicated and the lysate was centrifuged to remove debris, the fusion protein was cleaved with thrombin to remove thioredoxin from full-length ApoE3, ApoE4 or the 22- or 10-kDa fragment. For the full-length ApoE3 and ApoE4, the fusion protein was complexed with DMPC before it was cleaved with thrombin to protect the protease susceptible internal hinge region. After inactivation of the thrombin with β -mercaptoethanol, the mixture was lyophilized and delipidated, and the ApoE pellet was dissolved in 6 M guanidine-HCl, pH 7.4, containing 1% β -mercaptoethanol. The ApoE was isolated by gel filtration chromatography on a Sephacryl S-300 column. For further purification (>95%), the proteins were subjected to gel filtration with a Superdex 75 column or anion exchange chromatography with a HiTrap Q column.

When we used ApoE and ApoE fragments, the recombinant ApoEs were dissolved in 5 M guanidine-HCl. The resulting solutions were dialyzed against PBS at 4°C for 16 hr. Each ApoE level was determined with a BCA protein assay kit (Pierce, Rockford, IL).

Determination of Levels of Cholesterol and Phosphatidylcholine (PC) Released From Neurons and Astrocytes Labeled With [¹⁴C]acetate

Astrocytes plated in twelve-well dishes were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin solution for 72 hr. The cultures were then treated with 37 kBq/ml [¹⁴C]acetate (Moravek Biochemicals Inc., Brea, CA) for 48 hr. The astrocytes were washed in DMEM two times and treated with 0.3 μ M ApoE in DMEM for 24 hr. To analyze dose-dependent effects, the astrocytes were treated with 0.03 μ M, 0.1 μ M, 0.3 μ M and 1.0 μ M ApoE; to analyze time-dependent effects, the astrocytes were treated and maintained for 8, 24 and 48 hr. The culture medium was quickly removed and the astrocytes were dried at room temperature. 1.0 ml of the conditioned culture medium was extracted with 4.0 ml of hexane/isopropyl alcohol (3:2 v/v). For the extraction of intracellular lipids, dried astrocytes were incubated in hexane/isopropyl alcohol (3:2 v/v) for 1 hr at room temperature. The solvent from each plate was removed and dried under N_2 gas. The organic phase was

redissolved in 200 μ l of hexane/isopropyl alcohol (3:2 v/v), and 10 μ l of each sample was spotted on activated-silica-gel high-performance thin layer chromatography plates (Merck); the lipids were separated by sequential one-dimensional chromatography by using chloroform/methanol/acetic acid/water (25:15:4:2, v/v), followed by another run in hexane/diethyl ether/acetic acid (80:30:1). [14 C]Cholesterol and [14 C]PC 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). The levels of [14 C]cholesterol and [14 C]PC efflux were calculated by the following formula: % efflux = media $\times$ 100/(media + cell).

Dimerization of 22-kDa-ApoE3

The pure 22-kDa-ApoE3 was obtained by reduction with 10 mM dithiothreitol (DTT) in 5 M guanidine-HCl buffer. The dimer was formed by incubation of the monomer in oxygenated 5 M guanidine-HCl at 10 mg/ml for 2 weeks at 4°C. Residual monomer was removed by passage of the protein solution through a thiopropyl Sepharose 6B column (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

Dimerization of Intact ApoE3

The ApoE3 (1 mg/ml in 6 M urea, and 10 mM Tris-HCl) used was a mixture of monomeric and dimeric ApoEs. The mixture was reduced to generate monomeric ApoE3 by adding 10 mM DTT, incubated at 4°C for 16 hr, dialyzed against 6 M urea in 10 mM Tris-HCl solution, and oxidized by stirring at 4°C for 3 days. Then it was loaded onto a 2-ml-bed-volume prepacked 6% cross-linked beaded agarose gel column with a SulfoLink kit (Pierce, Rockford, IL) and equilibrated with PBS. Dimeric ApoE3 was eluted with PBS.

Western Blot Analysis of Dimeric 22-kDa-ApoE3 by Nonreducing Gel Electrophoresis

To determine the ratio of dimeric 22-kDa-ApoE3 in the solution, Western blot analysis was performed under nonreducing conditions. Dimeric 22-kDa-ApoE3 was mixed with the same volume of a 2 $\times$ nonreducing Laemmli buffer consisting of 100 mM Tris-HCl (pH 7.4), 10% glycerol, 4% SDS, and 0.01% bromophenol blue, and analyzed by 4–12% Tris/Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Daiichi Pure Chemicals Co., Tokyo, Japan). The separated proteins were transferred onto Immobilon membranes with a semidry electrophoretic transfer apparatus (Nihon Eido, Tokyo, Japan) with a transfer buffer (0.1 M Tris-HCl (pH 7.4), 0.192 M glycine, and 20% methanol). The blots were probed for 16 hr at 4°C with a goat anti-ApoE polyclonal antibody, AB947 (1:2,000; Chemicon, Temecula, CA). Band detection was carried out with an ECL kit (GE Healthcare UK Ltd., England).

Purification and Carboxamidomethylation of Recombinant Intact ApoE3

ApoE3 was dissolved in 5 M guanidine-HCl, 10 mM EDTA, 200 mM Tris-HCl (pH 8.5), and half of each protein solution was reduced with 10 mM DTT at room temperature for 2 hr and alkylated with 40 mM iodoacetamide in the dark at room temperature for 30 min, as described previously (Franceschini et al., 1990). All the proteins were then purified on an Aquapore RP300 column (2.1 $\times$ 30 mm; Applied Bio-systems; Foster City, CA) by reverse-phase high-performance liquid chromatography (HPLC; model 1100 Series; Agilent Technology, Waldbronn, Germany) with a linear gradient of 36–52% acetonitrile in 0.1% trifluoroacetic acid for 16 min and a linear gradient of 52–76% acetonitrile in 0.1% trifluoroacetic acid for 1 min at a flow rate of 0.2 ml/min. The HPLC-purified ApoE3 was lyophilized and kept at -30°C until use.

Effect of 22-hydroxycholesterol and Glyburide on 22-kDa-ApoEs-induced Lipid Efflux

Neuron cultures were prepared, maintained, and labeled with [^{14}C]acetate, and exposed to 22-kDa-ApoE3 dimer or 22-kDa-ApoE3 monomer at a concentration of 0.3 μM in the presence of 22-hydroxycholesterol (10 μM) or glyburide (500 μM) for 24 hr. The lipids released into the media and the lipids retained in the cells were then determined. The expression level of ABCA1 in the cultures for each treatment was determined by Western blot analysis with anti-ABCA1 antibody (Santa Cruz, Santa Cruz, CA) used as a primary antibody.

RNA Interference

To knock down the endogenous ABCA1, primary cultured neurons and astrocytes were transiently transfected with 50 nM of the synthesized small interfering RNAs (siRNAs) targeting ABCA1 or with the Stealth siRNA negative control (Invitrogen) with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. ABCA1 siRNA sequences is as follows: ABCA1-siRNA sense (5'-CA GGAUUUCCUGGUGGACAAUGAAA-3') and antisense (5'-UUUCAUUGUCCACCAGGAAAUCCUG-3').

Statistical Analysis

StatView computer software (Windows) was used for statistical analysis. The statistical significance of differences between samples was evaluated by multiple pairwise comparisons among the sets of data by ANOVA and the Bonferroni *t*-test.

RESULTS

Primary-cultured neurons and astrocytes were prepared and the levels of cholesterol and PC efflux were determined as described in Experimental Procedures. The levels of cholesterol and PC released from neurons in the medium treated with ApoE3 at 0.3 μM were significantly greater than those in the medium treated with ApoE4 (Fig. 1A). The level of cholesterol released by ApoE4 was 25% of that released by ApoE3. The reduced levels of cholesterol and PC efflux induced by ApoE4 increased significantly, although not to the level induced by ApoE3, when the neurons were incubated with the apoE4 (E255A) mutant (mt-ApoE4), which has altered amino- and carboxyl-terminal domain interaction because there is no electrostatic interaction between Arg61 and Glu255 (Dong and Weisgraber, 1996) (Fig. 1A). The partial recovery in the level of lipid efflux induced by mt-ApoE4 suggests that mechanisms other than the domain interaction are involved in ApoE-isoform-dependent lipid efflux from neurons in culture. Similar results were observed with cultured astrocytes. The levels of cholesterol and PC released from astrocytes in the medium treated with ApoE3 at 0.3 μM were significantly greater than those in the medium treated with ApoE4 (Fig. 1B). The levels of cholesterol and PC efflux induced by ApoE4 increased significantly, although not to the level induced by ApoE3, when the astrocytes were incubated with mt-ApoE4 (Fig. 1B).

We also examined the effect of the ApoE fragments on lipid efflux from cultured neurons and astrocytes to determine which part of the ApoE molecule is responsible for lipid efflux and ApoE isoform dependency. Interestingly, the 22-kDa fragment of ApoE (22-kDa-ApoE) induced cholesterol and PC efflux, which were ApoE-isoform-dependent (Fig. 1A,B). Unexpectedly, the carboxyl-terminal 10-kDa-ApoE and 12-kDa-ApoE, both of which contain the lipid binding site, have a very weak ability to release lipids (Fig. 1A,B).

Figure 2A shows the time-dependent cholesterol and PC efflux from neurons induced by 22-kDa-ApoE3 and 22-kDa-ApoE4 at 0.3 μM . The level of lipids released by 22-kDa-ApoE3

was significantly greater than that by 22-kDa-ApoE4 at time points of 24 and 48 hr (Fig. 2B). The level of cholesterol and PC efflux induced by 22-kDa-ApoE 24 hr after the treatment increased in an ApoE-concentration-dependent manner (Fig. 2B). The levels of cholesterol and PC efflux induced by 22-kDa-ApoE3 were greater than those released by 22-kDa-ApoE4 at 0.3 and 1.0 μ M (Fig. 2B). Time- and ApoE-dose-dependent lipid efflux was also examined with ApoE-deficient astrocyte cultures, and the similar results were observed (Fig. 2C,D).

The results in Figures 1 and 2 indicate that the amino-terminal domain, the 22-kDa fragment, induces lipid efflux in an ApoE-isoform dependent manner, whereas the carboxyl-terminal domain, the 10-kDa fragment, has a very weak ability to induce lipid efflux from cultured astrocytes. These results raise the question of how the amino-terminal and carboxyl-terminal domains contribute to lipid efflux induced by intact ApoE in an ApoE-isoform-dependent manner. To answer this question, we examined whether the carboxyl-terminal domain of ApoE modifies lipid efflux caused by 22-kDa-ApoE. The ApoEs used were 22-kDa-ApoE (amino acids, 1–191) and 22-kDa-ApoE with carboxyl-terminal fragment of various lengths, that is, ApoEs harboring amino acids 1–250, 1–260, 1–272, and 1–299 (intact ApoE). As shown in Figure 3A, cholesterol and PC efflux induced by ApoE3 species from cultured neurons depended on the carboxyl-terminal fragment length; that is, ApoE3 variants with a longer carboxyl-terminal region induced progressively more lipid efflux. However, such is not the case for ApoE4. The addition of a carboxyl-terminal region ending at amino acid 250 increased the level of lipid efflux significantly more than 22-kDa-ApoE4; however, 22-kDa-ApoE4 with a carboxyl-terminal region longer than 250 amino acid residues lost the additional effect of the carboxyl-terminal region on lipid efflux. The levels of cholesterol and PC efflux induced by mt-ApoE4 recovered significantly but partially, and they did not reach those induced by intact ApoE3, similar to the result shown in Figure 1. Similar results were observed when ApoE-deficient astrocyte cultures were used (Fig. 3B).

The above results suggest that the amino-terminal domain basically determines the ability of ApoE to induce lipid efflux and that the carboxyl-terminal region enhances this ability when the amino and carboxyl domain interaction is absent. Because the absence or presence of cysteine at position 112 in the amino-terminal domain differentiates ApoE3 from ApoE4, it is possible to assume that this one-amino-acid difference results in intra- or intermolecular structural differences leading to the domain interaction or dimerization, respectively. Thus, we examined the effect of the dimer formation of 22-kDa-ApoE3 through disulfide bonds on lipid efflux from neurons and cultured astrocytes. To determine directly whether the dimeric form of 22-kDa-ApoE3 induces greater lipid efflux from astrocytes than the monomeric form, the pure dimeric form of 22-kDa-ApoE3 was prepared as described in Experimental Procedures. To obtain a solution containing the pure monomeric form of 22-kDa-ApoE3, 22-kDa-ApoE3 was dissolved in 5 M guanidine-HCl and 10 mM DTT. The resulting solutions were dialyzed against PBS at 4°C for 16 hr and used for the experiment. The purity of dimer and monomer in each sample was confirmed by Western blot analysis (Fig. 4). We also used 22-kDa-ApoE4 as monomeric ApoE molecule for the experiment that used astrocyte cultures because 22-kDa-ApoE4 contains no cysteine and it remains monomeric (Fig. 4B). The results demonstrated that dimeric form of 22-kDa-ApoE3 induced greater lipid efflux than the monomeric form of 22-kDa-ApoE3 and 22-kDa-ApoE4 in both neuron and astrocyte cultures (Fig. 4). Importantly, regardless of ApoE isoform, monomeric 22-kDa-ApoEs induces similar level of lipid efflux (Fig. 4B).

To determine whether such is the case for intact ApoEs, we examined the effect of the dimer formation of ApoE3 through disulfide bonds on lipid efflux from cultured neurons. We obtained dimer-enriched ApoE3 solutions by using a SulfoLink kit as described under

Experimental Procedures. We also used ApoE3 solutions prepared without dimer enrichment, containing monomers and relatively few dimers. We also examined the effect of monomeric ApoEs, namely ApoE4 and ApoE3, whose cysteine was modified by carboxamidomethylation (ApoE3-CM). The levels of cholesterol and PC released from neurons treated with dimer-enriched ApoE3 were 2.9- and 7.6-fold greater than those released from neurons treated with ApoE3 and ApoE3 monomers (ApoE3-CM), respectively (Fig. 5). The effects of ApoE3-CM and ApoE4 on lipid efflux were similar. A Western blot analysis of each sample was performed and results show that the samples contained different amounts of dimers (Fig. 5B). The percentages of ApoE3 dimers as calculated by a densitometric analysis of the bands on the Western blot films were 64.5%, 36.9%, 1.8%, and 1.4% in the dimer-enriched ApoE3, non-dimer-enriched ApoE3, ApoE3-CM, and ApoE4 samples, respectively.

Next we determined the involvement of ABCA1 in 22-kDa-ApoEs-induced lipid efflux. The neuron cultures were treated with 22-kDa-ApoE3 dimers, 22-kDa-ApoE3 monomers, or 22-kDa-ApoE4 (monomers) concomitant with 10 μ M of 22-hydroxycholesterol, an LXR ligand to up-regulate *ABCA1* gene expression (Wang et al., 2001), and the cultures were maintained for 24 hr. After 24 hr incubation, the level of lipids released into the medium was determined. The ABCA1 expression level was enhanced when the neurons were treated with 22-hydroxycholesterol (Fig. 6A). The levels of lipids released by these ApoE fragments were significantly enhanced when the cultures were concomitantly treated with 22-hydroxycholesterol (Fig. 6B). These results suggest that ABCA1 plays a key role in 22-kDa-ApoEs-mediated lipid efflux in neurons. In support of this notion, we have observed that the treatment of neurons with glyburide, an inhibitor of the ABCA1 transporter, resulted in decreased levels of 22-kDa-ApoE3-mediated cholesterol efflux (Fig. 6C). We further examined the effect of ABCA1 knockdown on 22-kDa ApoEs-mediated lipid efflux by using specific siRNAs. The knockdown of ABCA1 in neurons significantly reduced lipid efflux induced by 22-kDa-ApoE3 dimers (Fig. 7).

DISCUSSION

We showed here that lipid efflux induced by ApoE is mainly mediated by the amino-terminal domain of ApoE and modified by the carboxyl-terminal domain. What we found are that the amino-terminal domain of ApoE induces lipid efflux in an isoform-dependent manner and the carboxyl-terminal domain enhances lipid efflux mediated by the amino-terminal domain of ApoE3. In contrast, the carboxyl-terminal domain does not strengthen the lipid efflux mediated by the amino-terminal domain of ApoE4 because of the domain interaction between the amino- and carboxyl-terminal domains. We also found that the lipid efflux induced by these ApoEs is mediated in an ABCA1-dependent manner.

Two of the main findings in this study are that the amino-terminal domain of ApoE, 22-kDa-ApoE, induces lipid efflux and that the extent of lipids released by the amino-terminal domain of ApoE, 22-kDa-ApoE3, is approximately 66% of that induced by intact ApoE3. The carboxyl-terminal domain synergistically and additively modifies lipid efflux mediated by 22-kDa-ApoE3. The additional contribution of the carboxyl-terminal domain is not observed in the case of ApoE4. Basically, 22-kDa-ApoE4 has a very weak ability to induce lipid efflux; moreover, the carboxyl-terminal region of ApoE does not effectively or additively enhance the lipid efflux mediated by 22-kDa-ApoE4. The lack of an additive effect by the carboxyl-terminal region on lipid efflux is likely due to the domain interaction, because an ApoE4 fragment ending at 250 (ApoE1–250) significantly gains in ability to release lipids; however, a carboxyl-terminal region longer than the amino acid 255, glutamate, which interacts with arginine at 61 (called the domain interaction; Dong and

Weisgraber, 1996), does not induce any additive effect on lipid efflux induced by 22-kDa-ApoE4.

Another important finding regarding the effect of the domain interaction is that lipid efflux induced by mt-ApoE4 shows only a partial recovery toward the level exhibited by ApoE3. This indicates that the presence or absence of the amino and carboxyl domain interaction cannot completely explain ApoE-isoform-dependent lipid efflux mediated by intact ApoE and that other mechanisms are responsible for such ApoE-isoform dependency. This is supported by the finding of this study that 22-kDa-ApoE, which has no carboxyl-terminal region and thus has no domain interaction, induces lipid release in an ApoE-isoform-dependent manner.

We have already shown that α -helix formation is required for the high-affinity binding of apolipoprotein A-I to lipids (Saito et al., 2004), and that the binding capacity of 22-kDa-ApoE3 is lower than that of 22-kDa-ApoE4 for lipid particles (Saito et al., 2003). On the basis of the facts that the structural stabilities of 22-kDa-ApoE3 and 22-kDa-ApoE4 determine their binding affinity to lipids (Morrow et al., 2002; Segall et al., 2002; Weers et al., 2003) and that 22-kDa-ApoE4 is less stable than 22-kDa-ApoE3 (Morrow et al., 2000), it is reasonable to predict that the level of lipid efflux induced by 22-kDa-ApoE4 would be greater than that induced by 22-kDa-ApoE3. However, our results show the opposite, indicating that ApoE-isoform-dependent cholesterol efflux is unlikely to be explained by a simple theory linking the structural difference between these two fragments with their binding affinity to lipids.

Therefore, the question arises as to what is the mechanism underlying the isoform dependency of 22-kDa-ApoE-induced lipid efflux. It is possible to assume that 22-kDa-ApoE induces lipid efflux, because the 22-kDa domain contains an amphipathic four-helix bundle (Wilson et al., 1991), and it can bind to and reorganize phospholipid vesicles to form discoidal complexes (Lu et al., 2000; Segall et al., 2002). Surprisingly, 22-kDa-ApoE-induced lipid efflux is ApoE-isoform dependent. Such isoform dependency is likely to be caused by the presence or absence of cysteine at residue 112, which may result in intra- or intermolecular structural changes, forming dimers of 22-kDa-ApoE through disulfide bonds. More direct evidence that the dimeric form of 22-kDa-ApoE3 induces greater lipid efflux from cultured neurons and astrocytes than the monomeric form (Fig. 4) supports this idea. Previous reports have demonstrated that cellular cholesterol efflux is induced by many apolipoproteins in their lipid-free form, including ApoA-I, ApoA-II, ApoA-IV, and ApoCIII in addition to ApoE, all of which harbor multiple segments of amphiphilic helices (Segrest et al., 1992); the reaction still occurs with shorter apolipoproteins but to a lesser extent and only at high concentrations (Bielicki et al., 1992). Synthetic amphipathic helical peptides that mimic the physical properties of amphipathic helical segments of apolipoproteins can also induce cholesterol efflux as long as the peptide has at least two such helical segments (Mendez et al., 1994; Yancey et al., 1995). Consistent with these lines of evidence, when human ApoA-II, a disulfide-linked dimer, is reduced to a carboxyamidomethylation monomeric form, the ability of ApoA-II to induce cholesterol efflux is significantly decreased (Hara et al., 1992). In addition, the disulfide-linked homodimer of ApoE3 has been identified not only in cell culture medium (Gong et al., 2002), but also in human plasma (Weisgraber and Shinto, 1991). The mechanism by which the ApoE and ApoA-II dimers gain their functions to release higher amounts of lipids than ApoE and ApoA-II monomers, respectively, remains to be elucidated.

ABCA1 is involved in apolipoprotein-induced lipid efflux, including that mediated by ApoA-I (Brooks-Wilson et al., 1999; Lawn et al., 1999) and ApoE (Remaley et al., 2001; Krimbou et al., 2004). Regarding its effect on lipid efflux, the carboxyl-terminal fragment of

ApoE (10-kDa-ApoE) induces a strong lipid efflux from non-CNS cells such as macrophages and ABCA1 plays a critical role in this efflux (Vedhachalam et al., 2007). Interestingly, contrary to these findings, 10-kDa-ApoE does not induce lipid efflux from the cultured neurons and astrocytes (Fig. 1). The reason for this discrepancy remains unknown; however, the cell type difference may be a likely reason. In support of this notion, even intact ApoE3 induces a very low level of lipid efflux from macrophages or fibroblasts, and ABCA1 transfection induces a marked lipid efflux mediated by intact ApoE3 in these cells (Smith et al., 1996; Remaley et al., 2001). In contrast, intact ApoE3 itself induces marked lipid efflux from astrocytes without ABCA1 transfection as shown in this study. This may be because apolipoprotein-mediated cholesterol efflux is only apparent in growth-arrested cells (Mendez, 1997).

We have observed that pretreatment with 22-hydroxylcholesterol enhanced ABCA1 expression in neurons and cholesterol efflux induced by ApoE, suggesting that ABCA1 is involved in ApoE- and 22-kDa-ApoE3-mediated cholesterol efflux (Fig. 6). The involvement of ABCA1 has been also demonstrated by the fact that the knockdown of ABCA1 significantly reduced lipid efflux induced by 22-kDa ApoEs (Fig. 7). One cannot exclude the possibility that factors other than ABCA1 that are relevant to biological mechanisms are involved because the involvement of ATP-binding cassette protein G1 has been reported previously (Karten et al., 2006; Kim et al., 2007); however, the lines of evidence in our present study suggest that lipid efflux from cultured neurons induced by ApoE or an ApoE fragment is mediated by ABCA1 function.

It is reasonable to assume that the disruption of the ApoE4 domain interaction by, for example, small molecules that create the ApoE3-like structure is a potential therapeutic target in neurodegenerative diseases including AD (Mahley et al., 2006). However, if the role of ApoE in HDL generation and its supply to neurons are critically involved in neurodegeneration in AD, other approaches that do not modulate the acceptor function, but modulate the cellular factors including ABCA1 expression and subsequent HDL generation, could also be candidate therapeutic targets.

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References

- Bielicki JK, Johnson WJ, Weinberg RB, Glick JM. Efflux of lipid from fibroblasts to apolipoproteins: dependence on elevated levels of cellular unesterified cholesterol. *J Lipid Res.* 1992; 33:1699–1709. [PubMed: 1464753]
- Borghini I, Barja F, Pometta D, James RW. Characterization of subpopulations of lipoprotein particles isolated from human cerebrospinal fluid. *Biochim Biophys Acta.* 1995; 1255:192–200. [PubMed: 7696334]
- Boyles JK, Pitas RE, Wilson E, Mahley RW, Taylor JM. Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J Clin Invest.* 1985; 76:1501–1513. [PubMed: 3932467]
- Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouellette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein

- JJ, Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 1999; 22:336–345. [PubMed: 10431236]
- Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science.* 1993; 261:921–923. [see comments]. [PubMed: 8346443]
- Demeester N, Castro G, Desrumaux C, De Geitere C, Fruchart JC, Santens P, Mulleners E, Engelborghs S, De Deyn PP, Vandekerckhove J, Rosseneu M, Labeur C. Characterization and functional studies of lipoproteins, lipid transfer proteins, and lecithin:cholesterol acyltransferase in CSF of normal individuals and patients with Alzheimer's disease. *J Lipid Res.* 2000; 41:963–974. [PubMed: 10828089]
- Dong LM, Weisgraber KH. Human apolipoprotein E4 domain interaction. Arginine 61 and glutamic acid 255 interact to direct the preference for very low density lipoproteins. *J Biol Chem.* 1996; 271:19053–19057. [PubMed: 8702576]
- Franceschini G, Calabresi L, Tosi C, Gianfranceschi G, Sirtori CR, Nichols AV. Apolipoprotein AII Milano. Disulfide-linked dimers increase high density lipoprotein stability and hinder particle interconversion in carrier plasma. *J Biol Chem.* 1990; 265:12224–12231. [PubMed: 2115514]
- Gong JS, Kobayashi M, Hayashi H, Zou K, Sawamura N, Fujita SC, Yanagisawa K, Michikawa M. Apolipoprotein E (ApoE) isoform-dependent lipid release from astrocytes prepared from human ApoE3 and ApoE4 knock-in mice. *J Biol Chem.* 2002; 277:29919–29926. [PubMed: 12042316]
- Hara H, Komaba A, Yokoyama S. Alpha-helical requirements for free apolipoproteins to generate HDL and to induce cellular lipid efflux. *Lipids.* 1992; 27:302–304. [PubMed: 1518389]
- Hatters DM, Budamagunta MS, Voss JC, Weisgraber KH. Modulation of apolipoprotein E structure by domain interaction: differences in lipid-bound and lipid-free forms. *J Biol Chem.* 2005; 280:34288–34295. [PubMed: 16076841]
- Karten B, Campenot RB, Vance D, Vance JE. Expression of ABCG1, but not ABCA1, correlates with cholesterol release by cerebellar astroglia. *J Biol Chem.* 2006; 281:4049–4057. [PubMed: 16352604]
- Kim WS, Rahmanto AS, Kamili A, Rye KA, Guillemin GJ, Gelissen IC, Jessup W, Hill AF, Garner B. Role of ABCG1 and ABCA1 in regulation of neuronal cholesterol efflux to apolipoprotein E discs and suppression of amyloid-beta peptide generation. *J Biol Chem.* 2007; 282:2851–2861. [PubMed: 17121837]
- Krimbou L, Denis M, Haidar B, Carrier M, Marcil M, Genest J Jr. Molecular interactions between apolipoprotein E and the ATP-binding cassette transporter A1 (ABCA1): impact on ApoE lipidation. *J Lipid Res.* 2004; 46:1457–1465. [PubMed: 15654121]
- LaDu MJ, Gilligan SM, Lukens JR, Cabana VG, Reardon CA, Van Eldik LJ, Holtzman DM. Nascent astrocyte particles differ from lipoproteins in CSF. *J Neurochem.* 1998; 70:2070–2081. [PubMed: 9572293]
- Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM, Oram JF. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *J Clin Invest.* 1999; 104:R25–R31. [PubMed: 10525055]
- Lu B, Morrow JA, Weisgraber KH. Conformational reorganization of the four-helix bundle of human apolipoprotein E in binding to phospholipid. *J Biol Chem.* 2000; 275:20775–20781. [PubMed: 10801877]
- Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E4: a causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc Natl Acad Sci U S A.* 2006; 103:5644–5651. [PubMed: 16567625]
- Mendez AJ. Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. *J Lipid Res.* 1997; 38:1807–1821. [PubMed: 9323590]
- Mendez AJ, Anantharamaiah GM, Segrest JP, Oram JF. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J Clin Invest.* 1994; 94:1698–1705. [PubMed: 7929849]
- Michikawa M, Fan QW, Isobe I, Yanagisawa K. Apolipoprotein E exhibits isoform-specific promotion of lipid efflux from astrocytes and neurons in culture. *J Neurochem.* 2000; 74:1008–1016. [PubMed: 10693931]

- Michikawa M, Gong JS, Fan QW, Sawamura N, Yanagisawa K. A novel action of Alzheimer's amyloid b-protein (Ab): oligomeric Ab promotes lipid release. *J Neurosci*. 2001; 21:7226–7235. [PubMed: 11549733]
- Molander-Melin M, Blennow K, Bogdanovic N, Dellheden B, Mansson JE, Fredman P. Structural membrane alterations in Alzheimer brains found to be associated with regional disease development; increased density of gangliosides GM1 and GM2 and loss of cholesterol in detergent-resistant membrane domains. *J Neurochem*. 2005; 92:171–182. [PubMed: 15606906]
- Morrow JA, Segall ML, Lund-Katz S, Phillips MC, Knapp M, Rupp B, Weisgraber KH. Differences in stability among the human apolipoprotein E isoforms determined by the amino-terminal domain. *Biochemistry*. 2000; 39:11657–11666. [PubMed: 10995233]
- Morrow JA, Hatters DM, Lu B, Hochtl P, Oberg KA, Rupp B, Weisgraber KH. Apolipoprotein E4 forms a molten globule. A potential basis for its association with disease. *J Biol Chem*. 2002; 277:50380–50385. [PubMed: 12393895]
- Nakai M, Kawamata T, Taniguchi T, Maeda K, Tanaka C. Expression of apolipoprotein E mRNA in rat microglia. *Neurosci Lett*. 1996; 211:41–44. [PubMed: 8809843]
- Pitas RE, Boyles JK, Lee SH, Foss D, Mahley RW. Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim Biophys Acta*. 1987a; 917:148–161. [PubMed: 3539206]
- Pitas RE, Boyles JK, Lee SH, Hui D, Weisgraber KH. Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. *J Biol Chem*. 1987b; 262:14352–14360. [PubMed: 3115992]
- Raffai RL, Dong LM, Farese RV Jr, Weisgraber KH. Introduction of human apolipoprotein E4 “domain interaction” into mouse apolipoprotein E. *Proc Natl Acad Sci U S A*. 2001; 98:11587–11591. [PubMed: 11553788]
- Ramaswamy G, Xu Q, Huang Y, Weisgraber KH. Effect of domain interaction on apolipoprotein E levels in mouse brain. *J Neurosci*. 2005; 25:10658–10663. [PubMed: 16291938]
- Remaley AT, Stonik JA, Demosky SJ, Neufeld EB, Bocharov AV, Vishnyakova TG, Eggerman TL, Patterson AP, Duverger NJ, Santamarina-Fojo S, Brewer HB Jr. Apolipoprotein specificity for lipid efflux by the human ABCA1 transporter. *Biochem Biophys Res Commun*. 2001; 280:818–823. [PubMed: 11162594]
- Roheim PS, Carey M, Forte T, Vega GL. Apolipoproteins in human cerebrospinal fluid. *Proc Natl Acad Sci U S A*. 1979; 76:4646–4649. [PubMed: 291993]
- Saito H, Dhanasekaran P, Baldwin F, Weisgraber KH, Lund-Katz S, Phillips MC. Lipid binding-induced conformational change in human apolipoprotein E. Evidence for two lipid-bound states on spherical particles. *J Biol Chem*. 2001; 276:40949–40954. [PubMed: 11533033]
- Saito H, Dhanasekaran P, Baldwin F, Weisgraber KH, Phillips MC, Lund-Katz S. Effects of polymorphism on the lipid interaction of human apolipoprotein E. *J Biol Chem*. 2003; 278:40723–40729. [PubMed: 12917433]
- Saito H, Dhanasekaran P, Nguyen D, Deridder E, Holvoet P, Lund-Katz S, Phillips MC. Alpha-helix formation is required for high affinity binding of human apolipoprotein A-I to lipids. *J Biol Chem*. 2004; 279:20974–20981. [PubMed: 15020600]
- Segall ML, Dhanasekaran P, Baldwin F, Anantharamaiah GM, Weisgraber KH, Phillips MC, Lund-Katz S. Influence of apoE domain structure and polymorphism on the kinetics of phospholipid vesicle solubilization. *J Lipid Res*. 2002; 43:1688–1700. [PubMed: 12364553]
- Segrest JP, Jones MK, De Loof H, Brouillette CG, Venkatachalapathi YV, Anantharamaiah GM. The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. *J Lipid Res*. 1992; 33:141–166. [PubMed: 1569369]
- Smith JD, Miyata M, Ginsberg M, Grigaux C, Shmookler E, Plump AS. Cyclic AMP induces apolipoprotein E binding activity and promotes cholesterol efflux from a macrophage cell line to apolipoprotein acceptors. *J Biol Chem*. 1996; 271:30647–30655. [PubMed: 8940040]
- Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in

- late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A*. 1993; 90:1977–1981. [PubMed: 8446617]
- Vedhachalam C, Narayanaswami V, Neto N, Forte TM, Phillips MC, Lund-Katz S, Bielicki JK. The C-terminal lipid-binding domain of apolipoprotein E is a highly efficient mediator of ABCA1-dependent cholesterol efflux that promotes the assembly of high-density lipoproteins. *Biochemistry*. 2007; 46:2583–2593. [PubMed: 17305370]
- Wang N, Silver DL, Thiele C, Tall AR. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem*. 2001; 276:23742–23747. [PubMed: 11309399]
- Weers PM, Narayanaswami V, Choy N, Luty R, Hicks L, Kay CM, Ryan RO. Lipid binding ability of human apolipoprotein E N-terminal domain isoforms: correlation with protein stability? *Biophys Chem*. 2003; 100:481–492. [PubMed: 12646385]
- Weisgraber KH. Apolipoprotein E distribution among human plasma lipoproteins: role of the cysteine–arginine interchange at residue 112. *J Lipid Res*. 1990; 31:1503–1511. [PubMed: 2280190]
- Weisgraber KH, Shinto LH. Identification of the disulfide-linked homodimer of apolipoprotein E3 in plasma. Impact on receptor binding activity. *J Biol Chem*. 1991; 266:12029–12034. [PubMed: 2050696]
- Weisgraber KH, Roses AD, Strittmatter WJ. The role of apolipoprotein E in the nervous system. *Curr Opin Lipidol*. 1994; 5:110–116. [PubMed: 8044413]
- Wetterau JR, Aggerbeck LP, Rall SC Jr, Weisgraber KH. Human apolipoprotein E3 in aqueous solution. I. Evidence for two structural domains. *J Biol Chem*. 1988; 263:6240–6248. [PubMed: 3360781]
- Wilson C, Wardell MR, Weisgraber KH, Mahley RW, Agard DA. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science*. 1991; 252:1817–1822. [PubMed: 2063194]
- Xu Q, Brecht WJ, Weisgraber KH, Mahley RW, Huang Y. Apolipoprotein E4 domain interaction occurs in living neuronal cells as determined by fluorescence resonance energy transfer. *J Biol Chem*. 2004; 279:25511–15516. [PubMed: 15054100]
- Yancey PG, Bielicki JK, Johnson WJ, Lund-Katz S, Palgunachari MN, Anantharamaiah GM, Segrest JP, Phillips MC, Rothblat GH. Efflux of cellular cholesterol and phospholipid to lipid-free apolipoproteins and class A amphipathic peptides. *Biochemistry*. 1995; 34:7955–7965. [PubMed: 7794908]

Fig. 1.

Cholesterol and PC efflux from cultured neurons and astrocytes induced by ApoEs. Neurons and astrocytes were prepared as described in Experimental Procedures. The neurons (**A**) and astrocytes (**B**) were cultured for 72 hr, labeled with [^{14}C]acetate for 48 hr, and exposed to intact, lipid-free, ApoE3(residues 1–299), ApoE4, mt-ApoE4(E255A), 22-kDa-ApoE3(residues 1–191), 22-kDa-ApoE4, 10-kDa-ApoE(residues 218–299), and 12-kDa-ApoE(residues 192–299) fragments at 0.3 μM for 24 hr. The lipids released into the media and the lipids retained in the cells were extracted and analyzed as described in Experimental Procedures. The percentages of released cholesterol and PC levels over the total levels were calculated. Data are means $\pm$ SE of four samples. * $P < 0.0001$ and ** $P < 0.0005$. Three

independent experiments showed similar results. The basal value of cholesterol and PC efflux in the absence of ApoEs are 1.0 ± 0.1 (%) and 0.4 ± 0.1 (%), respectively.

Fig. 2.

Time- and dose-dependent lipid efflux mediated by 22-kDa-ApoE fragment. Cultured neurons and astrocytes were prepared, maintained, and labeled with [^{14}C]acetate as described in the legend for Fig. 1. For determination of the time-dependent lipid release from neurons (**A**) and astrocytes (**C**), each culture was exposed to 22-kDa-ApoE3 (open circle) and 22-kDa-ApoE4 (closed circle) at 0.3 μM for 8, 24, and 48 hr. For determination of the dose-dependent lipid release from neurons (**B**) and astrocytes (**D**), each culture was exposed to 22-kDa-ApoE3 (open circle) and 22-kDa-ApoE4 (closed circle) at varying concentrations for 24 hr. The lipids released into the media and the lipids retained in the cells were determined as described in Experimental Procedures. (**B**) For determination of

Data are means $\pm$ SE of four samples. $*P < 0.05$ and $**P < 0.0005$ vs. 22-kDa-ApoE4 at each time point. The basal value of cholesterol and PC efflux in the absence of ApoEs are less than 0.8 ± 0.05 and 0.66 ± 0.11 , respectively (**A**), and 0.59 ± 0.04 and 0.39 ± 0.04 , respectively (**B**).

Fig. 3.

Cooperative effects of amino- and carboxyl-terminal regions of ApoE on lipid efflux. Neurons and astrocytes were prepared, maintained, and labeled with [^{14}C]acetate as described in the legend for Fig. 1. The neurons (**A**) and astrocytes (**B**) were then exposed to ApoE with carboxyl-terminal regions of various lengths including 1–250, 1–260, 1–272, and 1–299 (full-length ApoE) at 0.3 μM for 24 hr, and the lipids released into the media and the lipids retained in the cells were determined as described in Experimental Procedures. For comparison, the effect of mt-ApoE4 was also determined. Data are means $\pm$ SE of four samples. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0001$ vs. 22-kDa-ApoE (1–191). The basal value of cholesterol and PC efflux in the absence of ApoEs are less than 0.8 ± 0.05 and 0.66 ± 0.11 , respectively (A, B).

Fig. 4.

The ability of 22-kDa-ApoE to induce lipid efflux depends on the ApoE self-association state, i. e., monomer vs dimer. Preparation of 22-kDa-ApoE3 dimer (22-kDa-ApoE3-D), monomer (22-kDa-ApoE3-M), or 22-kDa-ApoE4 monomer was performed as described in Experimental Procedures. **A:** The same amount of 22-kDa-ApoE3-D or 22-kDa-ApoE3-M was subjected to SDS-PAGE under nonreducing conditions, and Western blot analysis was performed with the anti-ApoE antibody AB947. D; dimers, M; monomer. Neuron cultures were prepared, maintained, and labeled with [14 C]acetate and exposed to 22-kDa-ApoE3-D or 22-kDa-ApoE3-M at 0.3 μ M for 24 hr. The cholesterol and PC efflux by dimeric and monomeric 22-kDa-ApoE3 was determined. **B:** The same amount of 22-kDa-ApoE3 dimers,

22-kDa-ApoE3 monomers, and 22-kDa-ApoE4 monomers was subjected to SDS-PAGE under nonreducing conditions, and Western blot analysis was performed with the anti-ApoE antibody AB947. D; dimers, M; monomer. Astrocyte cultures were prepared, maintained, and labeled with [^{14}C]acetate and exposed to 22-kDa-ApoE3-D, 22-kDa-ApoE3-M, or 22-kDa-ApoE4 monomer at 0.3 μM for 24 hr. The cholesterol and PC efflux by dimeric and monomeric 22-kDa-ApoE3, and monomeric 22-kDa-ApoE4 was determined. Data are means $\pm$ SE of four samples. $*P < 0.0001$ vs. 22-kDa-ApoE3. The basal value of cholesterol and PC efflux in the absence of ApoEs are less than 1.41 ± 0.06 and 0.55 ± 0.03 , respectively (A, B).

Fig. 5.

Effect of intact ApoE dimers on lipid efflux from cultured neurons. A dimer-rich ApoE3 solution was obtained with a column that traps SH residues (monomeric ApoE3s). Carboxamidomethylated ApoE with iodoacetamide was prepared as described in Experimental Procedures. Neuronal cultures were prepared, maintained, and labeled with [^{14}C]acetate and exposed to dimer-enriched ApoE3, ApoE3, ApoE3-CM, and ApoE4 at 0.3 μM for 24 hr. **A:** The levels of cholesterol and PC released into the media and those retained in the cells were determined as described in Experimental Procedures. **B:** To determine the amount of dimers in the samples, Western blot analysis was performed under nonreducing conditions with anti-ApoE antibody AB947 used as the primary antibody. Data are means $\pm$

SE of four samples. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$. The basal value of cholesterol and PC efflux in the absence of ApoEs are 1.54 ± 0.05 and 0.56 ± 0.05 , respectively.

Fig. 6.

The effect of 22-hydroxycholesterol and glyburide on 22-kDa-ApoEs-induced lipid efflux. Neuron cultures were prepared, maintained, and labeled with [^{14}C]acetate as described in Fig. 1, and exposed to 22-kDa-ApoE3 dimer (22-kDa-ApoE3-D), 22-kDa-ApoE3 monomer (22-kDa-ApoE3-M), or 22-kDa-ApoE4 at a concentration of 0.3 μM in the presence of 22-hydroxycholesterol (HC) at a concentration of 10 μM for 24 hr. (A) The expression level of ABCA1 in the cultures for each treatment was determined by Western blot analysis, and (B) the lipids released into the media and the lipids retained in the cells were determined. (C) [^{14}C]acetate-labeled neuron cultures were exposed to 22-kDa-ApoE3 dimer (22-kDa-ApoE3-D), 22-kDa-ApoE3 monomer (22-kDa-ApoE3-M), or 22-kDa-ApoE4 at a

concentration of 0.3 μM in the presence of glyburide (500 μM) for 24 hr. The lipids released into the media and the lipids retained in the cells were determined. Data are means $\pm$ SE of four samples. $*P < 0.05$, $**P < 0.01$, and $***P < 0.0005$. Three independent experiments showed similar results. The basal value of cholesterol and PC efflux in the absence of ApoEs are 5.45 ± 0.51 and 0.58 ± 0.06 , respectively.

Fig. 7.

Lipid efflux by 22-kDa-ApoEs is mediated by ABCA1. The primary neurons, which had been labeled with [^{14}C]acetate and treated with siRNA against ABCA1 for 48 hr, were exposed to 22-kDa-ApoE3 dimer (22-kDa-ApoE3-D) or 22-kDa-ApoE3 monomer (22-kDa-ApoE3-M) at a concentration of 0.3 μM , and maintained for 24 hr. The lipids released into the media and the lipids retained in the cells were then determined as described in Experimental Procedures. Data are means $\pm$ SE of four samples. * $P < 0.0001$, ** $P < 0.002$, *** $P < 0.02$. Three independent experiments showed similar results. The basal value of cholesterol and PC efflux in the absence of ApoEs are 0.89 ± 0.08 and 0.97 ± 0.14 , respectively. Those values are 0.84 ± 0.08 and 0.46 ± 0.07 in the siABCA1 experiment.