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AN APOLIPOPROTEIN E4 FRAGMENT CAN PROMOTE INTRACELLULAR ACCUMULATION OF AMYLOID PEPTIDE BETA 42

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Abstract

Apolipoprotein E (apoE) plays a crucial role in lipid transport in circulation and the brain. The apoE4 isoform is a major risk factor for Alzheimer's disease (AD). ApoE4 is more susceptible to proteolysis than other apoE isoforms and apoE4 fragments have been found in brains of AD patients. These apoE4 fragments have been hypothesized to be involved in the pathogenesis of AD, although the mechanism is not clear. In this study we examined the effect of lipid-free apoE4 on amyloid precursor protein (APP) processing and A β 40 and A β 42 levels in human neuroblastoma SK-N-SH cells. We discovered that a specific apoE4 fragment, apoE4[Δ (166-299)], can promote the cellular uptake of extracellular A β 40 and A β 42 either generated after APP transfection or added exogenously. A longer length fragment, apoE4[Δ (186-299)], or full-length apoE4 failed to elicit this effect. ApoE4[Δ (166-299)] effected a 20% reduction of cellular sphingomyelin levels, as well as changes in cellular membrane micro-fluidity. Following uptake, approximately 50% of A β 42 remained within the cell for at least 24h, and led to increased formation of reactive oxygen species. Overall, our findings suggest a direct link between two early events in the pathogenesis of AD, apoE4 proteolysis and intraneuronal presence of A β .

Keywords

apolipoprotein E; Alzheimer's disease; apolipoprotein E4 fragments; amyloid peptide beta; intracellular accumulation; reactive oxygen species

Apolipoprotein E (apoE) is a major protein of the lipoprotein transport system that plays critical roles in atherosclerosis, dyslipidemia and Alzheimer's disease (AD) (Zannis et al. 2004; Mahley et al. 2006). In the brain, apoE is synthesised primarily by astrocytes and to a lesser extent by microglia and neurons (Pitas et al. 1987; Mori et al. 2004; Metzger et al. 1996). ApoE contains 299 residues and has three common isoforms (apoE2, apoE3, apoE4) each differing in the amino acid positions 112 and 158 (Zannis et al. 2004). ApoE4 has been associated with a variety

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of neuropathological processes, including AD (Mahley et al. 2006). ApoE4 is a major genetic risk factor for AD since 40% of all patients have at least one $\epsilon 4$ allele (Corder et al. 1993). Being homozygous or heterozygous for the $\epsilon 4$ allele increases the risk of AD four-fold and lowers the age of onset of late-onset AD (Corder et al. 1993; Myers et al. 1996).

Several studies have suggested that the production, oligomerization and deposition of β -amyloid peptide ($A\beta$) play central roles in AD (Haass and Selkoe 2007). $A\beta$ is a ~4 kDa peptide fragment generated by sequential proteolytic cleavage of the transmembrane amyloid precursor protein (APP). The $A\beta$ peptides can vary in length; the most common forms contain 38, 40 or 42 amino acids ($A\beta 38$, $A\beta 40$ or $A\beta 42$) (Haass and Selkoe 2007). $A\beta 42$ has been found to be more prone to fibril formation and more closely associated with the pathogenesis of AD than shorter $A\beta$ forms (Haass and Selkoe 2007). According to the amyloid cascade hypothesis, $A\beta$ forms soluble oligomers that affect synaptic structure and plasticity. In addition, $A\beta$ forms long insoluble amyloid fibrils that accumulate in neuritic plaques in the brains of AD patients and lead to widespread neuronal dysfunction and ultimate cell death (Haass and Selkoe 2007). Growing evidence from studies with humans and experimental animals suggests that $A\beta$ accumulates inside neurons. $A\beta$ accumulation occurs prior to extracellular amyloid formation and has been implicated in the onset of early cognitive alterations and may contribute to the pathological cascade of events that lead to neuronal dysfunction and eventually to AD (Bayer and Wirths 2008; LaFerla et al. 2007). Furthermore, $A\beta 42$ constitutes the majority of intraneuronal $A\beta$ (LaFerla et al. 2007).

A large number of studies have examined the association of apoE4 with sporadic AD. These studies suggested that apoE4 is involved in the modulation of plaque formation and clearance of $A\beta$, affects cholesterol homeostasis, alters phosphorylation of tau that leads to formation of neurofibrillary tangles, disrupts cytoskeleton structure and cause dysregulation of various signaling pathways (reviewed in (Mahley et al. 2006)). The molecular mechanisms of these pathological processes are still unclear. It is possible that several parallel pathways contribute to the pathogenic role of apoE4 in AD.

ApoE is sensitive to proteolytic cleavage. Bioactive carboxy-terminal truncated fragments of apoE4, which is much more susceptible to proteolysis than apoE3, have been found in brains of AD patients (25-30 and 14-22kDa) and transgenic mice that express apoE4 in neurons (29-30 and 14-20kDa) (Huang et al. 2001; Harris et al. 2003; Brecht et al. 2004; Wellnitz et al. 2005). Primary proteolytic cleavage sites of apoE have been proposed to be located at residues 268/272 (Harris et al. 2003), close to residue 187 (Wellnitz et al. 2005) or after residue 160 (Cho et al. 2001). The carboxy-terminal truncated apoE4 fragment apoE4[Δ (272-299)] has been associated with increased phosphorylation of tau, mitochondrial dysfunction and neurotoxicity in cultured neuronal cells and transgenic mice and could play a key role in the development of neuronal degeneration observed in AD (Huang et al. 2001; Harris et al. 2003; Brecht et al. 2004). In contrast, apoE4[Δ (241-299)] has been found to not be associated with increased mitochondrial dysfunction and neurotoxicity (Harris et al. 2003; Chang et al. 2005). This latter finding indicates that not all truncated apoE4 fragments have the same biological effects and that specific apoE4 fragments may be involved in separate processes associated with AD pathogenesis. Interestingly, the presence of apoE4 fragments in transgenic mice that express apoE4 in neurons is observed at 1 month of age, while the accumulation of phosphorylated tau starts at 5 months of age (Brecht et al. 2004).

Since apoE4 fragmentation has been suggested to be an early event in the pathogenesis of AD, we asked whether truncated apoE4 forms have any effect on $A\beta$ intracellular accumulation, an event that has been linked to early pathological processes that lead to AD. We examined the effect of two truncated apoE4 forms, apoE4[Δ (186-299)] (designated thereafter as apoE4-185) and apoE4[Δ (166-299)] (designated thereafter as apoE4-165) with molecular weights of 21

and 19 kDa respectively, on APP processing and A β levels in human neuroblastoma SK-N-SH and HEK293 cells transfected with human APP and on the uptake of exogenously added A β . We found that lipid-free apoE4-165, but not wild-type (WT) apoE4 or apoE4-185, significantly reduced the extracellular levels of A β 40 and A β 42, without affecting β -secretase activity. Furthermore, apoE4-165 stimulated the uptake of exogenously added A β 40 or A β 42 by SK-N-SH cells. Following uptake, approximately half of the internalized A β 42 remained in cells after 24h and led to formation of reactive oxygen species (ROS), while A β 40 is rapidly eliminated and did not lead to ROS formation. Our results indicate that a specific apoE4 fragment can promote the intracellular A β 42 accumulation, an event that has been linked to the pathogenesis of AD.

Materials and Methods

Materials

A β 40 was obtained from Chemicon/Millipore (Billerica, MA, USA) and A β 42 from Invitrogen (Carlsbad, CA, USA). The complete protease inhibitor cocktail and the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Methyl[3 H]choline chloride (1 mCi/mL; specific activity range, 66.7 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA). Dc Protein Assay Kit was from Bio-Rad (Hercules, CA, USA). Culture media and other reagents were purchased from Sigma Aldrich Corp., Invitrogen, Bio-Rad, Biochrom AG (Germany), Lonza (Belgium), Fisher Scientific (Germany) and GE Healthcare (Sweden).

Production and purification of recombinant apoE

All plasmids and recombinant adenoviruses containing the wild-type and mutated human apoE4 genes were constructed as described previously (Li et al. 2003). ApoE was purified from the culture medium of adenovirus-infected HTB-13 cells as described previously (Chroni et al. 2008).

Cell cultures and transfection

Human embryonic kidney 293 (HEK293) cells were cultured in DMEM (ultra-glutamine 1 and 4.5 g/L glucose) supplemented with 10% (v/v) FBS and antibiotics. Human neuroblastoma SK-N-SH cells (ATCC, Rockville, MD, USA) were cultured in Eagle medium supplemented with 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 1.5g/l sodium bicarbonate, 10%FBS and antibiotics.

Studies in HEK293 and SK-N-SH cells following transfection with an APP695 expression plasmid—Twenty-four hours before transfection, the HEK293 or SK-N-SH cells were plated on 100mm Petri dishes at a density of 1.5×10^6 cells/dish in culture medium without antibiotics. The next day, cells at about 90% confluence were transfected with a pcDNA3.1 plasmid encoding human APP695 or vector alone (mock) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. 6 h after DNA-Lipofectamine 2000 addition the cells were transferred on 24-well plates at a density of 1×10^5 cells/well in serum-free medium without antibiotics. Cells were incubated for 4 h to allow attachment to the plate and then incubated with 400 μ l serum-free medium in the presence or absence of 375 nM lipid-free WT or truncated apoE4 forms for 24 h. The culture medium was then collected, complete protease inhibitor cocktail was added and centrifuged in a microcentrifuge for 5 min. The cells were washed twice with ice-cold PBS, lysed at 4 °C with lysis buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 10 mM NaF, 1mM PMSF, 1 mM Na $_3$ VO $_4$, 1% Triton X-100 containing complete protease inhibitor cocktail) and centrifuged at 12,500 rpm in a microcentrifuge for 30 min at 4 °C. After centrifugation, supernatants were collected and their protein concentration was determined using the Dc Protein assay.

Studies in untransfected HEK293 and SK-N-SH cells—HEK293 or SK-N-SH cells were plated on 24-well plates at a density of 1×10^5 cells/well. After 4 h the cells were incubated with 400 μ l serum-free medium in the presence or absence of 375 nM lipid-free WT or truncated apoE4 forms and in the presence or absence of 25 ng/ml A β 40 or A β 42 for 24 h. At the end of this period culture media and cell lysates were collected as described above.

Western blotting

Detection of APP and sAPP α —Culture media and cell lysates, normalised for cell protein content, were analyzed by Western blotting. APP and sAPP α were detected using the mouse anti-amyloid beta monoclonal antibody 6E10 (Chemicon/Millipore), against residues 1-17 of A β , and a goat anti-mouse IgG coupled to horseradish peroxidase (HRP) (Chemicon/Millipore). To normalise the APP Western blot signal in cell lysates, blots were re-blotted with mouse anti- α -tubulin antibody DM 1A (Sigma) followed by incubation with goat anti-mouse IgG-HRP.

Detection of LDL receptor-related protein (LRP)—Cell lysates, normalised for cell protein content, were subjected to non-reducing 5% SDS-PAGE and analysed by Western blotting using the mouse anti-LRP monoclonal antibody 8G1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) that detects the 515 kDa extracellular domain of LRP.

Densitometric analysis was performed using the Image J image analysis software (NIH) (Abramoff et al. 2004).

Determination of A β levels

Extracellular A β levels—A β 40 and A β 42 levels in cell media, containing complete protease inhibitor cocktail (for A β 40) or 1 mM serine protease inhibitor AEBSF (for A β 42) were measured by sandwich Elisa using commercial kits (Invitrogen) following the manufacturer's instructions. The volume of cell medium or cell lysate loaded per well of the Elisa plate was adjusted according to the cell protein content.

Intracellular A β levels—Cells were washed with ice-cold PBS and lysed by sonication with the microtip of a sonifier (3 $\times$ 10sec) in standard diluent buffer containing 1 mM serine protease inhibitor AEBSF provided in the Human Amyloid High Sensitivity 1-40 or Human Amyloid 1-42 Elisa kits (Invitrogen). Finally, cell lysates were centrifuged at 12,500 rpm in a microcentrifuge for 30 min at 4 °C and the supernatants were collected. A β 40 or A β 42 in the whole volume of cell lysate were measured by the Elisa kits, according to manufacturer's instructions.

β -Secretase activity assay

APP695 expressing SK-N-SH cells were lysed by sonication in 0.3% Triton X-100/10mM Tris-HCl pH7.0 and β -secretase activity was assayed in cell lysates with a fluorogenic substrate (MCA-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Lys(DNP)-NH₂) (Calbiochem/Merck, Darmstadt, Germany) as described (Johnston et al. 2008). Enzyme reactions were carried in a final volume of 100 μ l of reaction buffer (50 mM CH₃COONa, pH 4.5) containing 10 μ g of total cell protein and 10 μ M of substrate. After 2 h of incubation at 37 °C in the dark, the increase in fluorescence at 393nm (excitation 325nm, emission 370-420nm) was measured in a Quantamaster 4 fluorescence spectrometer (PTI, New Jersey, USA) using a 0.5 nm excitation slit width and 2 nm emission slit width. β -Secretase activity was calculated by the increase in fluorescence per min per mg of cellular protein.

Confocal microscopy

SK-N-SH cells seeded on coverslips were incubated with or without A β and apoE4 forms. At the end of incubation, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS and incubated in blocking and permeabilization buffer (PBS containing 5% FBS and 0.05% Tween-20) for 30 min at room temperature. For A β 40 staining, cells were incubated with the mouse anti-A β monoclonal antibody 6E10 (1:200) overnight at 4°C. Cells were then washed with PBS and incubated with the secondary antibody FITC-conjugated goat anti-mouse IgG antibody (1:100, Cappel/ICN Pharmaceuticals, Frankfurt, Germany) for 1 h at room temperature. A β 40 was alternatively stained with the rabbit polyclonal anti-A β 40 antibody R163 which is specific for the carboxy-terminal region of A β 40 (1:150, a generous gift from Dr. P.D. Mehta, NY State Institute for Basic Research, NY, USA and Dr. S. Efthimiopoulos, University of Athens, Greece (Mehta et al. 1998)). As secondary antibody the Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (1:1000, Invitrogen) was used. For A β 42 staining, cells were incubated with the mouse anti-A β monoclonal antibody 6E10 (1:300) overnight at 4 °C, followed by incubation with a FITC-conjugated goat anti-mouse IgG antibody (1:100) for 1h at room temperature. For α -tubulin staining, cells were incubated with the mouse monoclonal anti- α -tubulin antibody DM 1A (1:500, Sigma) overnight at 4 °C, followed by incubation with a FITC - conjugated goat anti-mouse IgG antibody (1:100) for 1h at room temperature. For lysosomal and mitochondrial staining, 1h before the end of incubation of SK-N-SH cells with or without A β and apoE4 forms, 30 μ l of a stock solution of LysoTracker Red (Lonza, Walkersville, MD, USA) or MitoTracker Red CMXRos (Invitrogen) were added to cells in order to achieve a final concentration of 600 nM LysoTracker Red or 500 nM MitoTracker Red CMXRos and the incubation was continued for 1 h at 37 °C. Following labeling with LysoTracker Red or MitoTracker Red CMXRos the cells were washed, fixed, permeabilized and stained for A β 40 or A β 42, as described above.

Coverslips were mounted and pictures were taken using a Bio-Rad confocal microscope (MRC 1024 ES) equipped with Lasersharp software (BioRad) and a krypton-argon laser with a motor step of 0.5 μ m. Confocal images were merged with the Confocal Assistant software. Co-localization was visualized by suppressing all colors, except yellow, in superimposed images using Adobe Photoshop™ software.

Solid-phase Binding Studies

The interaction of A β 40 with WT and truncated apoE4 forms was evaluated by enzyme-linked immunosorbent solid phase assay as described before (Golabek et al. 1996) with some modifications. MaxiSorp 96-well immuno plates were coated with A β 40 (400 ng/100 μ L/well in 50 mM carbonate/bicarbonate buffer pH 9.0) for 2.5 h at 37°C. Under these conditions 10ng/well of peptide bound to plate (Golabek et al. 1996). Following blocking and wash, apoE4 forms were added at concentrations of 0-400 nM in PBS/1% Triton X-100 for 3 h at 37 °C. Subsequently, the plate was washed and incubated with mouse monoclonal anti-apoE antibody 6C5 (1:200, University of Ottawa Heart Institute, Canada) for 1 h at 37 °C, followed by wash and incubation with HRP-conjugated goat anti-mouse IgG (1:800) for 1h at room temperature. Bound apoE4 forms were detected spectrophotometrically at 490nm after addition of *o*-phenylenediamine dihydrochloride as substrate for HRP.

Sphingomyelin, phosphatidylcholine and cholesterol determination

SK-N-SH cells seeded on 24-well plates at a density of 1×10^5 cells/well were labeled with 0.5 ml of labeling medium (1 μ Ci methyl [3 H]choline chloride dissolved in 0.1% ethanol as the final concentration and dispersed into serum-free medium supplemented with 0.2% BSA). Following 24 h of labeling, cells were washed twice with serum-free medium and incubated in the absence or presence of 375 nM WT or truncated apoE4 forms for 24 h at 37 °C. At the

end of incubation, cell media were removed and total lipids were extracted from cells with chloroform and methanol (Bligh and Dyer 1959). Unlabeled sphingomyelin and phosphatidylcholine internal standards were added to lipid extracts and [^3H]choline phospholipids were separated by TLC on polysilica acid gel impregnated glass fiber sheets (Pall corporation, Ann Arbor, MI, USA) developed in $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ 65:35:4 (v/v/v). Finally, the radioactivity of sphingomyelin and phosphatidylcholine spots on TLC sheets was determined by liquid scintillation counting. The percentage of cellular [^3H] phosphatidylcholine or [^3H]sphingomyelin was calculated by dividing the phosphatidylcholine- or sphingomyelin-derived counts, respectively, by the sum of the total cellular [^3H]choline phospholipids counts.

For cellular cholesterol determination, cellular lipids were extracted with chloroform and methanol (Bligh and Dyer 1959), dried under N_2 and dissolved in isopropanol. Cellular cholesterol content was determined spectrophotometrically using the INFINITY cholesterol reagent (Thermo Electron, Melbourne, Australia), according to manufacturer's directions.

1-Pyrenedodecanoic acid fluorescence measurements

1-Pyrenedodecanoic acid labeling of plasma membranes and fluorescence measurements were carried out as described (Hashimoto et al. 1999; Galla and Luisetti 1980). SK-N-SH cells seeded on 24-well plates at a density of 1×10^5 cells/well, were incubated in the absence or presence of 375nM WT or truncated apoE4 forms for 24 h at 37 °C. At the end of incubation, cells were washed twice and suspended in PBS. 1-pyrenedodecanoic acid (Molecular probes/Invitrogen) was added to a fluorimeter cuvette containing cells suspension to a final concentration of 2 μM and incubated for 5 min in the dark. The fluorescence intensity was scanned, in a Quantamaster 4 fluorescence spectrometer (PTI, New Jersey, USA), from 380-580nm at an excitation wavelength of 340nm, using a 1 nm excitation slit width and 0.5 nm emission slit width. After scanning, the ratio of the maximum fluorescent intensities of excimer to pyrene monomer was calculated at 468 and 395 nm, respectively.

Measurement of ROS generation

Intracellular ROS generation was measured by a previously described method (Bae et al. 1997) modified for fluorescent microscopy. SK-N-SH cells, incubated with or without A β and apoE4 forms, were washed with DMEM and then incubated in the dark for 45 min at 37 °C in DMEM containing 25 μM 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Molecular probes/Invitrogen). At the end of this incubation period, cells were washed twice with preheated PBS at 37 °C and the production of ROS was detected by 2', 7'-dichlorofluorescein (DCF) fluorescence using the Axiovert 25 (Zeiss, Germany) inverted microscope equipped for fluorescence microscopy (excitation 450-490 nm, emission 520 nm). Forty five groups of 5 cells each were randomly selected from the images of each sample, the fluorescent intensity was measured for each group from the fluorescent image using the Image J image analysis software (Abramoff et al. 2004) and the relative fluorescent intensity was taken as average of the 45 values.

Results

Effect of truncated apoE4 forms on APP processing and A β levels in SK-N-SH and HEK293 cells transiently transfected with human APP

To study the effect of truncated apoE4 forms on APP processing and extracellular A β levels, human neuroblastoma SK-N-SH cells were transiently transfected with human APP695, which is the APP isoform mainly expressed in the human brain (Kang et al. 1987). After transfection, the cells were incubated in the presence or absence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165 for 24 h. The apoE4 forms were used at a concentration similar to the reported

apoE concentration in human cerebrospinal fluid (365-396 nM) (Kay et al. 2003; Wang et al. 2010). Treatment of cells with WT or carboxy-terminal truncated apoE4 forms had no effect on cellular APP or secreted sAPP α levels as assessed by Western blotting (Figure 1A and 1B). Similar data were obtained for APP and sAPP α levels in the non-neuronal cells HEK293 transfected with APP695 and incubated in the absence or presence of apoE4 forms (supporting information Figure S1). Incubation of SK-N-SH cells transfected with APP with WT apoE4 or apoE4-185 had no effect on extracellular A β 40 levels, as determined by sandwich Elisa. In contrast, incubation with apoE4-165 led to a 78% reduction of extracellular A β 40 levels as compared to untreated cells (Figure 1C). Similarly, incubation of APP-expressing HEK293 cells with apoE4-165 led to a 62% reduction of extracellular A β 40 levels as compared to untreated cells (supporting information Figure S1). These results suggest that apoE4-165 can affect the extracellular A β 40 levels in cultured cells.

To test whether apoE4-165 affects also the extracellular levels of A β 42, which is more closely associated with the pathogenesis of AD than the shorter A β 40 form (Haass and Selkoe 2007), we performed the same analysis as described above. Incubation of APP-expressing SK-N-SH cells with WT apoE4 or apoE4-185 did not affect the extracellular A β 42 levels as compared to untreated cells. Strikingly, no A β 42 was detectable after incubation of the cells with apoE4-165 (Figure 1D). These observations suggest that the apoE4-165 fragment can reduce the extracellular levels of A β 42, as well as of A β 40, from SK-N-SH cells.

To examine whether the reduction of the extracellular levels of A β 40 and A β 42 in APP-expressing SK-N-SH cells treated with apoE4-165 was due to reduced cellular β -secretase activity, we measured β -secretase activity using an assay based on the cleavage of the fluorogenic substrate (MCA-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Lys(DNP)-NH₂). We found no statistically significant change in β -secretase activity in APP-expressing SK-N-SH cells treated with WT or truncated apoE4 forms compared to untreated cells (data not shown). This finding suggests that the mechanism by which apoE4-165 affects A β 42 or A β 40 levels does not involve alterations of the rate of A β generation by β -secretase.

Effect of truncated apoE4 forms on the levels of exogenously added A β and on the uptake of A β from SK-N-SH cells

Since the reduction of extracellular A β 40 or A β 42 levels from cells treated with apoE4-165 was not due to decreased production by β -secretase, we asked whether A β 40 or A β 42 is actively eliminated from the medium. For this purpose, we examined whether apoE4-165 leads to reduction of A β that has been exogenously added to the cell medium. We incubated SK-N-SH or HEK293 cells with 25ng/ml (5.5nM) A β 40 in the absence or presence of 375nM WT or carboxy-terminal truncated apoE4 forms and measured the amount of remaining A β 40 in the cell medium after 24h by sandwich Elisa. The A β 40 concentration used was within the physiological range, as reported for human cerebrospinal fluid (4-44 ng/ml) (Nakamura et al. 1994; Mehta et al. 2001). As shown in Figure 2A and Figure S2, apoE4-165 reduced the levels of exogenously added A β 40 by 91% and 70% in SK-N-SH and HEK293 cells, respectively. Incubation of either cell line with WT apoE4 or apoE4-185 had no effect on the levels of exogenously added A β 40 (Figure 2A and Figure S2).

We then asked whether the reduction of extracellular A β 40 levels by apoE4-165 is due to A β 40 uptake by the cells. SK-N-SH cells incubated with 25 ng/ml exogenously added A β 40 in the presence of 375 nM WT or truncated apoE4 forms for 24 h were analyzed by fluorescence confocal laser scanning microscopy to detect intracellular A β 40. Staining of SK-N-SH cells with the R163 antibody, which is specific for A β 40 (Mehta et al. 1998), showed strong A β 40 immunoreactivity for cells treated with apoE4-165 (Figure 2E), while the A β 40 immunoreactivity was minimal in untreated cells or cells treated with WT apoE4 or apoE4-185 (Figures 2B, C and D). Similar results were obtained when cells were stained with the 6E10

antibody (supporting information Figure S3). In the absence of A β 40 or the primary antibody no immunoreactivity was observed (data not shown). To confirm the intracellular A β 40 immunostaining the cells were co-stained with the R163 antibody and an antibody against α -tubulin in order to view the outline of individual cells (Figures 2E, F). Merging the images of the A β 40 and α -tubulin stained cells confirmed that the detected A β 40 was inside the cells (Figure 2G).

Subsequently, we investigated whether apoE4-165 also promotes the uptake of A β 42. Incubation of SK-N-SH cells with apoE4-165 in the presence of 25 ng/ml A β 42 for 24 h reduced the levels of A β 42 by 56% as compared to the initial A β 42 levels (Figures 3A). Incubation of cells in the absence or presence of WT apoE4 or apoE4-185 had no effect on the levels of exogenously added A β 42 (Figures 3A). Incubation of SK-N-SH cells with exogenously added A β 42 followed by immunostaining and confocal microscopy showed strong intracellular A β 42 immunoreactivity for cells treated with apoE4-165 and minimal A β 42 immunoreactivity for cells treated with WT apoE4, apoE4-185 or untreated cells (Figures 3B-E). These results suggest that the extracellular levels of A β 40 and A β 42 in the presence of apoE4-165 are reduced due to increased uptake of the amyloid peptides by the cells.

Effect of truncated apoE4 form apoE4-165 on cellular LRP and lipid levels and on cellular membrane fluidity probed by 1-pyrenedodecanoic acid

In order to gain insights into the mechanism by which apoE4-165 promotes the uptake of A β 40 and A β 42 from cells several approaches were taken. First, we tested whether apoE4-165 could bind A β and transport it inside the cells. Determination of the capacity of WT apoE4, apoE4-185 and apoE4-165 to bind A β 40 by solid phase binding assay showed that only WT apoE4 bound to immobilised A β 40, while both apoE4-185 and apoE4-165 failed to bind to A β 40 (data not shown). It is therefore unlikely that apoE4-165 transports A β 40 or A β 42 inside the cell in the form of a molecular complex. Next, we tested whether apoE4-165 could increase LRP cellular levels leading to A β internalization via an LRP-dependent pathway. Previous studies have suggested that one of the mechanisms of A β uptake, independently of apoE, involves LRP that can bind and endocytose A β directly, thus facilitating A β cellular uptake (Zerbinatti et al. 2006; Deane et al. 2004). As shown in Figure 4A no statistically significant effects on LRP cellular levels were observed in SK-N-SH cells incubated with WT apoE4, apoE4-185 or apoE4-165 compared to the untreated cells. These results suggest that the apoE4-165-induced A β uptake is not dependent either on the direct binding of A β to apoE4-165 or upregulation of LRP gene expression.

Changes in brain cellular lipid levels have been associated with the internalization of A β and the pathogenesis of AD (Saavedra et al. 2007; Bandaru et al. 2009). Since apoE4 is a lipid-binding protein we asked whether apoE4-165 can affect the lipid composition of SK-N-SH cells. SK-N-SH cells labeled with [3 H]-choline were incubated in the presence or absence of 375nM WT or carboxy-terminal truncated apoE4 forms for 24h. Phospholipids were extracted from cells and separated by TLC, as described in the Materials and Methods section. As shown in Figure 4B incubation of SK-N-SH cells with apoE4-165 resulted in a statistically significant 20% reduction of cellular sphingomyelin levels as compared to untreated cells. WT apoE4 or apoE4-185 had no effect on cellular sphingomyelin levels. Incubation of cells in the absence or presence of WT apoE4, apoE4-185 or apoE4-165 had no effect on phosphatidylcholine levels (Figure 4B). In addition, there was no change in the cholesterol content of any of the treated cells (data not shown). Overall, the apoE4 fragment-specific effects on cellular sphingomyelin levels appear to mirror the A β 40 and A β 42 internalization.

The lateral and transversal fluidity of the cellular membrane can be altered by changes in the lipid composition of the membrane. 1-pyrenedodecanoic acid excimers break into monomers in the lipophilic interior of the membrane that give distinct fluorescence spectra and this

property can be used to gain insights on the micro-fluidic state of the membrane (Hashimoto et al. 1999; Galla and Luisetti 1980). To gain insight on the micro-fluidic properties of the cellular membrane of SK-N-SH cells when incubated with apoE4 we measured the fluorescence of 1-pyrenedodecanoic acid added onto cell suspension that had been treated with apoE4 forms. We found no statistically significant changes in the eximer/monomer ratio of the probe when the cells were incubated with WT apoE4 or apoE4-185 compared to untreated cells ($p>0.35$). In contrast, we found a statistically significant decrease of 10% in the eximer/monomer ratio of 1-pyrenedodecanoic acid when the probe was titrated onto cells previously treated with apoE4-165 ($p=0.001$). This observation suggests changes in the lateral and transversal mobility of the probe within the cellular membrane, a finding that may be related with changes in the lipid composition of cells treated with apoE4-165.

Preferential intracellular accumulation of A β 42 over A β 40 in SK-N-SH cells

We next examined the fate of the internalized A β . SK-N-SH cells were incubated with 25ng/ml A β 40 or A β 42 in the presence of 375nM apoE4-165 for 24 h. The cells were washed and incubated further in fresh A β and apoE4-165-free medium at 37 °C up to 24 h. The amount of intracellular A β was determined in cell lysates at various time points, by sandwich Elisa. As shown in Figure 5 A β 40 was rapidly eliminated since only 12-27% of the internalized A β 40 could be detected following 30min-6h incubation, while no A β 40 could be detected after 24 h in SK-N-SH cells. In contrast, the amount of the internalized A β 42 was approximately 50% of the initial A β 42 levels after 1.5 h of incubation of SK-N-SH cells and this amount remained the same after 24h (Figure 5). These findings indicate that internalized A β 42, but not A β 40, can accumulate inside SK-N-SH cells.

Previous studies have shown that intracellular A β can accumulate in lysosomes, late endosomes, mitochondria, but also outside of these organelles in the cytoplasm (LaFerla et al. 2007; Kandimalla et al. 2009). In order to determine the localization of A β internalized by SK-N-SH cells, the cells were simultaneously stained with 6E10 antibody and LysoTracker in order to label both A β and lysosomes or other acidic organelles such as late endosomes. In a separate experiment, the cells were stained with the 6E10 antibody and MitoTracker simultaneously in order to label both A β and mitochondria. Initially we examined the co-localization of A β 40 or A β 42 with LysoTracker or MitoTracker after incubation of SK-N-SH cells with apoE4-165 for 24h. Since we showed that A β 42 can accumulate inside SK-N-SH cells, we also examined the co-localization of A β 42 with LysoTracker or MitoTracker 24h after the removal of A β 42 and apoE4-165 from the cell medium. Fluorescence confocal laser scanning microscopy analysis showed only limited co-localization between intracellular A β 40 and LysoTracker (Figures 6A-D) in SK-N-SH cells incubated with A β 40 and apoE4-165 for 24h. Intracellular A β 42 also displayed limited co-localization with LysoTracker, in SK-N-SH cells incubated with A β 42 and apoE4-165 for 24h, although in 5-10% of the cells this co-localization was more pronounced (Figures 6E-H). Twenty-four hours after the removal of A β 42 and apoE4-165 from the cell medium the intracellular A β 42 still displayed only limited co-localization with LysoTracker and no cells with pronounced co-localization were observed (Figures 6I-L). Similar analysis showed only minimal co-localization between intracellular A β 40 or A β 42 and MitoTracker in SK-N-SH cells incubated with A β and apoE4-165 for 24h, as well as between intracellular A β 42 and MitoTracker in SK-N-SH cells 24h after the removal of A β 42 and apoE4-165 from the cell medium (Figures 7A-L). These data suggest that the majority of A β internalized by SK-N-SH cells treated with apoE4-165 and of A β 42 accumulated in SK-N-SH cells is dispersed throughout the whole cell, outside of lysosomes, late endosomes or mitochondria.

Effect of A β 40 and A β 42 following uptake by SK-N-SH cells treated with apoE4-165 on ROS generation

The accumulation of A β 42 inside SK-N-SH cells after apoE4-165 mediated internalization, raises questions whether this peptide can have an effect on oxidative stress in SK-N-SH cells. To address this question we tested whether intracellular A β 42 accumulation may lead to the formation of ROS, which are markers of oxidative stress. SK-N-SH cells were incubated with 25ng/ml A β 40 or A β 42 in the absence or presence of 375nM WT apoE4, apoE4-185 or apoE4-165 for 24h and subsequently the redox-sensitive dye DCFH-DA was added as described under “Materials and Methods”. This neutral dye is freely permeable to cells, but once it becomes internalized it is converted by cellular esterases to anionic 2', 7'-dichlorofluorescein (DCFH) which is no longer membrane permeable and is therefore trapped intracellularly. DCFH is able to interact with ROS inside the cell that convert it to the fluorescent DCF, which can be detected using a fluorescence microscope (LeBel et al. 1992). As shown in Figure 6B-F there was no statistically significant change in the fluorescence of cells incubated with A β 40 in the absence or presence of WT apoE4, apoE4-185 or apoE4-165. In contrast, incubation of cells with A β 42 in the presence of apoE4-165 resulted in 50% increase in ROS formation as compared to cells incubated in the absence of apoE4-165 (Figure 8A). Incubation of cells with A β 42 and apoE4 or apoE4-185 did not lead to enhanced ROS formation compared to cells incubated in the absence of apoE4 forms (Figure 8A). The fluorescence of cells in the absence of A β was minimal. Since A β 42 can accumulate inside SK-N-SH cells, we also measured the ROS levels in SK-N-SH cells that were incubated with A β 42 and apoE4-165 for 24h and then further incubated in the absence of A β 42 and apoE4-165 for twenty four more hours. As shown in Figure 8B, ROS levels were similar in SK-N-SH cells incubated with A β 42 and apoE4-165 for 24h and in SK-N-SH incubated without A β 42 and apoE4-165 for an additional twenty four hours. Overall, our data suggest that the intracellular accumulation of A β 42 in SK-N-SH cells incubated with apoE4-165 leads to increased ROS formation that can persist for at least 24h.

Discussion

Although apoE4 is considered a major risk factor for AD its exact role in the pathogenesis of AD has not been elucidated. ApoE4 has been shown to be more susceptible to proteolysis than apoE2 and apoE3 and carboxy-terminal truncated fragments of apoE4 accumulate in brains of AD patients and neurons of apoE4-transgenic mice (Huang et al. 2001; Harris et al. 2003; Brecht et al. 2004). Furthermore, studies in transgenic mice expressing apoE4 in neurons suggested that apoE proteolysis occurs in the secretory and not in the internalization pathway in neurons and that this apoE4 fragmentation is an early event in the pathogenesis of AD (Brecht et al. 2004).

In the current study we determined the effect of two truncated apoE4 forms, apoE4-185 and apoE4-165 on A β 40 and A β 42 metabolism and intracellular levels of SK-N-SH neuroblastoma cells. These apoE4 truncated forms have a molecular weight of 21 and 19 kDa, respectively, both within the range of molecular weights of carboxy-terminal apoE4 fragments found in brains of AD patients and apoE4 transgenic mice (25-30 and 14-22kDa) (Huang et al. 2001; Harris et al. 2003; Brecht et al. 2004). We show that one of the truncated apoE4 forms studied, apoE4-165, leads to intracellular A β 42 accumulation, an event that has been previously suggested to be an early pathologic feature of AD (Bayer and Wirths 2008; LaFerla et al. 2007). Approximately 50% of the internalized A β 42 persisted in the cells for at least 24h, while the internalized A β 40 was eliminated at a rapid rate. This is consistent with previous studies in nerve growth factor-differentiated PC12 cells and mouse cultured neurons which had also demonstrated that part of internalized A β 42 accumulates intracellularly, while A β 40 is eliminated (Burdick et al. 1997; Ditaranto et al. 2001). The majority of A β internalized by SK-

N-SH cells and of A β 42 accumulated in SK-N-SH cells can be found throughout the whole cell, outside of lysosomes, late endosomes or mitochondria.

It has been proposed that apoE can bind A β and undergo endocytosis via LRP (Gyls et al. 2003; Zerbinatti et al. 2006). Furthermore, it has been suggested that LRP also binds and endocytoses A β directly, facilitating A β cellular uptake (Zerbinatti et al. 2006; Deane et al. 2004). Therefore, we examined whether the A β uptake is mediated by direct interactions with apoE4-165 as well as LRP. We found that apoE4-165, as well as apoE4-185, failed to bind to A β , in accordance with previous studies showing that the carboxy-terminal region of apoE is necessary for A β binding (Aleshkov et al. 1999; Pillot et al. 1999). In addition, WT or truncated apoE4 forms did not have any effect on cellular LRP levels. These data suggest that uptake of A β from SK-N-SH cells treated with apoE4-165 is unlikely to proceed via direct binding of A β to apoE4-165 or to LRP upregulation.

Previous studies had suggested that changes in brain cellular lipid levels are associated with the internalization of A β and the pathogenesis of AD (Saavedra et al. 2007; Cutler et al. 2004; Bandaru et al. 2009). We demonstrated that apoE4-165 leads to 20% reduction of cellular sphingomyelin levels, while WT apoE4 and apoE4-185 have no such effect. Furthermore, we detected changes in the micro-fluidic properties of the cellular membrane of SK-N-SH neuroblastoma cells only when they were treated with apoE4-165. These two observations, taken together, suggest that exogenous apoE4-165 can affect the composition, biophysical state and possibly the functionality of the cellular membrane. In an earlier study it was shown that reduction of cellular sphingolipids by the ceramide synthesis inhibitor Fumonisin B1 led to increased A β 42 internalization by rat primary neurons (Saavedra et al. 2007). In that study the authors suggested that when A β is not complexed with apoE its internalization is not mediated by members of the LDL receptor family, but rather by an endocytic process affected by cellular cholesterol and sphingolipids levels (Saavedra et al. 2007). Decreased levels of sphingomyelin have also been measured in a brain region with extensive A β plaques and neurofibrillary tangles (middle frontal gyrus) of AD patients compared to controls (Cutler et al. 2004), as well as in the middle frontal gyrus grey matter of apoE4 AD patients compared to apoE3 AD patients (Bandaru et al. 2009). In another study it was proposed that A β 40 and A β 42, located mostly in cytoplasm of rat primary hippocampal neurons and differentiated PC12 cells, could be internalized via passive diffusion (Kandimalla et al. 2009). The passive diffusion mechanism of A β internalization was supported by studies showing that A β 40 can intercalate into the phospholipid bilayer of neuronal plasma membrane (Mason et al. 1999) and that interactions of A β with lipid bilayers are affected by the bilayer lipid composition (Waschuk et al. 2001). In the present study we showed by fluorescence confocal laser scanning microscopy that A β 42 and A β 40 internalized by SK-N-SH cells after incubation with apoE4-165 and A β for 24h were partially localized to lysosomes, indicating an endocytic uptake. However, a large fraction of A β 42 and a larger fraction of A β 40 were found in the cytoplasm of SK-N-SH cells, separate from LysoTracker-labelled intact lysosomes or other acidic organelles. This latter observation suggests a non-endocytotic uptake. Taken together our results suggest that the uptake of A β by SK-N-SH cells in the presence of apoE4-165 proceeds either by a LRP-independent endocytotic mechanism or by passive diffusion. However, internalization of A β by SK-N-SH cells through another receptor not identified in this study or a combination of the above mechanisms cannot be ruled out.

Oxidative stress has been proposed to be one of the earliest events in AD that plays important roles in the onset and progression of the disease. This stress has been suggested to be chronic in neurons (Zhu et al. 2003). Furthermore, A β has been shown to contribute to the oxidative stress in neurons (Smith et al. 2007). It has been proposed that ROS, that are considered markers of oxidative stress, are generated in the cytoplasm of neurons by the interaction of mitochondria, redox transition metals and other factors and contribute to the pathogenesis of

AD (Zhu et al. 2003). We show that incubation of SK-N-SH cells with apoE4-165 and A β 42 for 24h, but not A β 40, leads to a 50% increase in intracellular ROS levels. These levels remained unchanged for at least 24h after the removal of A β 42 and apoE4-165 from the cell medium. It is thus possible that the apoE4-165-induced uptake and accumulation of A β 42 from SK-N-SH neuroblastoma cells and chronic oxidative stress are linked.

Interestingly, the striking differences between the ability of apoE4-165 to promote A β uptake, compared to the apoE4-185 fragment can be correlated to structural differences between the two apoE4 fragments. ApoE in the lipid-free state is folded into two independent structural domains (Wetterau et al. 1988). Digestion with thrombin produces a 22-kD N-terminal fragment (residues 1 to 191) and a 10-kD C-terminal fragment (residues 216 to 299) (Wetterau et al. 1988). X-ray crystallographic analysis of the apoE N-terminal domain (residues 1-191) has revealed a 4 helix bundle spanning residues 24-164 that segregates the hydrophobic core of the 4 helices from the solvent (Wilson et al. 1991; Wilson et al. 1994). Unfolding of this N-terminal domain is thought to constitute a necessary conformational change for lipid binding and apoE function (Wilson et al. 1991; Wilson et al. 1994). In a previous study, we characterized the two apoE4 fragments by biophysical techniques and discovered that apoE4-165 is destabilized compared to apoE4-185 (Chroni et al. 2008). Furthermore, an additional solvent-exposed hydrophobic site was detected on apoE4-165 (Chroni et al. 2008). This site may be responsible for apoE4-165 destabilization and can mediate initial lipid interactions that subsequently lead to further conformational changes. It is conceivable that the lack of the 166-185 region may facilitate N-terminal core domain unfolding leading to a truncated apoE4 molecule that can interact more readily with hydrophobic sites of membrane-bound A β and lipids on the cell surface. Such interactions between apoE4-165 and the cellular membrane may effect changes in the membrane's structural and fluidic properties that mediate passive A β diffusion. Overall, the results described here are consistent with our previous study that suggested that apoE4 C-terminal truncations can have complex effects on the stability and dynamics of the remaining fragment (Chroni et al. 2008). It is therefore possible that not all apoE4 fragments found in AD patient's brains are equally bioactive and specific fragments alone can promote the pathogenesis of the disease.

Overall our findings provide an association between two molecular events, the proteolysis of apoE4 and the intraneuronal presence of A β , both of which are considered to be early events in the pathogenesis of AD. Intracellular accumulation of A β 42 in SK-N-SH neuroblastoma cells incubated in the presence of apoE4 carboxy-terminal truncated form apoE4-165 is associated with ROS formation and therefore increased oxidative stress, which is also an early event in AD. We therefore propose that specific short apoE4 proteolytic fragments produced in the brain under pathologic conditions may promote intraneuronal accumulation of A β 42 leading to neuronal dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Aβ	amyloid beta peptide
Aβ40	40-amino-acid A β variant
Aβ42	42-amino-acid A β variant
AD	Alzheimer's disease
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
apoE	apolipoprotein E
apoE4-185	apoE4[Δ (186-299)]
apoE4-165	apoE4[Δ (166-299)]
APP	amyloid precursor protein
DCF	2', 7'-dichlorofluorescein
DCFH	2', 7'-dichlorofluorescein
DCFH-DA	2', 7'-dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DNP	2,4-dinitrophenyl
Eagle	Minimum Essential Medium
EDTA	Ethylenediaminetetraacetic acid
Elisa	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HEK293 cells	human embryonic kidney 293 cells
HRP	horseradish peroxidase
LDL	low density lipoprotein
LRP	LDL receptor related protein
MCA	7-methoxycoumarin-4-acetic acid
PBS	phosphate buffered saline
PMSF	phenylmethylsulphonyl fluoride
ROS	reactive oxygen species
sAPPα	soluble amyloid precursor protein α
TLC	thin liquid chromatography
WT	wild type

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Figure 1A-D. Effect of WT and carboxy-terminal truncated apoE4 forms on cellular APP, secreted sAPPα and secreted Aβ levels in SK-N-SH cells expressing human APP

SK-N-SH cells transiently transfected with human APP695 were incubated in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165 for 24 h. Cellular APP levels normalized by the α-tubulin levels (A) and secreted sAPPα levels (B) were measured by immunoblotting as described under “Materials and Methods”. Western blots were scanned and quantified by ImageJ (lower panel). Secreted Aβ40 (C) or Aβ42 (D) levels were detected in cell medium by sandwich Elisa as described under “Materials and Methods”. Values represent the means ± SD of three experiments performed in triplicate or quadruplicate. *, $p < 0.0001$ vs. control. #, not detectable.

Figure 2A-G. Uptake of exogenous added Aβ40 by SK-N-SH cells in the presence of WT and carboxy-terminal truncated apoE4 forms

A, SK-N-SH cells were incubated with 25 ng/ml Aβ40 in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165 for 24h. The amount of remaining Aβ40 in the cell medium was measured by sandwich Elisa as described under “Materials and Methods”. The Aβ40 levels following 24 h of incubation are expressed as percent relative to the initial Aβ40 levels set to 100%. Values are the means $\pm$ SD of three experiments performed in duplicate or triplicate. *, $p < 0.0001$ vs. control. B-G, fluorescence confocal laser scanning microscopy of SK-N-SH cells incubated for 24 h with 25 ng/ml Aβ40 in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165. Aβ40 immunostaining of cells was detected with the antibody R163 followed by an Alexa Fluor 568-conjugated secondary antibody (B-E, red). α -tubulin was stained with a DM1A antibody followed by a FITC-conjugated secondary antibody (F, green). The merge of images E and F is shown in panel G.

Figure 3A-E. Uptake of exogenous added A β 42 by SK-N-SH cells in the presence of WT and carboxy-terminal truncated apoE4 forms

A, SK-N-SH cells were incubated with 25 ng/ml A β 42 in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165 for 24 h. The amount of remaining A β 42 in the cell medium was measured by sandwich Elisa as described under “Materials and Methods”. The A β 42 levels after 24 h of incubation are expressed as percent relative to the initial A β 42 levels set to 100%. Values are the means $\pm$ SD of three experiments performed in duplicate. *, $p < 0.0001$ vs. control. B-E, fluorescence confocal laser scanning microscopy of SK-N-SH cells incubated for 24h with 25 ng/ml A β 42 in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165. A β 42 immunostaining of cells was detected with the antibody 6E10 followed by a FITC-conjugated secondary antibody (green).

Figure 4A-C. LRP1 and phospholipid levels in SK-N-SH cells incubated with WT and carboxy-terminal truncated apoE4 forms. Changes in cellular membrane fluidity probed by 1-pyrenedodecanoic acid

A, SK-N-SH cells were incubated in the absence (control) or presence of 375 nM lipid-free WT apoE4, apoE4-185 and apoE4-165 for 24 h. Cellular LRP levels were measured by immunoblotting as described under “Materials and Methods” and quantified by ImageJ (lower panel). Values are the means $\pm$ SD of three experiments performed in duplicate. B, SK-N-SH cells were labeled with 1 μ Ci/ml [3 H]choline for 24 h. Cells were then incubated in the absence (control) or presence of 375 nM lipid-free apoE4 forms for 24 h. Media and cells were collected separately, the cellular [3 H]choline phospholipids were first extracted and [3 H]

phosphatidylcholine and [^3H]sphingomyelin were then separated by thin layer chromatography as described under “Materials and Methods”. Phosphatidylcholine and sphingomyelin levels of cells incubated with apoE4 forms are expressed as percent relative to phosphatidylcholine and sphingomyelin levels, respectively, of untreated (control) cells set as 100%. Values are the means $\pm$ SD of four experiments performed in duplicate. *, $p = 0.0002$ vs. control. *C, Left:* Fluorescence emission spectra of the probe 1-pyrenedodecanoic acid in the presence or absence of SK-N-SH cells that have been incubated in the absence (control) or presence of 375 nM lipid-free apoE4 forms for 24 h. The fluorescence spectrum of the probe exhibits two major peaks one at 468nm and one at 395nm corresponding to the excimer and monomer state of the probe. The monomer peak is barely detectable when the probe is in aqueous buffer but is significantly enhanced when the probe is added onto cell suspension. Spectra are averages of 6-9 separate experiments. *Right:* The ratio of excimer to monomer peak was measured in the presence of cells treated with different apoE4 forms. Values are the means $\pm$ SD of 6-9 separate experiments. * The p-value was calculated based on the measurements of control cells.

Figure 5. Intracellular levels of A β 40 and A β 42 in SK-N-SH cells incubated with apoE4-165 SK-N-SH cells were incubated with 25ng/ml A β 40 or A β 42 in the presence of 375 nM apoE4-165 for 24h. The cells were then washed and incubated in A β and apoE4-165 free medium up to 24 h. The amount of A β 40 or A β 42 was measured in cell lysates, at the indicated time points, by sandwich Elisa as described under “Materials and Methods”. The intracellular A β levels at various time points are expressed as percent relative to the initial A β levels set to 100%. Values are the means $\pm$ SD of three experiments performed in duplicate.

Figure 6A-L. Determination of lysosomal localization of A β 40 and A β 42 in SK-N-SH cells incubated with apoE4-165

Fluorescence confocal laser scanning microscopy of SK-N-SH cells incubated with 25 ng/ml A β 40 or A β 42 and 375 nM lipid-free apoE4-165 for 24 h (A-H), or 24h after the removal of A β 42 and apoE4-165 from the cell medium (I-L). Lysosomes or other acidic organelles were detected with LysoTracker Red (B, F, J, red) and A β 40 or A β 42 was detected with the antibody 6E10 followed by an FITC-conjugated secondary antibody (A, E, I, green). The merge of images A-B, E-F, and I-J showing the co-staining of cells for lysosomes and A β 40 or A β 42 is shown in panels C, G and K, respectively. Co-localization of lysosomes/A β 40 or lysosomes/A β 42 is shown in yellow in panels D, H and L. The co-localization was obtained by suppressing all colours, except yellow, in superimposed (merged) images using Adobe PhotoshopTM software.

Figure 7A-L. Determination of mitochondrial localization of A β 40 and A β 42 in SK-N-SH cells incubated with apoE4-165

Fluorescence confocal laser scanning microscopy of SK-N-SH cells incubated with 25 ng/ml A β 40 or A β 42 and 375 nM lipid-free apoE4-165 for 24 h (A-H), or 24h after the removal of A β 42 and apoE4-165 from the cell medium (I-L). Mitochondria were detected with MitoTracker Red CMXRos (B, F, J, red) and A β 40 or A β 42 was detected with the antibody 6E10 followed by an FITC-conjugated secondary antibody (A, E, I, green). The merge of images A-B, E-F, and I-J showing the co-staining of cells for mitochondria and A β 40 or A β 42 is shown in panels C, G and K, respectively. Co-localization of mitochondria/A β 40 or mitochondria/A β 42 is shown in yellow in panels D, H and L. The co-localization was obtained by suppressing all colours, except yellow, in superimposed (merged) images using Adobe Photoshop™ software.

Figure 8A,B. Effect of Aβ40 and Aβ42 following uptake by SK-N-SH cells treated with apoE4-165 on ROS formation

A, SK-N-SH cells were incubated with 25 ng/ml Aβ40 (white bars) or Aβ42 (black bars) in the absence (control) or presence of 375 nM WT apoE4, apoE4-185 or apoE4-165 for 24h. **B**, SK-N-SH cells were incubated with 25 ng/ml Aβ42 (black bars) in the absence (control) or presence of 375 nM WT apoE4-165 for 24h and then were further incubated in the absence of Aβ42 and apoE4-165 for 24h (grey bars). At the end of the incubation period the cells were treated with DCFH-DA for 45 min and the formation of ROS was measured by detection of DCF fluorescence emitted from cells using a fluorescence microscope, as described under “Materials and Methods”. From the obtained images the relative fluorescence intensity per cell was calculated by averaging the values for 45 groups, each containing 5 cells. The DCF fluorescence of cells incubated with Aβ40 or Aβ42 and apoE4 forms is shown relative to DCF fluorescence of control cells set as 100%. In the inset of *Panel A* two characteristic images of cells incubated for 24h with 25 ng/ml Aβ42 in the absence (control) or presence of 375 nM apoE4-165 are shown. Values are the means $\pm$ SD ($n=45$) of one representative of three similar experiments. *, $p < 0.0001$ vs. control.