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Following activation of the amyloid cascade, apolipoprotein E4 drives the in vivo oligomerization of amyloid- β resulting in neurodegeneration

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Abstract

According to the amyloid hypothesis, the accumulation of oligomerized A β is a primary event in the pathogenesis of Alzheimer's disease (AD). The trigger of the amyloid cascade and of A β oligomerization in sporadic AD, the most prevalent form of the disease, remains elusive. Here we examined the hypothesis that apolipoprotein E4 (apoE4), the most prevalent genetic risk factor for AD, triggers the accumulation of intraneuronal oligomerized A β following activation of the amyloid cascade. We investigated the intracellular organelles that are targeted by these processes and govern their pathological consequences. This revealed that activation of the amyloid cascade *in vivo* by inhibition of the A β degrading enzyme neprilysin specifically results in accumulation of A β and oligomerized A β and of apoE4 in CA1 neurons of apoE4 mice. This was accompanied by lysosomal and mitochondrial pathology and the co-localization of A β , oligomerized A β and apoE4 with enlarged lysosomes and of A β and oligomerized A β with mitochondria. The time course of the lysosomal effects paralleled that of the loss of CA1 neurons, whereas the mitochondrial effects reached a plateau earlier. These findings suggest that apoE4 potentiates the pathological effects of A β and the amyloid cascade by triggering the oligomerization of A β , which in turn, impairs intraneuronal mitochondria and lysosomes and drives neurodegeneration.

Keywords

apolipoprotein E4; beta amyloid; neurodegeneration; CA1 neurons; lysosomes; mitochondria

INTRODUCTION

According to the amyloid hypothesis, the accumulation of Ab is the primary event in the pathogenesis of Alzheimer's disease (AD) [1]. Recent findings suggest that A β undergoes a cascade of aggregation states of soluble and insoluble neurotoxic oligomers that mediate its pathological effects [2–4] and that accumulate intraneuronally [4–7]. This occurs at an early stage of the amyloid cascade and is associated with co-localization of intracellular A β with lysosomes, multi-vesicular bodies [8–10], and mitochondria [11, 12]. The trigger of the amyloid cascade in sporadic AD, the most prevalent form of the disease, remains elusive. Apolipoprotein E4 (apoE4), the most prevalent genetic risk factor for AD [13–15], is associated in AD with increased amyloid deposition [16]. Corresponding model studies revealed synergistic pathological interactions between A β and apoE4 [17–22], which are associated with cognitive deficits [23, 24]. This led to the suggestion that apoE4 potentiates the neurotoxic effects of A β and the amyloid cascade and drives them above a pathological threshold. The extent to which this cross talk is mediated via apoE4-driven oligomerization of A β is presently not known.

We have recently shown that activating the amyloid cascade *in vivo* by inhibiting the A β -degrading enzyme neprilysin [17] specifically stimulates the degeneration of hippocampal CA1 and of entorhinal and septal neurons in apoE4-targeted replacement mice. This is accompanied by the accumulation of intracellular A β and apoE4, as well as by lysosomal activation and cognitive deficits [23, 25]. The neprilysin inhibition paradigm also affects apoE3 mice, but this effect is markedly less pronounced and is delayed relative to that obtained in the apoE4 mice [23]. We presently employed this model to examine the possibility that the pathological synergistic interactions between A β and apoE4 drive the oligomerization of A β *in vivo*. In addition, we determined the sub-cellular localizations of A β , oligomerized A β , and apoE following activation of the amyloid cascade and their spatiotemporal correlation with the resulting neuronal pathology.

MATERIALS AND METHODS

Transgenic mice and implantation of Alzet Mini-osmotic pumps

APOE-targeted replacement mice (apoE mice), created by gene targeting [26], were purchased from Taconic. The mice were back-crossed to C57BL/6J mice for eight generations and were homozygous for either the *APOE3* (3/3) or the *APOE4* (4/4) allele. The experiments were performed utilizing 3- to 4-month-old male apoE3 and apoE4 mice whose genotypes were confirmed by PCR analysis [23]. All experiments were approved by the Tel Aviv University Animal Care Committee and every effort was made to minimize animal usage and reduce animal stress. Alzet® mini-osmotic pumps (model 2004, which delivers their contents at 0.25 μ l/h for up to 30 days) were loaded with the neprilysin inhibitor thiorphan (0.5 mM; Sigma) in artificial cerebrospinal fluid containing 1 mM ascorbic acid or with a similar solution without thiorphan ("sham"). The alzet pumps were implanted with a brain infusion cannula inserted into the lateral ventricle as previously described [23]

Immunofluorescence and confocal microscopy

Free-floating frozen coronal sections (30 μm) were immunostained, as previously described [23], with the following primary antibodies (Abs): biotinylated anti-A β mAb (monoclonal Ab) 4G8 (1:200; Signet, Dedham, MA); rabbit anti-cleaved caspase-3 (1:200; Asp175, Cell Signaling Technology, Danvers, MA); rabbit anti-A β 42 (1:500, Chemicon, Temecula, CA); mouse anti-A β 42, G2-11 (1:1000, Genetic Co., Schlieren, Switzerland); Ab I-11 (1:2000, provided by Dr. Rakez Kaye); rabbit anti-cathepsin D (1:500, Calbiochem, La Jolla, CA), and goat anti-cathepsin D (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); goat anti-apoE (1:5000, Calbiochem, La Jolla, CA), and goat anti-COX-1 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA). For A β staining, sections were first incubated in 70% formic acid for 7 min, and then washed with 10 mM phosphate-buffered saline (PBS), pH = 7.4. The bound primary antibodies were then visualized by incubating the sections with the appropriate fluorescently labeled second antibody, or with fluorescently labeled streptavidin as previously described [23]. The sections (between bregma -2.0 and -3.0) were visualized using a confocal scanning laser microscope (Zeiss, LSM 510). Images (1024 $\times$ 1024 pixels) were obtained by averaging eight scans per slice. All the images for each immunostaining were obtained utilizing identical conditions and their quantitative analyses were performed with no further handling. Moderate adjustments for contrast and brightness were performed on the images during the preparation of the figures but were the same for the images of the different apoE4 and apoE3 mice groups.

Image analysis

Utilizing the Image-Pro Plus system (version 5.1, Media Cybernetics), we calculated the intensities of immunohistochemical staining as the percentage of the area stained, as previously described [23]. Two sections were analyzed per brain and each staining was performed at least twice. The replicate experiments, one of which was quantified, yielded similar results. For the double- and triple-labeling co-localization experiment, each image was analyzed separately, first in terms of the percentage of the area stained and then of the percentage of the area that co-localizes with the other parameter. Control experiments revealed no co-localization in stained sections lacking the first antibody of each of the antigens. Measurements of the contribution of stochastic processes to the co-localization data were performed by shifting the channels of one of the images laterally by 1 μm^2 . This revealed that the contribution of stochastic processes in all the experiments was negligible except for the COX-1 (mitochondria), where it accounted for about 25% of the signal and accordingly was subtracted from the corresponding co-localization results.

Immunoblot analysis

Hippocampi were homogenized (20% w/v) in PBS, pH 7.4, with protease inhibitor cocktail (Roche, # 1 836 153) and centrifuged at 10,000 rpm for 5 min, after which the supernatant (PBS extract) was collected. The resulting pellet was extracted by re-suspension in an equal volume of 10 mM Tris pH 7.6 containing 150 mM NaCl, 2 mM EDTA, protease inhibitor cocktail (Roche), and 1% NP-40, after which it was centrifuged for 5 min at 10,000 rpm. The resulting supernatant (NP-40 extract) was collected. The pellet was further extracted with 70% formic acid. Protein contents of the samples were determined by the BCA Protein

Assay Kit (Pierce Biotechnology, #23225). For SDS gel electrophoresis, 30 µg and 4 µg protein of the PBS and NP-40 extracts were used, respectively, and the gels were blotted and immunoreacted with mAb 266 (1:1000 Elan pharmaceuticals). Dot-blot experiments utilizing 0.125 µg of the PBS extract and 30 µg of the NP-40 extract per dot and I-11 or OC (1:1000, provided by Dr. Rakez Kaye) were performed as previously described [27]. Intensities of the immunoblot bands and the dot blots were quantified by using EZQuant-Gel software (EZQuant, Tel-Aviv, Israel).

Electron microscopy and immunogold labeling

90-nm sections (ultramicrotome, Leica) prepared from brain vibratome sections and treated with osmium tetroxide were collected into copper grids and analyzed with a Zeiss OM 10 electron microscope as previously described [28]. Aβ immunogold EM was performed as previously described [28]. Accordingly, the sections were treated with Antigen retrieval solution (sodium periodate saturated in water) followed by blocking with 3% bovine serum albumin, incubation with the primary anti-Aβ mAb 4G8 (Sigma, Saint Louis, MO) and subsequently with the secondary antibody, IgG-anti-rabbit/10-nm gold particles (AURION ImmunoGold Reagents). The grids were then analyzed using a Zeiss OM 10 electron microscope. Both for the EM and the immunogold labeling, an average of 100 micrographs from pyramidal neurons were randomly acquired from three grids at a magnification of 25,000 each, and analyzed with the Image Quant program to estimate the average numbers of gold particles in lysosomes, mitochondria, synapses, and nuclei.

Statistical analysis

Statistical analysis was performed using SPSS version 14. The two groups (apoE3 and apoE4) and two treatments (thiorphan and sham) were compared by two-way ANOVA. Kinetic studies were subjected to ANOVA in terms of two groups (apoE3 and apoE4) and subjected to four time treatments (0, 4, 7, and 10 days). When appropriate, the results were further subjected to post-hoc analysis by Student's *t*-test utilizing Bonferroni correction for multiple comparisons.

RESULTS

ApoE4 triggers the accumulation of intraneuronal oligomerized Aβ following activation of the amyloid cascade

The extent to which the isoform-specific accumulation of apoE4 and Aβ in CA1 neurons following activation of the amyloid cascade [23, 25] is associated with oligomerization of the accumulated Aβ was first examined immunohistochemically utilizing mAb I-11, which is directed against distinct epitopes of the oligomeric amyloid backbone [27]. Importantly, this revealed that I-11 immunoreactivity, like that of Aβ, was significantly higher in the CA1 neurons of the thiorphan-treated apoE4 mice than in either the corresponding apoE3 mice or the sham-treated apoE3 and apoE4 mice (Fig. 1A, respectively $P < 0.005$). Previous experiments revealed that I-11 also recognizes amyloid-like structures derived from non-Aβ peptides [29]. Accordingly, the levels of bound I-11 immunoreactivity that correspond to oligomerized Aβ42 were determined by double-labeling confocal experiments utilizing I-11 and anti-Aβ42. This revealed significant co-localization of these stains (Fig. 1B) and showed

that the merged image, which we will term here “oligo-A β 42” is localized predominantly in the perikarya of the CA1 neurons. By day 7, ~15% of the I-11-positive and ~15% of the A β 42-positive staining were co-localized in the thiorphan-treated apoE4 mice. The I-11 immunostaining that did not co-localize with A β 42 represents amyloid-like epitopes of other proteins. Kinetic studies revealed that the accumulation of A β and oligo-A β in CA1 neurons had a similar time course that paralleled the activation of Caspase-3 and preceded the loss of CA1 neurons (see Supplementary Fig. S1).

Complementary biochemical experiments revealed that the soluble PBS extracts of the CA1 subfield of thiorphan-treated apoE3 and apoE4 mice had elevated levels of A β ($P < 0.04$ for treatment), and no effect of either group or group $\times$ treatment which were slightly but not significantly higher in the thiorphan-treated apoE3 mice (Fig. 1C upper left panel). A β levels in the NP-40 extractable fraction of CA1 homogenates were also increased by thiorphan ($P < 0.001$ for treatment and $P < 0.01$ for group $\times$ treatment) and were higher in the thiorphan-treated apoE4 than in the treated apoE3 mice ($P < 0.02$ for post hoc comparison of the thiorphan-treated apoE4 and apoE3 mice; Figure 1C upper right panel). Importantly, the total PBS-soluble and NP-40-extractable A β levels of the CA1 homogenates in the apoE3 and apoE4 mice were similar in the sham-treated mice (45 ± 4 pg A β /hippocampus and 47 ± 3 pg A β /hippocampus, respectively) and were similarly elevated in the thiorphan-treated mice (95 ± 10 pg A β and 79 ± 10 pg A β /hippocampus, respectively). The hippocampal extracts taken from the apoE3 and apoE4 mice did not contain additional formic acid extractable insoluble A β . Dot-blot experiments utilizing mAb I-11 which specifically recognizes oligomerized A β (see supplementary Fig. 2S) revealed, in accordance with the morphological results, higher levels of I-11-stained material in the NP-40 extract from the thiorphan-treated apoE4 than in all other mouse groups (Fig. 1C, lower panels).

Immunostaining and dot-blot experiments with OC antibodies, which are directed against a different oligomeric form of A β [27, 29], revealed no effect of either apoE genotype or thiorphan (see supplementary Fig. S3).

Identifying the intracellular compartments that accumulate A β , oligomerized A β , and apoE4 and that are affected by them

Ultrastructural analysis—Electron microscopy (EM) was employed for unbiased screening of the intracellular pathology induced by apoE4 in CA1 neurons following activation of the amyloid cascade. This disclosed marked enlargement and deformation of lysosomes, mitochondria, and synaptic vesicles, which were specific to the thiorphan-treated apoE4 mice (Fig. 2A). In contrast, the Golgi apparatus and endoplasmic reticulum of the CA1 neurons in the apoE4 mice exhibited minimal dilations (see supplementary Fig. S4A). EM immunogold labeling showed that A β in the CA1 neurons of the apoE4 mice was co-localized with lysosomes, mitochondria, and synaptic vesicles (Fig. 2B). A β was also present in the cytoplasm of thiorphan-treated apoE4 mice in the form of amorphous and fibrillar deposits (Fig. 2C) and in secondary lysosomal/autophagic structures (see supplementary Fig. S4B). Control experiments where brain sections were incubated with the gold-secondary antibody in the absence of primary antibody, or where the anti-A β antibody was adsorbed with excess A β , revealed that no gold particles were associated with

organelles in samples from either the apoE4 or apoE3 thiorphan-treated mice. Moreover, only background levels of gold particles associated with organelles were observed in wild-type mice incubated with the anti-A β antibody (not shown).

These results suggest that lysosomes and mitochondria are prominent intracellular targets of the accumulated A β in CA1 neurons of the apoE4 mice following activation of the amyloid cascade. Next, the spatiotemporal relationships regarding the accumulation of A β 42, oligomerized A β , and apoE4 in these organelles were studied by confocal microscopy of I-11, A β 42, apoE, and the lysosomal and mitochondrial markers Cathepsin D (CatD) and cytochrome oxidase (COX-1).

Lysosomal pathology—In accordance with the EM results, thiorphan specifically elevated the levels of punctuate CatD-positive lysosomes and increased their sizes in the CA1 neurons of apoE4 mice (Fig. 3A, 3B). Quantification of the effects of apoE4 on the levels of small (0.2–0.5 μm^2) and large (0.5–2 μm^2) lysosomes at different time points after initiation of the thiorphan treatment revealed elevated levels of both small and large lysosomes of the apoE4 CA1 neurons, which were detectable by day 4 and reached a plateau by day 7 (Fig. 3C; $P < 0.004$ for the treated apoE4 compared to the other mouse groups). Measurements of the extent to which A β 42 co-localizes with the lysosomes revealed that it co-localized preferentially with the large lysosomes of the treated apoE4 mice (Fig. 4A, $P < 0.001$ for treated apoE4 compared to the other mouse groups), and hardly at all with the small lysosomes. About 6% of the intracellular A β 42 co-localized with the large lysosomes on day 4 and 15% on days 7 and 10. Oligomerization of the lysosomal A β 42 was measured by the co-localization of oligo-A β 42 and CatD, where oligo-A β 42 is the masked image of I-11 and A β 42 which contains only pixels stained positively for both I-11 and A β 42. Oligo-A β 42 co-localized with large lysosomes of the CA1 neurons in the treated apoE4 mice (Fig. 4B; $P < 0.03$ for the treated apoE4 compared to the other mouse groups), but not with the small lysosomes. The fraction of oligo-A β 42 that co-localized with large lysosomes increased from 10% on day 4 to about 25% at the plateau.

Activation of the amyloid cascade is also accompanied by the accumulation of apoE4 in the CA1 neurons [25]. We next examined whether apoE4, like A β and oligo-A β 42, also co-localizes with the large lysosomes. As shown in Figure 4C, apoE accumulated specifically in the CA1 neurons of the thiorphan-treated apoE4 mice ($P < 0.03$ for treated apoE4 compared to other mouse groups), and the accumulated apoE4 co-localized preferentially with the large lysosomes ($P < 0.03$ for treated apoE4 compared to the other mouse groups) but not with the small ones. About 6% of the accumulated intracellular apoE4 co-localized with the large lysosomes. As shown in Figure 4D, quadruple labeling (A β 42, I-11, CatD, and apoE4) revealed co-localization of lysosomal apoE with A β 42 (yellow arrowheads) and with oligo-A β 42 (yellow arrows). Furthermore, co-localization of these aggregates with large lysosomes was specifically elevated in the thiorphan-treated apoE4 mice ($P < 0.02$ for the treated apoE4 compared to other mouse groups) (Fig. 4D).

Mitochondrial pathology—To monitor the accumulation of A β 42 and oligo-A β 42 in mitochondria, we employed confocal microscopy, utilizing the mitochondrial marker COX-1. Levels of COX-1 staining were elevated isoform-specifically in the thiorphan-

treated apoE4 mice (Fig. 5A). This effect was faster but less pronounced than that observed with the lysosomes (compare Figs. 3 and 5A). It did not reach significance when the results obtained at each of the different time points were analyzed separately but was significant when the overall effects of the thiorphan treatment on the apoE4 mice at days 4, 7, and 10 was compared to the corresponding results which were obtained with the apoE3 mice ($P < 0.01$ by 1 way ANOVA). Both A β 42 and oligo-A β 42 co-localized specifically with COX-1 in the CA1 neurons of the thiorphan-treated apoE4 mice, and their levels reached a plateau by 4 days following the thiorphan treatment (Fig. 5B, 5C). Similarly to the COX-1 results, these effects were significant when the overall results of the thiorphan treated apoE4 mice at days 4, 7, and 10 were compared to those of the corresponding apoE3 mice ($P < 0.005$ and $P < 0.02$ for respectively A β 42 and oligo A β 42 which co-localized with COX-1). About 8% of the accumulated A β 42 and 23% of the oligo-A β 42 in the thiorphan-treated apoE4 mice co-localized with the mitochondria. Importantly, thiorphan treatment did not induce the co-localization of apoE4 with the mitochondria in any of the mouse groups (Fig. 5D).

The extents to which the different A β 42 and apoE complexes were associated with the large lysosomes and the mitochondria were compared in terms of the percentages of these organelles in CA1 neurons of the thiorphan-treated apoE4 mice, which were occupied by the different A β and apoE species. This comparison revealed that by day 4 about 4% of the mitochondria and no lysosomes were occupied by A β , and that at this time point these organelles did not contain either oligo-A β 42 or apoE4. In contrast, at later time points (e.g. at day 7), occupancy of the large lysosomes by A β and apoE was greater than that of the mitochondria ($P < 0.005$ and $P < 0.01$, respectively), and both organelles contained similar amounts of oligo-A β 42 (Fig. 6).

Taken together, the above findings suggest that apoE4 drives the accumulation of A β 42 and oligomerized A β 42 in the mitochondria and lysosomes of CA1 neurons. This consequently triggers mitochondrial and lysosomal pathology, which induce neuronal cell death.

DISCUSSION

Activation of the amyloid cascade via inhibition of the A β -degrading enzyme neprilysin resulted in the isoform-specific accumulation of A β , oligomerized A β , and apoE in the CA1 neurons of apoE4 mice. This was accompanied by lysosomal and mitochondrial pathology and by the co-localization of A β 42, oligomerized A β 42, with enlarged lysosomes or with the mitochondria. Neprilysin inhibition also induced the accumulation of apoE4 in CA1 neurons of the apoE4 which, however, co-localized with the enlarged lysosomes but not with the mitochondria. The time course of the lysosomal effects paralleled that of the loss of the CA1 neurons; however, the mitochondrial effect reached a plateau earlier. These findings are in accordance with the reported co-localization of A β in activated lysosomes and in mitochondria in AD and in amyloid precursor protein (APP) transgenic mice [8, 30–34] and with findings that these effects are specifically exacerbated by apoE4 [35, 36]. Importantly, the extracellular deposition of A β , which also occurs in the neprilysin inhibition model, evolves much later [17] and is not specific to the hippocampus.

The mechanisms underlying the apoE4-driven accumulation of A β and oligomerized A β 42 in the lysosomes and mitochondria of the CA1 neurons are not fully understood. Lysosomes are endpoints of the two major processing and degradation pathways, autophagy and endocytosis, and it was reported that A β can accumulate in lysosomes via both of these pathways [37, 38]. Studies *in vitro* have shown that A β induces lysosomal leakage and cellular toxicity via distinct apoE receptor-mediated mechanisms [36, 39–41]. Preliminary findings revealed that the level of the apoE receptor, LRP1, is up regulated in CA1 neurons of the apoE4 mice following inhibition of neprilysin and down regulated in the corresponding CA1 neurons of the apoE3 mice (not shown). Thus, it is possible that A β accumulates in the apoE4 CA1 lysosomes via the LRP-mediated endocytotic pathway. Lysosomal accumulation of A β might be mediated by endocytosis of extracellular A β or via intra-lysosomal degradation of APP [42]. Since neprilysin is localized in the plasma membrane and can degrade extracellular peptides [43], it is likely that at least some of the accumulated lysosomal A β is extracellular. EM findings suggest that the endoplasmic reticulum in the CA1 neurons of thiorphan-treated apoE4 mice possesses A β immunoreactivity (not shown), suggesting that the free intra-neuronal and the mitochondrial A β pools are synthesized intra-neuronally [44, 45]. However, since extracellularly applied A β accumulates in the mitochondria of neuronal cultures [46], the free and mitochondrial A β could accumulate via this route.

Previous studies revealed that apoE4 is endocytosed preferentially by neurons [47, 48], suggesting that accumulation of apoE4 in the CA1 neurons is also mediated via endocytosis. However, since the extent of co-localization of apoE4 and A β in the CA1 neurons is small [25], most of these molecules are probably sorted differentially. ApoE can also be synthesized by neurons [49], and it is therefore possible that some of the CA1 neuronal apoE4 is produced in these cells.

Oligomerization of A β in AD and in corresponding transgenic models is accelerated by endosomal-lysosomal membranes [50, 51], suggesting that much of the oligomerization of A β in the CA1 neurons is produced within the endosomal-lysosomal system. Since, extracellular oligomeric A β can bind specifically to distinct surface proteins, such as prion protein [52], it is also possible that some of the intracellular cytosolic and mitochondrial oligomeric A β accumulate via this route. Oligomerization of A β has been shown to occur via a prefibrillar pathway whose products are recognized by I-11 or via a different fibrillar route whose products are specifically recognized by the OC antibody and which appear to be small fragments of fibrils or fibril protofilamnets [27, 53, 54]. Thus, our finding that the immunoreactivity of I-11, but not that of OC, was elevated following inhibition of neprilysin suggests that the prefibrillar A β oligomerization pathway is specifically and selectively activated by apoE4. These A β oligomers, which can serve as a template and seed the conversion of A β monomers directly into prefibrillar oligomers, are the most toxic class of amyloids and are structurally distinct from amyloid fibrils [27, 53].

The observed lysosomal pathology is probably due to the high resistance of A β , and in particular of oligomerized A β , to degradation [55], which leads to their clogging and impairment. *In vitro* studies utilizing isolated mitochondria disclosed that A β also induces morphological and functional pathology of mitochondria [56–61]. It is therefore possible

that the morphological and histochemical mitochondrial impairments observed in this study were induced by direct interactions of A β with the mitochondria. However, the possibility that apoE4 or fragments thereof also play a direct role in the mitochondrial pathology cannot be excluded [62, 63].

Both the lysosomal and the mitochondrial pathologies probably contributed to the observed neuronal pathology and to the subsequent death of the CA1 neurons. The preferential and pronounced accumulation of A β and apoE in the lysosomes, as well as our finding that the resulting oligomerization of A β and lysosomal activation followed a time course similar to that of the loss of CA1 neurons (Figs. 3–4 and see supplementary Fig. S1), suggests that these events are directly and possibly causally related. The findings that the mitochondrial pathology and the accumulation of A β in this organelle preceded the lysosomal pathology (Figs. 4 and 5) suggest that mitochondrial dysfunction might have a priming effect either on the lysosomes [59, 60, 64] or on other cellular targets whose downstream consequences contribute to neuronal loss. This pathological cascade is presented schematically in Supplementary Figure S5. Importantly, the extracellular deposition of A β in this model does not seem to be related to the observed neuronal loss as it occurs after the accumulation of oligomerized A β in the CA1 neurons and is not specific to this hippocampal subfield [17].

In conclusion, this study shows that the synergistic pathological effects of apoE4 following activation of the amyloid cascade *in vivo* were induced via the accumulation and oligomerization of A β , and their co-localization with lysosomes and mitochondria of CA1 hippocampal neurons. This resulted in mitochondrial pathology and lysosomal activation, and subsequent degradation of the CA1 neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effects of apoE4 on the levels of Aβ and oligomerized Aβ in CA1 hippocampal neurons following inhibition of neprilysin. ApoE3 and apoE4 male mice were injected i.c.v. with the neprilysin inhibitor thiorphan or sham-treated for 7 days. The mice were then killed and their brains subjected to anti-Aβ and anti-I-11 immunohistochemistry and to Aβ immunoblotting and I-11 dot-blot experiments, as described in Materials and Methods. **(A)** Representative coronal sections of thiorphan-treated apoE3 and apoE4 mice immunostained with anti-Aβ42 and I-11 are shown on the left (bar = 50 μm). Quantification of the density of Aβ42 and I-11 staining (mean ± SEM; *n* = 4–5 mice/group) in the CA1 neurons of the thiorphan- and sham-treated apoE3 and apoE4 mice is shown on the right. **P* < 0.03 for the effect of group × treatment by two-way ANOVA, and *P* < 0.005 for a comparison of the results of the thiorphan-treated apoE4 with those of the other mouse groups. **(B)** Representative confocal images of Aβ42 and I-11 of the CA1 area of apoE4 mice treated 7 days with thiorphan, and of their merged image (bar = 25 μm). **(C)** Immunoblots and dot blots of Aβ, and I-11 of hippocampal CA1 extracts (*n* = 4–5 mice/group). Upper panels depict immunoblots of PBS (left) and NP40 (right) extracts of the indicated mouse groups and their quantification, whereas the corresponding dot blots are shown in the lower panels. For the PBS extract immunoblot results, *P* < 0.05 for the effect of treatment by two-way ANOVA. For the NP-40 extract results, immunoblot, and dot blot results, **P* < 0.001 for the effect of group × treatment, and *P* < 0.02 for a comparison of the results of the thiorphan-treated apoE4 with those of the other mouse groups.

Figure 2.

Ultrastructural analysis of the pathology and co-localization of Aβ in distinct sub-cellular organelles in CA1 neurons of ApoE4 mice following inhibition of neprilysin. ApoE3 and ApoE4 male mice were injected i.c.v. with thiorphan or sham-treated for 7 days. The mice were then killed and their brains subjected to EM and Aβ EM immunogold analysis, as described in Materials and Methods. (A) Representative EM images of thiorphan-treated and sham-treated ApoE3 and ApoE4 mice depicting primary lysosomes, mitochondria, and synaptic vesicles. Quantification of the sizes of the organelles in the indicated sub-cellular compartments (mean ± SEM; $n = 3$ mice/group) are shown on the right (* $P < 0.04$ for the effect of group × treatment, and for a comparison of the results of the thiorphan-treated ApoE4 to those of the other mouse groups) (B) Representative EM Aβ immunogold images of the corresponding organelles in CA1 neurons are shown on the left. The numbers of Aβ immunogold particles that co-localized with the indicated organelles (mean ± SEM; $n = 3$ mice/group) were quantified, and are presented on the right (* $P < 0.03$ for the effect of group × treatment, and $P < 0.01$ for a comparison of the results of the thiorphan-treated ApoE4 to those of the other mouse groups). (C) EM Aβ immunogold images depicting amorphous and fibril-like deposits of Aβ in CA1 neurons of thiorphan-treated ApoE4 mice.

Figure 3.

Effects of apoE4 on lysosomal activation in CA1 hippocampal neurons following inhibition of neprilysin. ApoE3 and apoE4 male mice were injected i.c.v. with the neprilysin inhibitor thiorphan or sham-treated for the indicated times. Their brains were then processed and stained for the lysosomal marker CatD, as described in Materials and Methods. **(A)** Representative coronal sections of thiorphan-treated and sham-treated apoE4 and apoE3 mice immunostained with CatD (bar = 50 μm). **(B)** Analysis of size distribution of the lysosomes of the four mouse groups. (●), (○) correspond to the apoE4 mice and (▲), (△) to the apoE3 mice; filled and empty symbols correspond, respectively, to thiorphan-treated and sham-treated mice. Results shown are means $\pm$ SEM; $n = 4-5$ mice/group. $*P < 0.02$ for the effect of group $\times$ treatment and $P < 0.005$ for a comparison of the results for thiorphan-treated apoE4 mice with those for the other mouse groups. **(C)** Time course of lysosomal activation of the thiorphan-treated apoE4 (●) and apoE3 (▲) mice. Panels on the left and right correspond, respectively, to the amounts of small ($0.2-0.5 \mu\text{m}^2$) and large ($0.5-2 \mu\text{m}^2$) lysosomes. $*P < 0.04$ for the effect of group $\times$ treatment and $P < 0.004$ for a comparison of the results for thiorphan-treated apoE4 mice with those of the other mouse groups.

Figure 4.

Co-localization of A β and oligomerized A β and apoE with lysosomes in CA1 neurons of apoE4 mice following inhibition of neprilysin. ApoE3 and apoE4 male mice were processed for confocal immunohistochemistry at the indicated times following i.c.v. injection of thiorphan, as described in Materials and Methods. (A) Representative confocal images of A β , CatD, and their merged image in CA1 neurons of apoE4 mice injected i.c.v. with the neprilysin inhibitor thiorphan for 7 days (bar = 25 μ m) and quantitative analysis of the accumulated A β that co-localized with small and large lysosomes and of non-lysosomal A β ("free A β "). (B) Representative confocal images of oligo-A β 42 (a masked merged image of A β 42 and I-11), CatD, and their merged image (bar = 25 μ m) and quantitative analysis of the accumulated oligo-A β 42 that co-localized with small and large lysosomes and of non-lysosomal oligo-A β 42 ("free oligo-A β 42"). (C) Corresponding results of the accumulation of apoE and its co-localization with small and large lysosomes and of non-lysosomal apoE (free apoE). Results (mean $\pm$ SEM; n = 4–5 mice/group) were quantified as described in Materials and Methods. In (A), (B) and (C), (●), (▲) correspond, respectively, to thiorphan-treated apoE4 and apoE3 mice. * P < 0.03, # P < 0.001 for group $\times$ treatment, whereas * P < 0.03, # P < 0.004 for post hoc comparisons of the results of the free and large lysosomal A β 42 and oligo-A β 42 pools of the thiorphan-treated apoE4 mice to the corresponding values of the other mouse groups. (D) Representative confocal images of A β , oligo-A β 42, CatD, and apoE (bar = 25 μ m). The arrowhead denotes co-localized A β , apoE, and CatD, and arrows indicate the co-localized oligo-A β 42, apoE, and CatD. The panel on the right depicts the levels of apoE-A β and apoE-oligo-A β 42 species that co-localize with the large

lysosomes in the CA1 neurons of thiorphan-treated apoE4 (black bars) and apoE3 mice (white bars). * $P < 0.002$ for a comparison of the results of the treated apoE4 and apoE3 mice.

Figure 5.

Effects of apoE4 and inhibition of neprilysin on the mitochondria of CA1 neurons and on their interaction with Aβ and oligo-Aβ. ApoE3 and apoE4 male mice were processed for confocal immunohistochemistry at the indicated times following i.c.v. injection of thiorphan, as described in Materials and Methods. (A) Left, representative coronal sections of the indicated mouse groups immunostained with COX-1 (bar = 50 μm). Right, quantification (mean ± SEM; $n = 4-5$ mice/group) of the levels of COX-1 immunostaining in CA1 neurons of the thiorphan-treated apoE3 (▲) and apoE4 mice (●) at the indicated time points. (B) Left, representative merged confocal images of thiorphan-treated apoE4 mice double-labeled for Aβ₄₂ and COX-1 (bar = 25 μm). Right, quantification of the kinetics of accumulation of mitochondrial Aβ₄₂ in the thiorphan-treated apoE4 (●) and apoE3 (▲) mice. (C) Co-localization of oligo-Aβ₄₂ and COX-1. Left, a representative merged confocal image of oligo-Aβ₄₂ and COX-1 in CA1 neurons of thiorphan-treated apoE4 mice (bar = 25 μm). Right, quantification of the kinetics of accumulation of mitochondrial oligo-Aβ₄₂ in the thiorphan-treated apoE4 (●) and apoE3 (▲) mice. * Corresponds to comparisons of the results of the thiorphan-treated apoE4 mice at days 4, 7 and 10 to the corresponding results of the apoE3 mice. $P < 0.01$ for the COX-1 results (Fig. 5A) and $P < 0.005$ and $P < 0.02$ for the Aβ₄₂ and oligo-Aβ₄₂ co-localization results (Fig. 5B,C). (D) Representative confocal images of apoE, COX-1, and their merged image in the CA1 area of thiorphan-treated apoE4 mice (bar = 50 μm). As shown apoE4, unlike Aβ₄₂ and oligo-Aβ₄₂, did not co-localize with the mitochondria

Figure 6.

Comparison of the co-localization of the different Aβ and apoE aggregates with large lysosomes and mitochondria in apoE4 mice following inhibition of neprilysin. Results shown (mean ± SEM; 4–5 mice/group) were taken from Figures 4–5. Accordingly, black bars correspond to areas of the large lysosomes that co-localized with the indicated Aβ and apoE species in each image of the treated apoE4 mice from which the average of the corresponding area of the treated apoE3 mice was subtracted. The resulting apoE4-specific staining was then divided by the total area of the large lysosomes of the same image of the treated apoE4 mice. Gray bars depict the corresponding mitochondrial co-localization results. * $P < 0.005$ and $P < 0.01$ for comparison of lysosomal and mitochondrial Aβ42 results of the apoE4 mice and $P < 0.03$ for the corresponding comparisons of the apoE4-containing species.