

ApoE4 upregulates the activity of mitochondria-associated ER membranes

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Abstract

In addition to the appearance of senile plaques and neurofibrillary tangles, Alzheimer's disease (AD) is characterized by aberrant lipid metabolism and early mitochondrial dysfunction. We recently showed that there was increased functionality of mitochondria-associated endoplasmic reticulum (ER) membranes (MAM), a subdomain of the ER involved in lipid and cholesterol homeostasis, in presenilin-deficient cells and in fibroblasts from familial and sporadic AD patients. Individuals carrying the $\epsilon 4$ allele of apolipoprotein E (ApoE4) are at increased risk for developing AD compared to those carrying ApoE3. While the reason for this increased risk is unknown, we hypothesized that it might be associated with elevated MAM function. Using an astrocyte-conditioned media (ACM) model, we now show that ER-mitochondrial communication and MAM function—as measured by the synthesis of phospholipids and of cholesteryl esters, respectively—are increased significantly in cells treated with ApoE4-containing ACM as compared to those treated with ApoE3-containing ACM. Notably, this effect was seen with lipoprotein-enriched preparations, but not with lipid-free ApoE protein. These data are consistent with a role of upregulated MAM function in the pathogenesis of AD and may help explain, in part, the contribution of ApoE4 as a risk factor in the disease.

Keywords ApoE; cholesterol; cholesteryl esters; endoplasmic reticulum; lipoproteins; MAM; mitochondria; phospholipids

Subject Categories Membrane & Intracellular Transport; Molecular Biology of Disease

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Introduction

Alzheimer's disease (AD), the most common form of dementia in the elderly, is characterized by progressive neuronal loss

accompanied by the formation of extracellular plaques containing β -amyloid ($A\beta$) and of intracellular tangles composed of hyperphosphorylated tau [1]. $A\beta$ results from the processing of the C-terminus of the amyloid precursor protein (APP), first by β -secretase (BACE1) and then by γ -secretase. The active components of γ -secretase are presenilin-1 (PS1) and/or presenilin-2 (PS2), which are aspartyl proteases [1].

In addition to plaques and tangles, AD is associated with an array of biochemical alterations, including perturbations in cholesterol homeostasis [2,3], phospholipid metabolism [4], calcium trafficking [5], and mitochondrial function [6]. Notably, these functions share a common feature in that they are associated with a subcompartment of the endoplasmic reticulum (ER) known as mitochondria-associated ER membranes (MAM). MAM is a region of the ER that communicates with mitochondria, both physically and biochemically, but it has different biophysical properties than bulk ER (e.g., it is a lipid raft-like domain [7–9]). We recently showed that PS1 and PS2, and γ -secretase activity itself, are localized predominantly in MAM [10] and that pathogenic mutations in, or genetic ablation of, PS1 and PS2 increase MAM activity significantly [7]. The convergence of these upregulated MAM functions in AD, and the finding that MAM contains active γ -secretase complexes, has led us to propose that MAM dysfunction is a key event in the pathogenesis AD [7,11,12].

Mutations in APP, PS1, or PS2 result in the early-onset or familial form of AD (FAD), although these mutations account for only 1% of total AD incidence; the vast majority of AD cases are late-onset or sporadic (SAD), and are not inherited in an autosomal dominant manner, as is the case with FAD [13]. Nevertheless, SAD is characterized by the inheritance of genetically determined risk factors, polymorphisms that do not positively determine AD but rather confer an increased likelihood of the disease.

Different isoforms of *APOE* are the most common and validated of these risk factors [14]. *APOE* encodes apolipoprotein E (ApoE), a component of the lipoproteins that transport cholesterol and lipids throughout the body. In the brain, cholesterol is synthesized mainly by astrocytes but not neurons, with astrocyte-derived cholesterol delivered to neurons via high-density lipoproteins (HDL) [15,16].

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The $\epsilon 4$ variant of *APOE*, denoted here as ApoE4 (arginines at positions 112 and 158; allele frequency in Europeans of ~10–30% [17]), confers an increased gene dose-dependent risk for developing AD of ~fourfold with one copy and ~12-fold with 2 copies compared to individuals carrying ApoE3, the most common isotype (Cys112/Arg158; allele frequency of ~70–90%), while the rarest variant, ApoE2 (Cys112/Cys158; allele frequency of ~3–12%), is protective against AD [18]. While it is unclear exactly how the two coding mutations that determine the *APOE* genotype modulate AD risk, a proposed mechanism implicates differential ApoE-mediated aggregation and clearance of A β [14], but it is still unknown whether the normal physiological function of ApoE in cholesterol and lipid homeostasis relates to increased AD risk [14].

Given that ApoE4 is the main genetic risk factor for AD and that MAM alterations in cholesterol and lipid homeostasis are features of AD, we investigated the possibility that relative to ApoE3, ApoE4 exerts effects that lead to MAM dysfunction. Specifically, we compared MAM activity of cells treated with astrocyte-conditioned media (ACM) generated from gene replacement mouse astrocytes expressing either ApoE3 or ApoE4. We found that, compared to ApoE3 ACM, ApoE4 ACM increased the MAM activity of target cells significantly. These findings imply that ApoE can modulate ER–mitochondrial communication, which may help explain, in part, the contribution of ApoE4 to the risk of developing AD.

Results and Discussion

We developed a protocol to treat fibroblasts with ACM derived from knock-in mice expressing either human ApoE3 or ApoE4 under the control of the endogenous mouse ApoE promoter [19]. We cultured the astrocytes for 3 days [20], and then incubated human fibroblasts, which, like neurons, normally do not express ApoE [21] (Fig EV1), in ACM for 1 day [22] (see scheme in Fig 1A). Prior to all experiments, we measured the amount of ApoE present in the ACM, which typically contained more ApoE3 than ApoE4, as expected [23,24] (Fig EV1), and then applied the ACM's on an "equal ApoE" basis. Following treatment with the ACM's, we measured various aspects of MAM function.

Phospholipid synthesis is a major function of MAM [25]. In particular, phosphatidylserine (PtdSer) is synthesized in MAM [26]. PtdSer is then transported from the ER to mitochondria, where it is decarboxylated to produce phosphatidylethanolamine (PtdEtn); PtdEtn can then be transported back to the ER, where it is either methylated to produce phosphatidylcholine (PtdCho) or distributed to other membranes of the cell [27]. Although some PtdEtn is produced on the cytosolic face of the ER by CDP-ethanolamine exchange via the Kennedy pathway [28], the majority of PtdEtn is produced by the conversion of PtdSer to PtdEtn in mitochondria [25]; this conversion is an established marker for ER–mitochondrial communication [25].

We had shown previously that presenilin-mutant cells, including AD fibroblasts, synthesized significantly more PtdSer and PtdEtn via the MAM pathway than did wild-type controls [7]. We therefore incubated normal human fibroblasts in ACM medium containing ^3H -Ser and measured the incorporation of the label into newly synthesized ^3H -PtdSer and ^3H -PtdEtn [29].

Relative to the values obtained with ApoE3 ACM, we found a significant increase in the synthesis and transport of both ^3H -PtdSer (fold increase of $\sim 2.0 \pm 0.3$) and ^3H -PtdEtn ($\sim 2.2 \pm 0.3$) in human fibroblasts treated with ApoE4 ACM (Fig 1B). We also performed the same experiment on explanted hippocampal neurons derived from 1- to 3-day-old mice and found a significant increase in ApoE4-mediated PtdEtn synthesis ($\sim 1.8 \pm 0.5$), while the increase in PtdSer trended to significance ($\sim 1.7 \pm 0.7$) (Fig 1B). Notably, this increase in PtdEtn synthesis did not appear to be the result of increased expression of the PtdEtn biosynthetic machinery, as the expression of phosphatidylserine decarboxylase (PISD), a key enzyme of PtdEtn synthesis that converts PtdSer to PtdEtn within the mitochondrial matrix [30], was similar in ApoE3 and ApoE4 ACM-treated fibroblasts (Fig EV2).

To establish that the increase in phospholipid production was indeed mediated by MAM, we performed the same assay using mouse embryonic fibroblasts (MEFs) in which the mitofusin 2 gene (*Mfn2*) had been knocked out [30]. *Mfn2*, in addition to its role in mitochondrial fusion, tethers ER to mitochondria [31]; importantly, genetic ablation of *Mfn2* reduces this connectivity [31]. Consistent with this loss in interorganellar communication, we had previously established that PtdSer and PtdEtn production is reduced significantly in *Mfn2*-KO MEFs [7]. We therefore asked whether the ApoE4-mediated increase in phospholipid synthesis could be abrogated in *Mfn2*-KO cells. Similar to what we observed in the human fibroblasts [31], treatment of wild-type MEFs with ApoE4 ACM resulted in an increase in PtdSer ($\sim 1.5 \pm 0.3$) and PtdEtn ($\sim 1.6 \pm 0.2$) production compared to ApoE3 ACM (Fig 1C). However, we found no such increase in either PtdSer ($\sim 1.0 \pm 0.2$) or PtdEtn ($\sim 0.9 \pm 0.1$) production in the *Mfn2*-KO MEFs (Fig 1C), strongly suggesting that the ApoE4-mediated effect on phospholipid metabolism was indeed the result of increased ER–mitochondrial communication.

Phospholipids produced at the MAM, including PtdEtn, are subsequently transported to the other membranous compartments of the cell, including the plasma membrane. Cell surface PtdEtn can be detected using cinnamycin and duramycin, two highly related lantibiotics that bind specifically to PtdEtn on the cell surface and induce cell death in a PtdEtn concentration-dependent manner [32,33]. We had previously shown that the elevated levels of PtdEtn produced by presenilin-mutant cells, including AD fibroblasts, were significantly more sensitive to both cinnamycin and duramycin than were wild-type controls [7]. To test whether there was also increased lantibiotic sensitivity in our experimental paradigm, we applied duramycin to ACM-treated fibroblasts and measured cell death. We found that ApoE4 ACM-treated fibroblasts were significantly more sensitive to duramycin treatment ($\sim 50 \pm 20\%$ cell death) than were ApoE3 ACM-treated cells ($\sim 15 \pm 7\%$; Fig 1D). The ability of duramycin to induce cell death was abrogated by the addition of exogenous PtdEtn to the medium (Fig 1D), supporting the specificity of the assay. Thus, these data are consistent with the ^3H -serine incorporation data and support the conclusion that ApoE4 plays a role in MAM-mediated phospholipid synthesis and transport.

ApoE is present in the brain as a component of HDL-like particles that are secreted by astrocytes and are taken up by neurons by various lipoprotein receptors [15,16]. Following receptor-mediated endocytosis, the ApoE-containing HDL-like particles are hydrolyzed

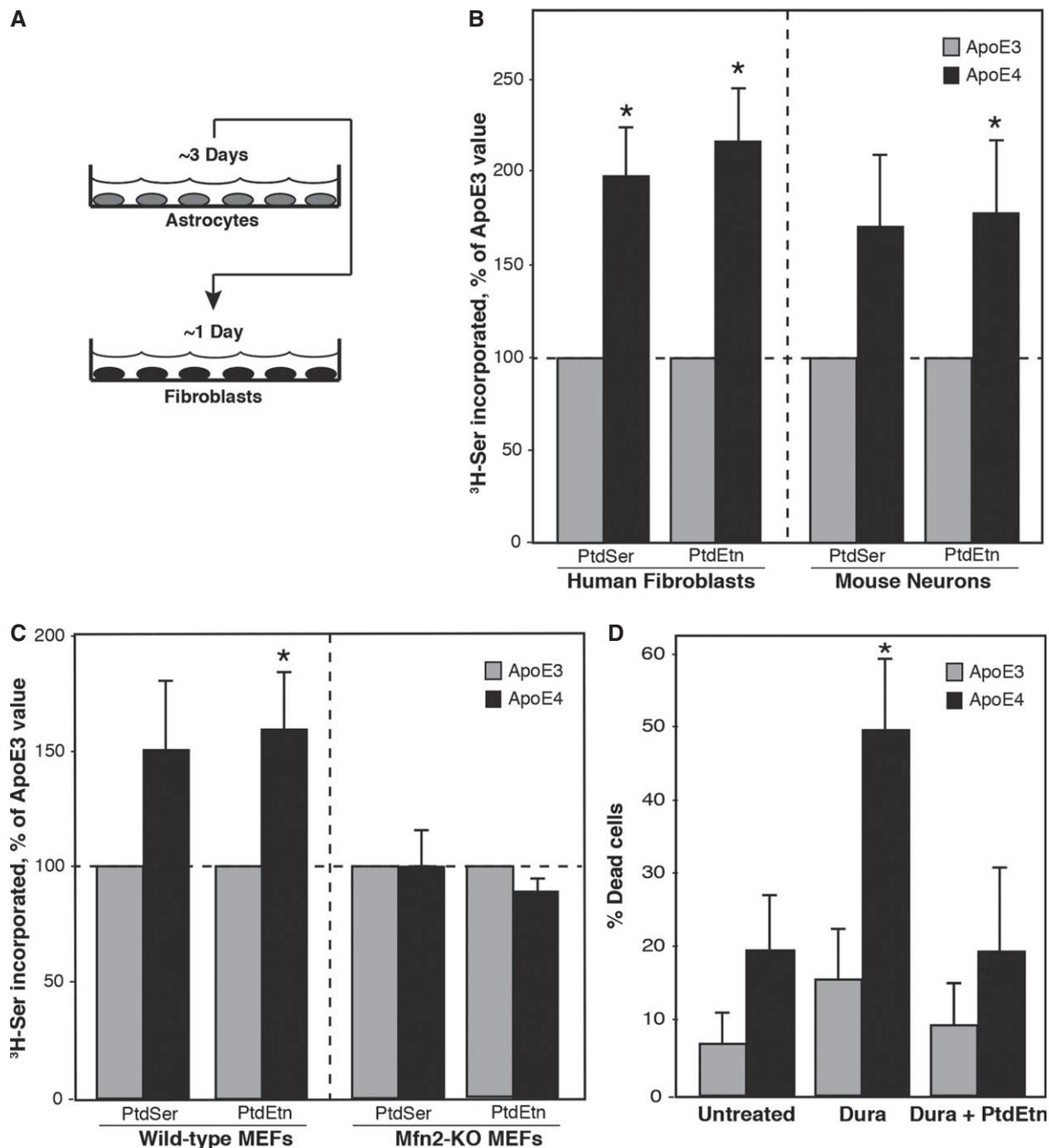


Figure 1. Phospholipid synthesis in ApoE ACM-treated cells.

A Experimental scheme. See text for details.

B Phospholipid transport/synthesis, as measured by ³H-serine incorporation, in human fibroblasts (mean ± SE; n = 9, with 5 replicates/experiment) and in primary mouse hippocampal neurons (mean ± SE; n = 3, with 4 or 5 replicates/experiment). *P < 0.05 vs. E3.

C Comparison of ³H-serine incorporation in ApoE ACM-treated WT and *Mfn2*-KO MEFs (mean ± SE; n = 3, with 3 replicates/experiment). *P < 0.05 vs. E3.

D Duramycin sensitivity in ApoE ACM-treated fibroblasts. Note increased sensitivity after 20 min to 5 μM duramycin in ApoE4-treated cells, which was blocked upon the addition of 15 μM exogenous PtdEtn (mean ± SD; n = 3, with 3 replicates/experiment). *P < 0.05 vs. E3.

in the lysosome, and the remaining ApoE is either degraded intracellularly or re-lipidated in the secretory pathway or at the cell surface [34]. The activity of ApoE largely depends upon its lipidation state, as the lipid-free form of ApoE (“free ApoE”) affects

cellular activities differently than does ApoE as a component of assembled lipoproteins [34–36].

We therefore examined whether the increase in MAM-mediated phospholipid synthesis seen in ApoE4 ACM-treated cells was the

result of the effect of ApoE4 as a component of HDLs as opposed to an effect due to ApoE as the free protein. Specifically, we prepared lipoprotein-rich and lipoprotein-free fractions from ApoE3 ACM and ApoE4 ACM by density ultracentrifugation (purification scheme in Fig 2A) [20], isolated successive fractions from the gradient, and then added selected fractions either containing (e.g., fraction 5, Fig 2B) or not containing (e.g., fraction 3, Fig 2B) ApoE (Fig EV1) to apolipoprotein-free medium, in order to generate a “surrogate” ACM that was then applied to the cells, followed by assays for phospholipid synthesis as before. Compared to a lipoprotein-rich fraction derived from ApoE3 ACM (i.e., fraction 5), lipoproteins enriched from ApoE4 ACM increased PtdEtn synthesis significantly ($\sim 2.4 \pm 0.5$) (Fig 2C). Importantly, this effect was not seen with a lipoprotein-negative fraction from the same preparations (i.e., fraction 3), nor when equimolar amounts of free (i.e., unlipidated) recombinant ApoE3 and ApoE4 were applied to the cells (Fig 2C). Thus, we ascribe ApoE4’s role in increasing MAM activity (as measured

by phospholipid synthesis) to its function as a component of lipoproteins.

Mitochondria-associated ER membranes have the properties of an intracellular lipid raft that is rich in cholesterol and sphingolipids [7–9]. Membrane cholesterol is under tight control, and excess cholesterol may either be oxidized by cholesterol hydroxylases (e.g., CYP46A1) or converted into cholesteryl esters (CE) by acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1; gene *SOAT1*), a resident MAM protein [37]. ACAT1 controls the equilibrium between membrane-bound free cholesterol and CE stored in cytoplasmic lipid droplets [38]. Remarkably, that equilibrium plays a key role in regulating the production of A β , via a currently unknown mechanism [39]. We also note that skin fibroblasts [2,7] and neurons [40] from AD patients contain more lipid droplets than controls.

We had shown previously that presenilin-mutant cells, including AD fibroblasts, synthesized significantly more CE and lipid droplets than did wild-type controls [7]. To test the contribution of

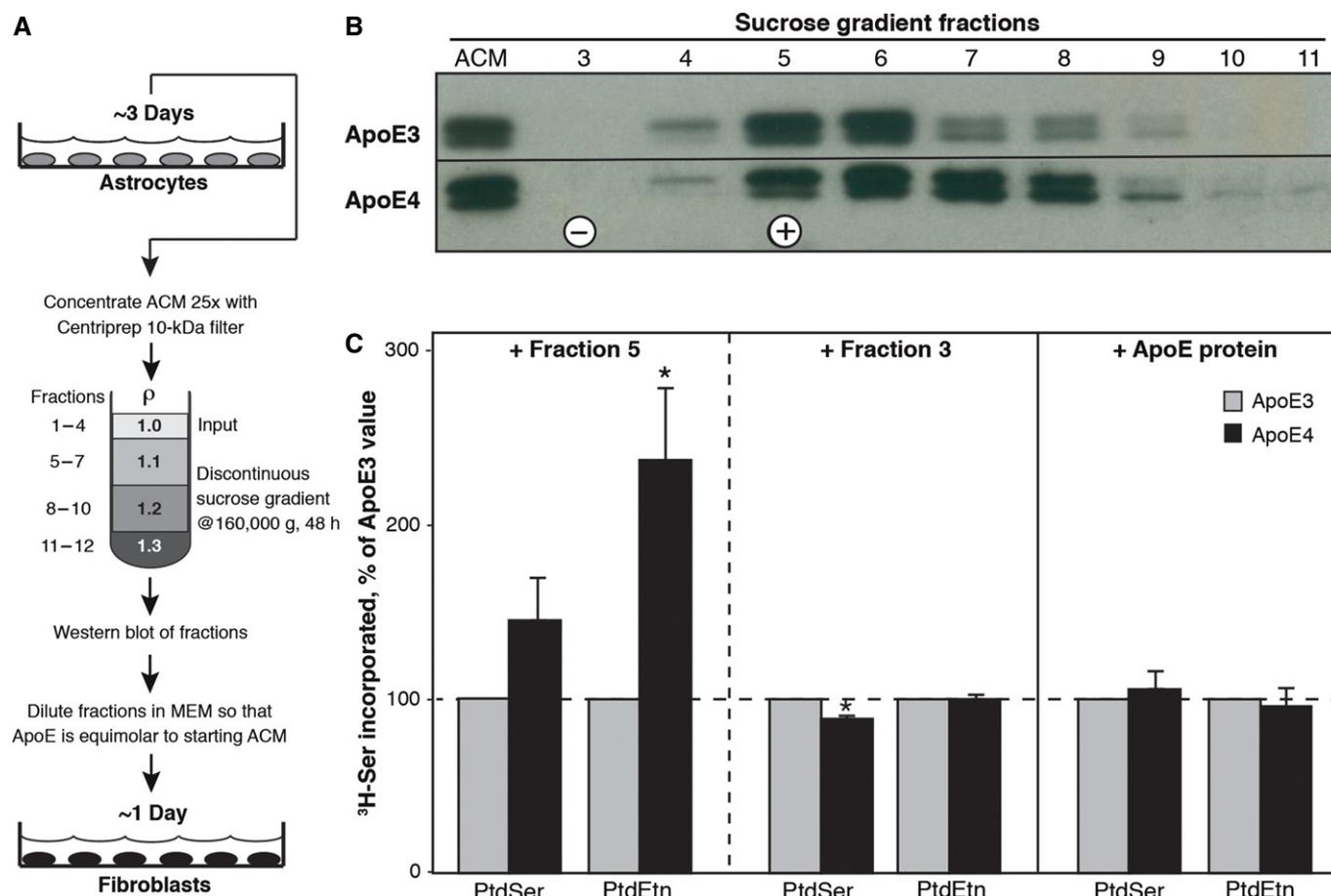


Figure 2. Effect of lipoprotein-rich and lipoprotein-poor fractions on phospholipid synthesis.

A Experimental scheme. See text for details.

B Fractions from the discontinuous gradient were blotted with anti-ApoE (WUE-4) antibody; the doublet is a characteristic feature on ApoE Western blots due to ApoE sialylation [16].

C An ApoE-containing fraction (fraction 5 [+]), an ApoE-negative fraction (fraction 3 [-]), and recombinant lipid-free ApoE protein were applied to human fibroblasts. Note increase in phospholipid synthesis using an ApoE4-containing lipoprotein fraction, but not with either an ApoE4-negative fraction or with lipoprotein-free recombinant ApoE3 or ApoE4 protein (mean \pm SE; $n = 3$, with 3 replicates/experiment). * $P < 0.05$ vs. E3.

ApoE to CE synthesis, we applied ACM to wild-type fibroblasts as above and determined CE production (via incorporation of labeled oleate into CE) and lipid droplet formation (via staining with LipidTox™). We added ³H-oleate to ACM-treated fibroblasts for 24 h, extracted lipids from these samples, and separated cholesteryl esters from triglycerides by thin layer chromatography (TLC). Compared to ApoE3 ACM-treated fibroblasts, there was a significant increase in the levels of ³H-cholesteryl oleate in the ApoE4 ACM-treated cells (~4.1 ± 1.2) (Fig 3A). Notably, this increase in CE synthesis did not appear to be the result of increased ACAT1

expression, as the levels of this key cholesterol metabolism enzyme were similar in ApoE3 and ApoE4 ACM-treated fibroblasts (Fig EV2). As with the phospholipid assays, we had established previously that cholesteryl ester synthesis is reduced significantly in *Mfn2*-KO MEFs [7]. Consistent with this observation, the ApoE4-mediated increase in cholesteryl ester synthesis was abrogated in *Mfn2*-KO cells (Fig 3B), supporting the view that the ApoE4-mediated effect on cholesterol metabolism was indeed the result of increased MAM functionality. In addition, the ApoE4 ACM-treated cells contained significantly more lipid droplets per cell (~39 ± 4) than did the ApoE3 ACM-treated samples (~25 ± 3) (Fig 3C). LipidTox also stains triglycerides, another component of lipid droplets. Importantly, we detected essentially no difference in the incorporation of ³H-oleate into ³H-triglycerides (Fig 3A), indicating that the ApoE4-specific increase in lipid droplets was primarily the result of elevated CE production (Fig 3C).

The stability and capacity of mitochondrial oxidative phosphorylation (OxPhos) complexes (which is reduced in AD cells [6]) can be affected by the phospholipid composition of the mitochondrial inner membrane, especially by PtdEtn and the related molecule cardiolipin [41]. Nevertheless, we found that respiration in ApoE4 ACM-treated cells was similar to that in ApoE3 ACM-treated cells (Fig EV3).

We had shown previously that ER-mitochondrial apposition is increased in AD fibroblasts [42]. We therefore measured ER-mitochondrial co-localization in cells after 24 h of treatment with ApoE ACM, using confocal microscopy to visualize ER in green (transfection with GFP-Sec61-β) and mitochondria in red (transfection with DsRed-Mito). While there was no significant difference in the overall degree of co-localization between ApoE3 ACM vs. ApoE4 ACM treatment after 24 h, there was a clear trend indicating that the median degree of co-localization was higher in ApoE4-treated cells as compared to ApoE3-treated cells (Fig 4).

The role of ApoE4 as a major risk factor in AD has been enigmatic since the initial discovery of its linkage to the disease in 1993 [42]. Broadly speaking, studies of ApoE's role in AD pathogenesis have focused on amyloid-dependent or amyloid-independent pathways. Amyloid-dependent theories make use of the fact that ApoE, either by direct binding to Aβ [42] or by competition with Aβ receptors, such as the lipoprotein receptor-related protein-1 [43], is able to modify Aβ production [44] or clearance [45]. Amyloid-independent theories implicate the cytotoxicity of ApoE4 fragments [46], its role in cholesterol homeostasis [35], neuroprotection/apoptosis [20], neurite outgrowth [19], inflammation [47], and vascular integrity [48]. AD is clearly a multifaceted disease, with changes in amyloid metabolism occurring alongside these amyloid-unrelated phenomena.

We reported previously that PS1, PS2, and γ-secretase activities are highly enriched in the MAM [10] and that ER-mitochondrial communication and MAM function, including phospholipid and cholesteryl ester synthesis, are increased dramatically in presenilin-mutant and presenilin-deficient cells and in cells from AD patients [7]. Both lines of evidence support the view that PS1 and PS2, in addition to generating Aβ, are negative regulators of MAM function [7] and that these alterations play a critical role in the pathogenesis of the disease (the “MAM hypothesis”) [11,12]. If the hypothesis has any validity, it should accommodate ApoE4's role as a risk

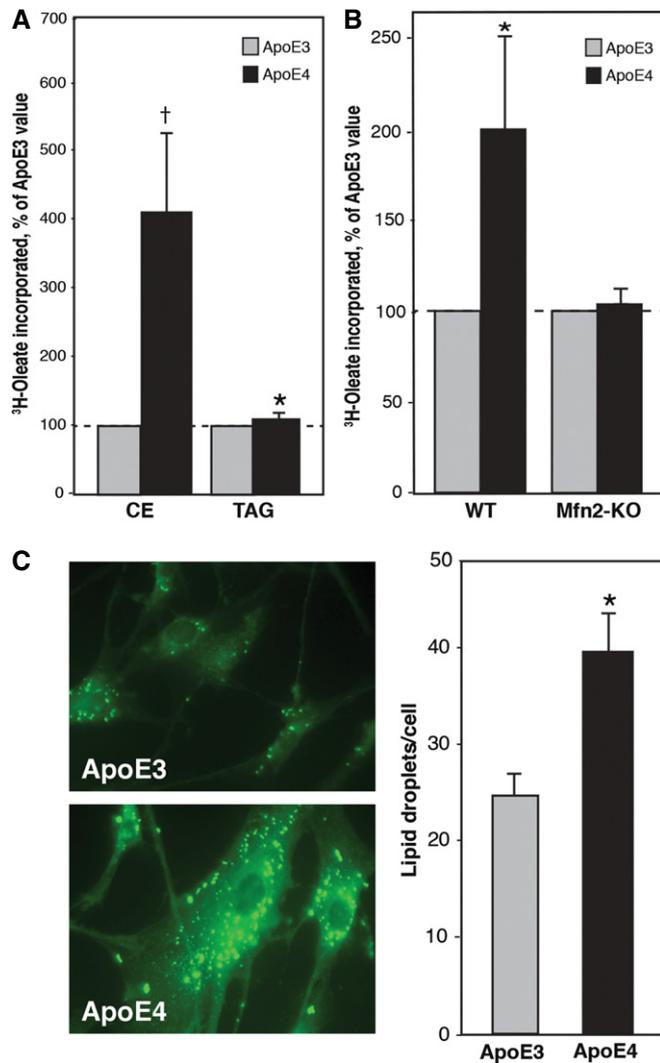


Figure 3. Cholesteryl ester production in ApoE ACM-treated cells.

A Cholesteryl ester synthesis, as measured by ³H-oleate incorporation. Note increased CE in ApoE4-treated cells, whereas there was essentially no difference in triglyceride (TAG) production (mean ± SE; n = 3, with 3 replicates/experiment). *P < 0.05, †P = 0.06 vs. E3.
 B Comparison of ³H-oleate incorporation in ApoE ACM-treated WT and *Mfn2*-KO MEFs (mean ± SE; n = 3, with 5 replicates/experiment). *P < 0.05 vs. E3.
 C ApoE ACM-treated fibroblasts were stained with LipidTox green neutral lipid stain, and lipid droplets were visualized (example at left) and counted (right). Note significantly more lipid droplets in ApoE4-treated cells (mean ± SD, n = 3). *P < 0.05 vs. E3.

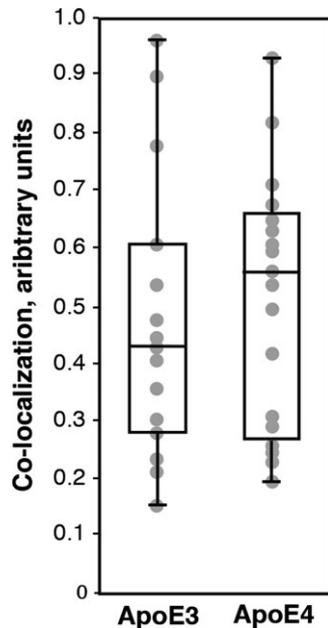


Figure 4. ER-mitochondrial co-localization in ApoE ACM-treated cells.

HeLa cells were transfected with plasmids to visualize ER (in green) and mitochondria (in red) and then were treated with ApoE3 ACM and ApoE4 ACM for 24 h. Shown is a box-and-whisker plot (showing the median and upper and lower quartiles) comparing co-localization in ApoE3 ACM ($n = 15$ images analyzed; gray circles, average of co-localization in ~5 cells in each image field) compared to that in ApoE4 ACM ($n = 18$ images).

factor in the disease, that is, ApoE4 should play a role in mediating MAM behavior, either directly or indirectly. We therefore treated cells with ApoE4 vs. ApoE3 ACM and measured two key aspects of MAM behavior, namely ER-mitochondrial communication and MAM function, as measured by the synthesis of phospholipids and of cholesteryl esters, respectively. We found that in both cases, ApoE4 ACM, like the presenilin mutants, was indeed able to increase MAM functionality. We note that in general, the degrees of increase in phospholipid and cholesterol ester synthesis, while significant, were not as pronounced as those reported previously in presenilin-mutant cells [7], consistent with ApoE4's role as a risk factor, not a determinative factor, in AD. This latter point may also help explain why the degree of ER-mitochondrial apposition, as measured by confocal microscopy, showed only a trend toward greater median apposition in ApoE4-treated cells as compared to ApoE3-treated cells.

The mechanism of this ApoE4-mediated increase in MAM activity is unclear. However, given that ApoE4 exerted its effects on MAM when incorporated into lipoprotein particles, but not when present as the "free" protein, we suspect that the lipoprotein metabolism pathway plays a role in ApoE4-mediated pathogenesis. Support for this idea comes from the numerous points of intersection between lipoprotein metabolism and alterations in APP metabolism by γ -secretase, which is a known regulator of MAM function [7] and which is central to AD pathology [42].

Isoform-specific differences in lipoprotein metabolism, and, by association, cholesterol trafficking, may provide some insight as to how ApoE4 leads to MAM dysfunction. One of these differences is

the relative affinity of each isoform for the lipoprotein receptors at the cell surface, thereby affecting the total amount of cholesterol imported [14]. Upon receptor-mediated endocytosis, the ApoE-containing HDL-like particles are transported to lysosomes, where they are hydrolyzed. Some ApoE is degraded and some is recycled back to the cell surface [34,35], where it is re-lipidated by ABCA1 or ABCG1 and secreted. Notably, ApoE4 is recycled much less efficiently than is ApoE3 [34,35], resulting in an accumulation of cholesterol in endosomes and lysosomes, reminiscent of Niemann-Pick disease type C, a neurodegenerative disorder characterized by the accumulation of cholesterol in lysosomes. Thus, it may be that the increase in cholesteryl esters seen in ApoE4 ACM-treated cells reflects a concomitant increase in available substrate (i.e., cholesterol) at the MAM.

Cellular cholesterol handling affects APP processing at numerous points in cell metabolism [49] and *vice versa* [50]. First, the internalization of cholesterol-containing lipoproteins from the extracellular space may affect the rate of APP internalization, which is a requirement for its amyloidogenic processing [51]. Indeed, the bulk uptake of cholesterol is important in AD, as diet-induced hypercholesterolemia in rabbits resulted in amyloid pathology [52]. Second, the cholesterol content of intracellular membranes likely affects APP processing, since the C-terminal fragment of APP binds cholesterol [53,54]. Third, each of the three proteases that process APP is also influenced by cholesterol. A fraction of BACE1 localizes to, and is active in, lipid rafts, and an increase in cholesterol enhances the β -cleavage of APP [55]. γ -Secretase is also localized to rafts [56] and is affected by cholesterol levels, particularly by the ratio of free cholesterol:cholesteryl esters, as inhibition of ACAT1, either pharmacologically or by genetic ablation, reduces A β production [39]. Conversely, the non-amyloidogenic arm of APP processing by α -secretase is inhibited by the addition of exogenous cholesterol [57]. These observations point to a complex biology in which the subcellular amount and distribution of cholesterol are linked to APP metabolism and MAM function.

It is unclear at present why ApoE4 should exert differential effects on MAM function compared to ApoE3. We note that the two main risk factors for developing AD are aging and ApoE4. Perhaps, the two are related, with potentially increased ApoE4-mediated cholesterol levels affecting cellular processes that are known to be altered in aging (and in AD) [58].

We also note that from an evolutionary point of view, the ApoE4 genotype is apparently present only in the primate line, including ancestors to humans [59], implying that the maintenance of the $\epsilon 4$ allele in this genus may actually confer a selective advantage. Besides the aforementioned role of ApoE4 in apoptosis [20] (a MAM-mediated function [60]), another possible advantage might be in resistance to infection [61,62]. Since it is known that MAM mediates the innate immune response [63] and that pathogens gain entry into the host cell via lipid rafts [64], perhaps ApoE4 mitigates infectivity [65]. More research on the relationship of MAM to infection is needed to elucidate the differential contribution of ApoE isoforms to this process.

In summary, we have demonstrated that ApoE4-containing ACM increases MAM function relative to ApoE3. This report extends our previous findings demonstrating upregulation of MAM function in AD and may help explain why ApoE4 is a risk factor in the disease.

Materials and Methods

Cells and reagents

Telomerase (hTERT)-transformed human fibroblasts were a kind gift of Drs. Orhan Akman and Sonja Brosel (Columbia University). Immortalized *APOE3*- and *APOE4*-targeted gene replacement astrocytes [66] were prepared as described [67]. Cells were cultured in minimal essential media (MEM [lacking serine]) (Gibco 11095) or Dulbecco's MEM (DMEM [containing serine]) (Gibco 11995-065). Immortalized cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (Sigma F2442) and 1% antibiotic-antimycotic (Gibco 15240).

Mouse hippocampal neuronal culture techniques were adapted from our previous approaches [68]. The hippocampus was dissected from 1- to 3-day-old wild-type C57BL6/J pups, the tissue digested in a papain solution (Worthington Biochemical, Lakewood, NJ) at 20 units/ml, the cells triturated, spun down, and resuspended in NbActiv4 media (Brain Bits Nb4-500) supplemented with 1% heat-inactivated FBS (Life Technologies 16140-063). Cells were plated at a density of ~320,000 cells per 35-mm diameter petri dishes pre-coated with laminin (Millipore, 10 µg/ml) for 1 h. The medium was changed 3 days after plating the cells. Cultures were maintained at 37°C and 5% CO₂. For all experiments with ACM, the cultured neurons were incubated for 10 days prior to assay.

We used antibodies against ACAT1 (polyclonal aa6-23; Abcam 39327), ApoE (monoclonal aa140-160; Novus WUE-4 NB110-60531), clusterin (polyclonal C-terminus; Novus NBP1-19637), β-actin (monoclonal; Sigma AC-15 A5541), and PISD (Sigma HPA031091), which were used according to manufacturers' instructions. Thin layer chromatography (TLC) silica plates were from EMD Biosciences (5748-7). Phosphatidylserine (PtdSer) (Sigma P7769), phosphatidylethanolamine (PtdEtn) (Sigma 60660), cholesterol palmitate (Sigma C6072), and soybean phospholipid mixtures (Sigma P3817) were used as standards for TLC in experiments with radiolabeled ³H-serine (Perkin Elmer NET248005MC) and ³H-oleate (Perkin Elmer, NET289001MC). Bovine serum albumin (Sigma A3803) was used to solubilize ³H-oleate [69].

Astrocyte-conditioned media generation and lipoprotein enrichment

APOE3 and *APOE4* astrocytes were grown to confluency, washed once with MEM, and incubated in MEM for 3 days. Astrocyte-conditioned medium (ACM) was spun at 3,000 g for 3 min to remove debris and analyzed first by Western blot against ApoE to confirm the presence of lipoproteins. Prior to all experiments, we measured the amount of ApoE present in the ACM by dot blot (Bio-Rad Bio-Dot™ Apparatus) (it typically contained more ApoE3 than ApoE4, consistent with the finding that the concentration of ApoE3 in the brain homogenates [23] and in the interstitial fluid of human ApoE knock-in mice [24] is ~50% greater than that of ApoE4) and then added the ACM on an "equal ApoE" basis, by adding MEM to the ApoE3 ACM as appropriate.

Density ultracentrifugation was used to prepare enriched fractions of lipoproteins [70]. Briefly, 100 ml ACM was concentrated to ~4 ml using Centriprep 10K filters (EMD Millipore). Four milliliters of ACM concentrate was layered on top of 3 ml 1.1 g/ml, 3 ml 1.2 g/ml, 2 ml 1.3 g/ml discontinuous sucrose gradient. Samples

were centrifuged at 160,000 g in a Sw44-Ti rotor (Beckman) for 48 h at 4°C. One-millilitre fractions were removed from the top of the gradient, and ApoE content was analyzed by Western blot.

Analysis of phospholipid synthesis

Fibroblasts were grown to confluency (5 replica plates per condition) and washed once with MEM. ACM, recombinant ApoE, or enriched lipoprotein fractions containing 2.5 µCi/ml of ³H-serine (30.5 Ci/mmol) were applied to the cells grown in 60-mm dishes for 24 h. The cells were washed twice with 1 ml ice-cold phosphate-buffered saline (PBS) and collected on ice in 1 ml PBS, pelleted at 3,000 g for 3 min at 4°C, and resuspended in 0.5 ml water. Twenty microliters of suspension was stored at -20°C for protein quantification by Bradford analysis. Total lipids were extracted as follows: Three volumes of 2:1 chloroform:methanol v/v were added to the samples which were then vortexed and centrifuged at 8,000 g for 5 min, and the aqueous phase was discarded. The organic phase was washed with two volumes of 1:1 methanol/water v/v, centrifuged at 8,000 g for 5 min, and then washed again in the same manner. The organic phase was then transferred to a fresh tube and incubated at 50°C until dry. After resuspension in 40 µl of chloroform, total lipids were spotted onto a TLC plate. Plates were run through a series of two solvents, first 84:15:1 petroleum ether/diethyl ether/acetic acid v/v/v, and then 60:50:1:4 chloroform/methanol/acetic acid/water v/v/v/v. Separated phospholipids and the corresponding standards were visualized after overnight exposure to iodine vapor. Phospholipid species were cut from the plate, suspended in scintillation liquid (Ultima Gold XR Perkin Elmer), and counted by scintillation to detect ³H (Packard Tri-Carb 2900TR).

Duramycin assay

Fibroblasts were treated with ACM for 24 h, washed once with MEM, and treated with the specified concentration of duramycin (Sigma D3168) or duramycin and PtdEtn diluted in MEM (1:3 duramycin:PtdEtn mol/mol) for 20 min at 37°C. Cells were washed twice with PBS and stained with the LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies L3224) according to manufacturer's instructions. Cells were visualized by fluorescent microscopy and hand-scored. Percent death was calculated as a ratio of ethidium-stained red cells to total cells.

Analysis of cholesteryl ester synthesis

Fibroblasts were grown to confluency and washed once with MEM. ACM containing 2.5 µCi/ml of ³H-oleate (54.6 Ci/mmol) solubilized in MEM-2% BSA were applied to the cells for 24 h. Sample processing and total lipid extraction were performed as described above. TLC plates were run using 190:9:1 chloroform/methanol/acetic acid v/v/v as a solvent. After exposure to iodine vapors, spots corresponding to the cholesteryl ester standards were scraped and counted by scintillation as described above.

Lipid droplet staining

Fibroblasts were grown to ~80% confluency on glass coverslips, washed once with MEM, and treated with either ApoE3 or ApoE4

ACM for 24 h. Cells were washed once in PBS, and lipid droplets were stained with HCS LipidTox™ Deep Green neutral lipid stain (Invitrogen H34475) according to manufacturer's instructions. Lipid droplets were visualized by fluorescent microscopy, and the number of lipid droplets per cell was quantified by hand.

Mitochondrial respiration measurements

Mitochondrial respiration was determined using the Seahorse XF-24 analyzer (Seahorse Bioscience). WT fibroblasts were seeded at 8×10^4 cells per well in a XF24 cell culture microplate and treated with ACM for 24 h. Cells were washed with XF Assay Media (Seahorse Bioscience), pre-incubated for 1 h at 37°C and 0% CO₂ in XF Assay Media, and supplemented with 2 mM pyruvate and 25 mM glucose. For the assay, cells were treated sequentially with 1 μM oligomycin, 0.75 μM carbonyl cyanide-4-trifluoromethoxy phenylhydrazone, and 1 μM rotenone plus 1 μM antimycin A.

Analysis of ER-mitochondrial apposition

ER-mitochondrial apposition was assayed as described previously [7]. In brief, following ACM treatment for 24 h, HeLa cells were co-transfected with GFP-Sec61-β (Addgene plasmid #15108) and DsRed-Mito (Clontech, #632421) at a 1:1 ratio in serum-free DMEM, and 12 h post-transfection were analyzed in a single-plane with a Zeiss LSM510 microscope. Interactions between ER (green) and mitochondria (red) were calculated using ImageJ software (<http://rsbweb.nih.gov/ij/>), determining the area occupied by one organelle and using its signal as a mask for the other one. The co-localization data sets were compared using Mander's coefficient.

Expanded View for this article is available online.

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Author contributions

MDT, EA-G, and EAS designed the research; MDT, MP, HY, and CG-L performed the experiments; MDT, MP, and EAS analyzed the data; EK, DS, DH, and HY provided key reagents and critical advice on their use and preparation; MDT and EAS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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