Apolipoprotein E modulates γ-secretase cleavage of the amyloid precursor protein

Michael C. Irizarry,*1 Amy Deng,*1 Alberto Lleo,* Oksana Berezovska,* Christine A. F. von Arnim,* Matthew Martin-Rehrmann,* Arlene Manelli,† Mary Jo LaDu,† Bradley T. Hyman* and G. William Rebeck‡

*Alzheimer Disease Research Unit, Massachusetts General Hospital–East, Charlestown, Massachusetts, USA
†ENH Research Institute, Evanston, Illinois, USA
‡Department of Neuroscience, Georgetown University, NW, Washington, DC, USA

Abstract
Polymorphisms in the apolipoprotein E (APOE) gene affect the risk of Alzheimer disease and the amount of amyloid β-protein (Aβ) deposited in the brain. The apoE protein reduces Aβ levels in conditioned media from cells in culture, possibly through Aβ clearance mechanisms. To explore this effect, we treated multiple neural and non-neural cell lines for 24 h with apoE at concentrations similar to those found in the cerebrospinal fluid (1–5 μg/mL). The apoE treatment reduced Aβ40 by 60–80% and Aβ42 to a lesser extent (20–30%) in the conditioned media. Surprisingly, apoE treatment resulted in an accumulation of amyloid precursor protein (APP)-C-terminal fragments in cell extracts and a marked reduction of APP intracellular domain-mediated signaling, consistent with diminished γ-secretase processing of APP. All three isoforms of apoE, E2, E3 and E4, had similar effects on Aβ and APP-C-terminal fragments, and the effects were independent of the low-density lipoprotein receptor family. Apolipoprotein E had minimal effects on Notch cleavage and signaling in cell-based assays. These data suggest that apoE reduces γ-secretase cleavage of APP, lowering secreted Aβ levels, with stronger effects on Aβ40. The apoE modulation of Aβ production and APP signaling is a potential mechanism affecting Alzheimer disease risk.

Keywords: amyloid β-protein, amyloid precursor protein, apolipoprotein E, γ-secretase.


The apolipoprotein E (APOE) ε4 allele is a genetic risk factor for sporadic Alzheimer disease (AD) (Rebeck et al. 1993; Saunders et al. 1993; Strittmatter et al. 1993); however, the mechanism of this increased risk is unclear. Apolipoprotein E in the CNS is implicated in repair, synaptogenesis, nerve growth and development (Mahley 1988; Poirier et al. 1993). Human studies indicate that the APOE ε4 allele affects amyloid pathology, resulting in increased amyloid deposition in AD (Rebeck et al. 1993; Saunders et al. 1993) and increased brain levels of the amyloid β-protein (Aβ) species ending at val-40 (Aβ40) (Gearing et al. 1996; Ishii et al. 1997; Mann et al. 1997; McNamara et al. 1998). Apolipoprotein E colocalizes with amyloid plaques (Rebeck et al. 1995) and apoE has been characterized as a ‘pathological molecular chaperone’, mediating β-pleated sheet formation of polypeptide fragments (Wisniewski and Frangione 1992). Studies of amyloid precursor protein (APP) transgenic mice lacking APOE support a role for apoE in affecting Aβ fibril formation.

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Address correspondence and reprint requests to G. William Rebeck, Department of Neuroscience, Georgetown University, Box 571464, 3970 Reservoir Road, NW, Washington, DC 20057, USA.

E-mail: gwr2@georgetown.edu

*These authors contributed equally to this article.

Abbreviations used: Aβ, amyloid β-protein; AD, Alzheimer disease; AICD, APP intracellular domain; apoE, apolipoprotein E; APP, amyloid precursor protein; CHO, Chinese hamster ovary; CTF, C-terminal fragment; E141–149Δ2, tandem double sequence containing amino acids 141–149 of the receptor-binding domain of apoE; HDL, high density lipoprotein; HEK, human embryonic kidney; LDL, low-density lipoprotein; LRP, LDL receptor-related protein; NAEc, construct containing Notch bp 5374–7836 lacking the extracellular domain but containing the membrane-spanning region, including the γ-secretase cleavage site, and the intracellular domain; NICD, Notch intracellular domain; PS-1, presenilin-1; RAP, LRP receptor-associated protein; sAPP, soluble extracellular APP domain.
Knockout of APOE in APP transgenic mice eliminates thioflavin-S staining fibrillar amyloid deposits, although Aβ-immunoreactive deposits still occur (Bales et al. 1997; Bales et al. 1999; Irizarry et al. 2000). In addition to modulating Aβ aggregation, apoE may also influence Aβ clearance; low-density lipoprotein (LDL) receptor-related protein (LRP), an apoE receptor, interacts with apoE/Aβ complexes and can clear Aβ from cell culture media (Beffert et al. 1999; Kang et al. 2000).

Amyloid precursor protein undergoes two major proteolytic processing pathways. A non-amyloidogenic pathway is initiated by γ-secretase cleavage within the Aβ region, precluding Aβ formation and resulting in a secreted N-terminal fragment soluble extracellular APP domain (sAPPα), as well as a membrane-bound C-terminal fragment (APP α-CTF, C83). The amyloidogenic pathway is initiated by cleavage of APP at the N-terminus of Aβ by the β-site APP-cleaving enzyme (β-secretase, EC 3.4.23.46), resulting in a sAPPβ and a β-cleaved CTF (APP β-CTF, C99) (Vassar et al. 1999). The γ-secretase enzyme complex, composed of presenilin-1 (PS-1), aph-1, pen-2 and nicastrin, can cleave both APP α-CTF, producing a non-pathogenic p3 fragment, and APP β-CTF, producing Aβ (Francis et al. 2002). Inhibition of γ-secretase results in accumulation of APP α- and β-CTFs (Wolfe et al. 1999). The γ-secretase cleavage also produces the APP intracellular domain (AICD) implicated in nuclear signaling, which is rapidly degraded (Cao and Sudhof 2001; Kinoshita et al. 2002). Other substrates for γ-secretase include Notch, Jagged-2 and Delta-1 (De Strooper et al. 1999; Ikeuchi and Sisodia 2003). γ-secretase cleaves within the intramembrane domain of Notch, yielding the transcriptionally active Notch intracellular domain (NICD) (De Strooper et al. 1999).

In the course of experiments studying the clearance of Aβ, we surprisingly found that the presence of apoE in cultured cells reduced the γ-secretase cleavage of APP in an LRP-independent fashion. Using simple and sensitive measures of Aβ and APP-CTFs, we demonstrate that physiological concentrations of apoE reduce Aβ and AICD signaling and increase intracellular APP-CTFs in non-neuronal and neural cell lines. These data suggest that modulation of APP processing is an additional mechanism whereby apoE affects AD-related processes.

Experimental procedures

Chemicals

Human recombinant apoE (E2, E3 and E4) was purchased from Panvera (Madison, WI, USA). Human high density lipoprotein (HDL) was purchased from Biodesign (Saco, ME, USA). The γ-secretase inhibitor N-(N-(3,5-difluorophenacetyl)-l-alanyl)-l-phenylglycine t-butyl ester (Dovey et al. 2001) was obtained from M.S. Wolfe (Brigham and Women’s Hospital, Boston, MA, USA). Purified LRP receptor-associated protein (RAP) was obtained from D. Strickland (American Red Cross, Rockville, MD, USA) (Williams et al. 1992). Synthetic peptide E141–149 is a tandem double sequence containing amino acids 141–149 of the receptor-binding domain of apoE obtained from K. Crutcher (University Cincinnati College of Medicine, OH, USA) (Tolar et al. 1997; Qiu et al. 2003).

Antibodies

Several antibodies specific for the C-terminus of APP were utilized: polyclonal C8, anti-APP676–695 (D. Selkoe, Brigham and Women’s Hospital) (Selkoe et al. 1988), polyclonal 369 (P. Greengard, Rockefeller University, New York, NY, USA) (Buxbaum et al. 1990) and monoclonal c1/6.1 antiAPP676–695 (A. Cataldo, McLean Hospital, Belmont, MA, USA) (Mathews et al. 2002). 6E10 anti-Aβ1–17 was used to recognize sAPPα and APP β-CTF (C99). Antibody 22C11 (anti-APP N-terminus; Chemicon, Temecula, CA, USA) was used for analysis of full-length APP, sAPPα and sAPPβ. Antibody MAB5308 was used to recognize β-site APP-cleaving enzyme (Chemicon) and antibody MAB5232 was used to recognize the PS-1 CTF (Chemicon). Rabbit polyclonal antibody against the NICD fragment produced from γ-secretase cleavage of the Notch receptor was obtained from Cell Signaling Technology (Beverly, MA, USA).

Plasmids

Plasmids for the APP-Fe65 transactivation assay were: APP-Gal1 (a kind gift from T. Sudhof, UT-Dallas, TX, USA) (Cao and Sudhof 2001), consisting of APP695 with the Gal4 sequence spliced to the cytoplasmic tail, Fe65-myc (Kinoshita et al. 2001), consisting of APP695 with the Gal4 sequence spanning region, including the active forms of Notch1, NACE (containing the membrane-spanning region, including the γ-secretase cleavage site, and the intracellular domain, 5374–7836 bp) and NICD (active Notch1 intracellular signal domain alone, 5476–7836 bp), were cloned into pBos vectors (G. Weinmaster, UCLA, Los Angeles, CA, USA) (Berezovska et al. 2000; Jack et al. 2001; Lleo et al. 2003). The CBF1-luc construct (JH23) was obtained from D. Hayward (Johns Hopkins School of Medicine, Baltimore, MD, USA) (Hsieh et al. 1996).

Cell lines

Stably transfected Chinese hamster ovary (CHO) cell lines overexpressing the following were utilized for these studies: (i) APP751 (CHO-APP751, line 7W; D. Selkoe) (Wolfe et al. 1999); (ii) APP751 and PS1 (CHO-APP751/PS1, line PS70; D. Selkoe) (Wolfe et al. 1999); (iii) APP751 and PS1 D257A (CHO-APP751/PS1D257A, containing a PS1 mutant lacking γ-secretase activity; D. Selkoe) (Wolfe et al. 1999); (iv) APP751 and lacking LRP (CHO-APP751/LRP –); CHO line 13-5-1 lacking LRP (Fitzgerald et al. 1995) stably transfected with APP751; D. Strickland, American Red Cross (Ulery et al. 2000) and (v) 13-5-1 cells stably transfected with APP751 and LRP (CHO-APP751/LRP +).

Neural cells used were the IMR-32 human neuroblastoma cell line (American Type Culture Collection, Manassas, VA, USA). Human embryonic kidney (HEK)293 cell lines were utilized as an additional human cell line. Mouse primary neuronal cultures were
generated from 16-day Swiss-Webster mice as described in Qiu et al. (1999) and Fukumoto et al. (2002).

Cell culture
Cell lines were cultured in Opti-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum in 12-well plates at a density of 10^5 cells/well and incubated at 37°C with 5% CO_2.

Mouse primary neuronal cultures were obtained from dissociation of 16-day-old Swiss-Webster mouse cortex in calcium-free saline and plating onto poly-L-lysine-coated tissue culture dishes at a density of 1.5 x 10^5 cell/mL; primary neurons were cultured in neurobasal medium (Invitrogen) with 10% fetal bovine serum for 1 h and then maintained in medium containing B27 supplement (Invitrogen) (Qiu et al. 1999; Fukumoto et al. 2002).

Twenty-four hours after plating, the medium was replaced with fresh Opti-MEM containing 3% fetal bovine serum with or without apoE or other treatments. Serum was included in the medium to provide a lipid source for apoE binding. In addition, HDL particles were preincubated with purified apoE3 at 37°C for 1 h and then added to cells to model the effects of apoE-containing CSF lipoprotein particles (Pitas et al. 1987; Rebeck et al. 1998). Conditioned media and cells were collected 1.5–24 h later. Conditioned medium was analyzed for sAPP, Aβ and lactate dehydrogenase activity (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Amyloid β-protein quantitation
Aβ40 and Aβ42 levels in the conditioned media were determined by ELISA using either (i) BNT77 (anti-Aβ1–28) or BAN50 (anti-Aβ1–18) as the capture antibody and horseradish peroxidase-linked BA27 for Aβ40 or BC05 for Aβ42 as detection antibodies (Takeda Chemical Company, Osaka, Japan) (Suzuki et al. 1994; Fukumoto et al. 1999) or (ii) a commercial BioSource (Camarillo, CA, USA) Aβ40 and Aβ42 ELISA kit. Consistent results were obtained using both methods. Purified Aβ40 and Aβ42 (Bachem, King of Prussia, PA, USA) served as standards. The ELISAs are well characterized (Asami-Odaka et al. 1995; Scheuner et al. 1996; Kosaka et al. 1997) and are sensitive to 1 pM Aβ. The specificity of the BNT77/BAN50/BA27/BC05 antibodies was confirmed relative to western blot and reverse phase (RP)-HPLC and mass spectroscopy of IMR-32 and primary rat neuronal culture conditioned media (Asami-Odaka et al. 1995; Fukumoto et al. 1999) and plasma (Scheuner et al. 1996). Serum and plasma proteins do not affect detection of Aβ due to the high affinity of the antibodies (Kosaka et al. 1997; Fukumoto et al. 2003a). The correlation between conditioned media spiked and not spiked with 1–10 μg/mL apoE was >95%, indicating that apoE did not interfere with the ELISAs.

Western blot
Cells were lysed in 250 mM sucrose, 10 mM HEPES (pH 7.4) containing protease inhibitors (Complete; Roche Molecular Biochemicals) or in 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2. Cell lysates (50 μg) were separated on 4–12% Bis-Tris polyacrylamide gels (NuPAGE; Invitrogen) by electrophoresis under denaturing, reducing conditions using MES or MOPS running buffer (Invitrogen) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Blots were blocked in 5% non-fat dry milk in Tris-buffered saline, pH 7.4, with 0.05% Tween 20 and sequentially probed with primary and secondary antibodies (horse radish peroxidase goat antinimouse or antirabbit IgG; Jackson Immuno-Research, West Grove, PA, USA) and visualized by enhanced chemiluminescence (Perkin-Elmer, Boston, MA, USA). Cells lysed in the sucrose/HEPES and ristocetin-induced platelet agglutination buffers both showed a similar pattern of APP-CTF blotting.

Fe65-dependent amyloid precursor protein luciferase transactivation assay
The assay was performed according to the method of Cao and Sudhof (2001) (Lleo et al. 2003). HEK293 cells were cotransfected with the APP-Gal4 construct and Fe65-myc as well as the pGSE1B-luciferase reporter plasmid (to measure transactivation) and a β-galactosidase plasmid (to normalize transfection efficiency). Luciferase activity from cell lysates was determined in triplicate using the luciferase assay kit (Promega, Madison, WI, USA) by LKB 1251 Luminometer. Results were normalized to β-galactosidase expression levels by β-galactosidase enzyme assay kit (Promega).

Notch cleavage assay
In transient transfections, 1 μg of DNA (NAEC or NICD) was introduced into HEK293 cells using FuGENE 6 reagents (Roche Molecular Biochemicals) according to the manufacturer’s instructions. At 5 h post-transfection, the cells were washed once in growth medium and replaced with fresh Opti-MEM containing 3% fetal bovine serum and the different treatments. Cells transfected with NICD served as a positive control and cells transfected with empty pBos were used as a negative control. The NICD generation was assessed by western blot of cells lyzed in PBS containing 1% Triton X-100.

CBF1-luciferase assay
Activation of the Notch signaling pathway was also monitored using the CBF1-luciferase assay as described previously (Berezovska et al. 2000). Cells were cotransfected with NAEC (or NICD), the CBF1 luciferase reporter construct (Hsieh et al. 1996) and β-galactosidase as an internal control for transfection efficiency and analyzed 24 h post-transfection using an LKB 1251 Luminometer.

Results
Apolipoprotein E reduces secreted amyloid β-protein levels
To investigate the effect of apoE on Aβ levels, we treated CHO-APP751 (Fig. 1a), CHO-APP751/PS1 (Fig. 2), IMR32 (Fig. 1b) and primary neuronal cultures (Fig. 1c) with 1.0–5.0 μg/mL of apoE2, apoE3 or apoE4 for 24 h and measured Aβ in conditioned media by ELISA. These doses of apoE were chosen because they represent the range of apoE levels in human CSF (Hesse et al. 2000; Fukumoto et al. 2003b). Apolipoprotein E2, apoE3 and apoE4 resulted in a dose-dependent reduction of Aβ in the conditioned...
media (Figs 1 and 2). In all experiments, Aβ40 levels declined more than Aβ42 levels, with a 50–63% reduction in Aβ40 levels obtained at doses of 5 μg/mL (p < 0.01) and a 17–36% reduction in Aβ42 levels (p < 0.05). The three apoE isoforms appeared to be equally effective in reducing Aβ levels (Figs 1 and 2). While lipid-poor apoE is receptor competent (Narita et al. 2002), we confirmed the effect of apoE in lipid particles similar to those in CSF by preincubating apoE3 with HDL for 1 h at 37°C prior to application to CHO-APP751 cells. Apolipoprotein E preincubated with HDL reproduced the effect of apoE alone (Fig. 1e). In time-course experiments, reduction of Aβ in the conditioned media was detected within 1.5 h of exposure to apoE (Fig. 1d).

No cellular toxicity was observed morphologically or by lactate dehydrogenase determination with these doses of apoE and apoE did not interfere with the ELISA used for detection of Aβ. We interpreted these results to be consistent with either apoE-mediated clearance and degradation of extracellular Aβ, an alteration in Aβ generation or both.

We investigated whether the reduction in Aβ was due to a decline in cellular levels of proteins responsible for Aβ production, APP, PS-1 and β-site APP-cleaving enzyme, or an increase in the alternative nonamyloidogenic α-secretase processing of APP as determined by sAPPα levels in the conditioned media (Fig. 3). After treatment of CHO

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**Fig. 1** Apolipoprotein E (apoE) inhibition of secreted amyloid β-protein (Aβ) levels. Triplicate cultures of (a) CHO-APP751, (b) IMR32 and (c) primary neurons were treated with apoE3 and apoE4 protein for 24 h. Conditioned media were analyzed for Aβ40 and Aβ42 and compared with levels found in sister cultures treated only with vehicle [control (ctl), defined as 100%]. In all cell lines studied, apoE3 and apoE4 treatment resulted in a dose-dependent reduction of Aβ40 and, to a lesser extent, Aβ42 (left panels). Both apoE3 and apoE4 isoforms reduced Aβ equally (right panels, different experiment from left panels). (d) apoE3 reduced Aβ40 and Aβ42 in conditioned media of CHO-APP751 cells within 1.5 h. (e) apoE3 preincubated with high density lipoprotein (HDL) reproduced the Aβ40- and Aβ42-lowering effect of apoE3 alone. *Significant reduction (p < 0.05) compared with vehicle-treated controls.

**Fig. 2** Apolipoprotein E (apoE)2, apoE3 and apoE4 reduce amyloid β-protein (Aβ)40 more than Aβ42. Triplicate cultures of CHO-APP751/PS1 were treated for 24 h with increasing doses of (a) apoE2, (b) apoE3 or (c) apoE4. Each isoform had similar effects in reducing Aβ40 more than Aβ42 in the conditioned media in a dose-dependent manner. *Significant reduction (p < 0.05) compared with vehicle-treated controls.
Apolipoprotein E (apoE) does not affect levels of amyloid precursor protein (APP), presenilin-1 (PS-1) and β-site APP-cleaving enzyme (BACE). CHO-APP751 cells were treated with apoE (5 μg/mL). Conditioned media were analyzed for total soluble extracellular APP by C8 and 6E10 antibodies, and conditioned media were analyzed for total soluble extracellular APP by C8 and 6E10 antibodies, and cell lysates were assessed for full-length APP (C8 and 6E10), PS-1-C-terminal fragment (CTF) and BACE. ApoE treatment had no effect on secreted sAPP in the conditioned media or on cell-associated full-length APP, PS1-CTF or BACE.

APP751 cells with 5 μg/mL apoE3, western blotting of cell lysates did not show any consistent change in intensity of the 98-kDa APP bands (by C8 and 6E10 antibodies), 62-kDa β-site APP-cleaving enzyme protein or the 16–18-kDa PS-1 CTF (Fig. 3). Apolipoprotein E treatment did not alter the amount of secreted sAPPα in the conditioned media by western blot with 6E10 or of total sAPP by 22C11 (Fig. 3). Therefore, we conclude that the levels or activity of α- or β-secretases were not appreciably affected by apoE treatment.

Apolipoprotein E causes accumulation of amyloid precursor protein-C-terminal fragments

To test whether apoE treatment changed APP processing in addition to Aβ levels, we examined cellular APP and APP-CTFs in CHO-APP751, CHO-APP751/PS1 and HEK293 cells after apoE2, E3 or E4 treatment. Amyloid precursor protein cleavage by α- or β-secretase generates APP-CTFs that serve as substrates for γ-secretase to generate the AICD fragment; inhibition of γ-secretase results in accumulation of APP-CTFs (Wolfe et al. 1999) and reduction in AICD generation (Kimberly et al. 2003). We found that exposure of each cell line to apoE2, apoE3 or apoE4 induced a dose-dependent increase in the 7–9-kDa APP α- and β-CTFs by western blot of cell extracts probed with three different APP C-terminal antibodies: 369 (Buxbaum et al. 1990) (Fig. 4a), c1/6.1 (Mathews et al. 2002) (Fig. 4b–e,h) and C8 (Selkoe et al. 1988) (Fig. 4g,i,j); β-CTF accumulation after apoE treatment was confirmed with the antibody 6E10 (Fig. 4f).

No consistent differences were observed between cells treated with apoE3 and those treated with apoE4. The apoE-induced accumulation of APP-CTF also occurred when apoE3 was preincubated with HDL (Fig. 4g). In time-course experiments, accumulation of APP-CTF was faintly detectable by 1.5 h after treatment with apoE, although the effects were more robust by 7–28 h (Fig. 4h). Similar increases in APP-CTFs were obtained with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester (500 nm) (Fig. 4i), which also reduced secreted Aβ40 and Aβ42 levels by >75%. Apolipoprotein E did not further increase APP-CTFs in the CHO-APP751/PS1D257A line where γ-secretase is already non-functional and secreted Aβ was undetectable (Fig. 4j). These results indicate that apoE inhibits APP processing at or immediately preceding the γ-secretase cleavage.

Apolipoprotein E reduces Fe65-dependent amyloid precursor protein luciferase transactivation

The increase in APP-CTFs after apoE treatment suggested that there may be a concomitant decrease in the γ-secretase-mediated production of AICD. We utilized an Fe65-dependent APP luciferase transactivation assay to assess AICD generation in the presence and absence of apoE. The assay is based on transfection with an APP-Gal4 construct that is cleaved by endogenous γ-secretase to generate AICD-Gal4; in the presence of cotransfected Fe65 adaptor protein, AICD-Gal4 activates Gal4-dependent transactivation of a luciferase plasmid reporter (Cao and Sudhof 2001; Lleo et al. 2003). HEK293 cells were cotransfected with: APP fused to the DNA binding domains of the Gal4 transcription factor, APP-Gal4, which is constitutively processed by γ-secretase to release AICD-Gal4; Fe65, a transcriptional coactivator that stimulates APP-mediated transactivation; Gal4 luciferase reporter construct, which is activated by AICD-Gal4 signaling through Fe65 and β-galactosidase to normalize to transfection efficiency (Cao and Sudhof 2001). As expected, APP-Gal4 with Fe65 robustly increased Gal4 luciferase activity compared with APP-Gal4 alone (Fig. 5). A 24-h treatment of transfected cells with 2.5 and 5 μg/mL apoE3 or apoE4 resulted in an over 50% reduction of transactivation. Similar reductions in AICD signaling were found after apoE treatment of CHO cells and H4 human neuroglioma cells (data not shown). These data indicate that apoE impairs Fe65-dependent AICD-mediated signaling at or immediately preceding the γ-secretase cleavage of APP.
Effects of apolipoprotein E on amyloid β-protein and amyloid precursor protein-C-terminal fragments are independent of the low-density lipoprotein receptor family

Amyloid precursor protein is known to interact with LRP, an apoE receptor (Kounnas et al. 1995; Ulery et al. 2000). To determine if the apoE-mediated effect on APP-CTF generation required the LRP–APP interaction, we treated CHO cells expressing APP751 that lacked LRP (CHO-APP751/LRP –) and cells expressing LRP (CHO-APP751/LRP +) with apoE (Figs 6a and b). Apolipoprotein E (2.5 and 5 μg/mL treatments) resulted in a dose-dependent accumulation of APP-CTFs (a–i) over 24 h. (a) Antibodies against the C-terminus of APP could identify accumulation of both β-CTF (C83) and β-CTF (C99). (f) Western blot with the 6E10 antibody confirmed accumulation of β-CTF (C99). (h) Accumulation of APP-CTFs was detectable within 1.5 h of 5.0 μg/mL apoE treatment of CHO-APP751 cells, although this was more robust by 7–24 h. (i) Identical APP-CTFs accumulated when cells were treated with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-α-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (0.5 μM). ApoE could not potentiate accumulation of APP-CTFs after maximal γ-secretase inhibition by DAPT (i) or in CHO-APP751/PS1D257A cells lacking functional γ-secretase (j).

Apolipoprotein E minimally affects Notch cleavage

As apoE altered γ-secretase cleavage of APP, we examined whether apoE also inhibited γ-secretase cleavage of Notch (De Strooper et al. 1999). HEK293 cells were cotransfected with: constitutively active NΔEC, which requires γ-secretase cleavage to release NICD; β-galactosidase, to normalize for

Fig. 4 Apolipoprotein E (apoE)-induced accumulation of amyloid precursor protein (APP)-C-terminal fragments (CTFs). Treatment of CHO-APP751 (a, g and h), CHO-APP751/PS1 (b, c, e–g) or HEK293 (d) cells with apoE2 (c), apoE3 (a, b, d–j), apoE3 + high density lipoprotein (HDL) (g) or apoE4 (b) resulted in a dose-dependent accumulation of APP-CTFs (a–i) over 24 h. (a) Antibodies against the C-terminus of APP could identify accumulation of both β-CTF (C83) and β-CTF (C99). (f) Western blot with the 6E10 antibody confirmed accumulation of β-CTF (C99). (h) Accumulation of APP-CTFs was detectable within 1.5 h of 5.0 μg/mL apoE treatment of CHO-APP751 cells, although this was more robust by 7–24 h. (i) Identical APP-CTFs accumulated when cells were treated with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-α-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (0.5 μM). ApoE could not potentiate accumulation of APP-CTFs after maximal γ-secretase inhibition by DAPT (i) or in CHO-APP751/PS1D257A cells lacking functional γ-secretase (j).

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transfection efficiency and CBF1 luciferase reporter construct (Hsieh et al. 1996; Berezovska et al. 2000). As expected, NICD and NAEC robustly increased CBF1 luciferase activity compared with empty vector (Fig. 7a). A 24-h treatment of NAEC-transfected cells with 5 μg/mL of apoE3 or apoE4 did not affect NICD-induced activation of the downstream transcription factor CBF1 in HEK293 cells (Fig. 7a). Furthermore, apoE isoforms did not affect generation of NICD by western blot of cells transfected with NΔEC in the Notch cleavage assay (Fig. 7b).

**Discussion**

We investigated the effect of apoE on levels of endogenous Aβ in conditioned media, which represents a balance between cellular Aβ secretion and Aβ clearance. Sensitive methods for assaying APP cleavage fragments, specifically Aβ40, Aβ42, APP-CTFs and AICD-mediated signaling, allowed us to examine whether apoE altered APP processing. Treatment of multiple cell lines and primary neurons with physiological concentrations of apoE resulted in a dose-dependent reduction of Aβ, particularly Aβ40, in the conditioned media. These cell lines demonstrated consistent results, even though some expressed only endogenous APP, some overexpressed APP and one also overexpressed PS-1. While we cannot directly distinguish the relative contribution of reduced Aβ production and increased Aβ uptake to the decline in

Fig. 5 Apolipoprotein E (apoE) reduces Fe65-dependent amyloid precursor protein (APP) luciferase transactivation. Transfection of HEK293 cells with APP-Gal4 and Fe65 activates a Gal4-dependent luciferase plasmid (APP-Gal4 + Fe65) while APP-Gal4 alone is not transcriptionally active (APP-Gal4), (a) ApoE3 affects transactivation at 2.5 and 5.0 μg/mL, and (b) ApoE3 and apoE4 similarly decreased transactivation. *Significant reductions (p < 0.05) of Fe65-dependent APP luciferase transactivation by apoE.

![Fig. 5](image)

Fig. 6 Apolipoprotein E (apoE) effects are independent of low-density lipoprotein-related protein (LRP). (a and b) Triplicate cultures of 13-5-1 CHO-APP751 cells lacking LRP [LRP (−)] and expressing LRP [LRP (+)] were treated with apoE or the LRP-binding peptide E(141-149)2, (b) Conditioned media collected after 24 h of apoE treatment demonstrated a dose-dependent reduction of amyloid β-protein (Aβ)40 and Aβ42 and cell lysates demonstrated accumulation of amyloid precursor protein (APP)-C-terminal fragments (CTFs) using antibody c16.1. The E(141-149)2 peptide had no significant effect on APP-CTF accumulation. Triplicate cultures of CHO-APP751 cells were treated with apoE in the presence or absence of (c) 0.25 μM LRP receptor-associated protein (RAP) and (d) 1.0 μM RAP. The dose-dependent (c) reduction of secreted Aβ and (d) accumulation of APP-CTFs using antibody C8 by apoE was unaffected by cotreatment with both doses of RAP. *Significant reductions (p < 0.05) of Aβ by apoE treatment compared with controls.
steady-state Aβ levels in conditioned media after apoE treatment, the changes that we detected in APP processing indicate an effect of apoE on Aβ production. Surprisingly, in apoE-treated cells, we found an accumulation of APP-CTFs and marked reduction of AICD-mediated signaling, with minimal effects on Notch cleavage/signaling. The accumulation of APP-CTFs could not be blocked by RAP, and occurred in LRP-deficient cells, indicating that apoE affects APP processing at or prior to the γ-secretase step and does not require the LDL receptor family. These data suggest that apoE modulates γ-secretase cleavage of APP.

The effects of apoE isoforms on APP metabolism have been investigated in several cell culture systems with inconsistent results on cellular APP and secreted sAPP and Aβ (Biere et al. 1995; Wolozin et al. 1996; Hass et al. 1998; Oron et al. 2000; Cedazo-Minguez et al. 2001; Vincent and Smith 2001). Surprisingly, no published studies have measured Aβ40, Aβ42 or APP-CTFs after the addition of exogenous purified apoE. Prior studies adding purified apoE to cells in culture only evaluated APP or sAPP (Wolozin et al. 1996; Hass et al. 1998; Oron et al. 2000; Cedazo-Minguez et al. 2001; Vincent and Smith 2001) and not Aβ or APP-CTF. The most detailed study assessing the interaction of apoE with APP evaluated CHO cells and a human glioma cell line double transfected with apoE isoforms and APP695 (Biere et al. 1995). There were no consistent changes in APP and APP-CTFs within cells or sAPP, Aβ and p3 in conditioned media of cells stably transfected with APP695 and apoE isoforms; treatment of human neuroblastoma cells expressing APP695 with apoE2-, E3- or E4-containing conditioned media from glioma cells had no effect on APP, sAPP, APP-CTF, Aβ and p3 (Biere et al. 1995). The short time-course of the metabolic labeling study and the necessity for 100-fold concentration of the media may have obscured subtle early changes in APP processing. Nonetheless, Biere et al. (1995) do note the accumulation of a 12-kDa CTF in human glioma cells coexpressing APP695 and apoE. Our study utilized purified apoE and could thus separate out effects that may have been confounded by the stable transfection, changes in cholesterol metabolism within the apoE-transfected cells and changes in lipids within the conditioned media from apoE-overexpressing cells. Interestingly, a study of HEK cells overexpressing APP found a marked reduction in Aβ in the conditioned media when apoE was coexpressed (LaDu et al. 1994). Apolipoprotein E coexpression also caused a dramatic reduction in the p3 peptide, the product of α- and γ-secretase cleavage of APP, consistent with the mechanism proposed in our current study. In the experiments described herein, we utilized several different types of cells, added purified apoE and included 3% serum in culture and HDL to bind the apoE and to preserve cell viability. Assays specific for Aβ40 and Aβ42, APP-CTFs and AICD transactivation demonstrated that the apoE-induced reduction of Aβ in conditioned media was accompanied by cellular accumulation of APP-CTFs and reduced functional AICD generation indicating a reduction of APP γ-secretase cleavage. These findings were consistent and robust; in particular, the increase in APP-CTFs seen in western blots was striking and easily reproducible.

Many of the neuronal effects of apoE are mediated by LRP (Hyman et al. 2000) which is a multifunctional receptor with multiple ligand-binding domains for apoE, lipid-related proteins, proteases, protease inhibitors (including APP with the Kunitz protease inhibitor domain) and other proteins. Ligand binding induces endocytosis and degradation. The LRP interacts with APP, modulates APP processing (Ulery et al. 2000; Kinoshita et al. 2001; Rebeck et al. 2001; Pietrzik et al. 2002) and can clear Aβ (Kang et al. 2000). In our study, however, the accumulation of APP-CTFs induced by apoE treatment was independent of LRP or the LDL receptor family; the apoE effect could not be inhibited by blocking LRP with 250 nM RAP, blocking the family of LDL receptors with 1 μM RAP or eliminating LRP in a CHO-APP751 cell line. The effect was still observed with apoE2, which interacts poorly with the LDL receptor (Weisgraber et al. 1982). Furthermore, treatment with an LRP receptor-
binding fragment of apoE that induces LRP-mediated uptake and degradation did not reproduce the effects of full-length apoE, demonstrating that LRP stimulation alone is not sufficient to modulate \( \gamma \)-secretase.

The lipid-binding domain of apoE may, therefore, be critical for modulation of \( \gamma \)-secretase. Apolipoprotein E-induced subcellular redistribution of APP may restrict access to compartments containing \( \gamma \)-secretase. Amyloid precursor protein processing to form A\( \beta \) and AICD appears to occur in cholesterol-rich microdomains of the membrane (lipid rafts) (Lee et al. 1998). Cellular and membrane redistribution of cholesterol affects the composition of lipid rafts and A\( \beta \) production (Simons et al. 1998; Fassbender et al. 2001; Puglielli et al. 2001; Fukumoto et al. 2002).

Treatment of cells in culture with apoE promotes cholesterol efflux (Michikawa et al. 2000) and the lipid-binding domain of apoE may alter the properties of cholesterol microdomains (Igbavboa et al. 1997) affecting access of \( \gamma \)-secretase to APP. For example, the presence of apoE increased the proportion of A\( \beta \) within detergent-insoluble glycoprotein-enriched microdomains in APP transgenic mice relative to mice lacking apoE (Fagan et al. 2002). Apolipoprotein E affected APP processing in APP transgenic mice; however, in contrast to our cell culture results, APP transgenic mice lacking apoE appeared to accumulate APP-CTFs in the brain (Dodart et al. 2002). These effects in transgenic mice may be mediated by derangements resulting from lack of apoE \textit{in vivo}, such as hypercholesterolemia (Zhang et al. 1992), rather than the direct effects of apoE \textit{per se}. However, there is also evidence for differences in mouse and human apoE. When human apoE3 or apoE4 is genetically reintroduced into APP transgenic mice lacking mouse apoE, A\( \beta \) deposition is further reduced (Holtzman et al. 1999).

Alternatively, the effect of apoE on \( \gamma \)-secretase cleavage of APP could involve other mechanisms. The \( \gamma \)-secretase complex consists of several proteins, including presenilins, nicastrin, aph-1 and pen-2 (Francis et al. 2002; Hu and Fortini 2003), and apoE could affect the levels or distribution of any of these transmembrane proteins. Apolipoprotein E has been shown to bind directly to APP and could potentially alter its accessibility to \( \gamma \)-cleavage (Hass et al. 1998, 2001). Apolipoprotein E could also affect the levels or distribution of APP or of interacting proteins that affect \( \gamma \)-cleavage, such as Fe65 (Chang et al. 2003), although a direct interaction seems unlikely.

The reduction in secreted A\( \beta \) by apoE is mainly in the form of A\( \beta \)40. While we detected no apoE isoform-specific effects, our data suggest that the absolute level of apoE can modulate A\( \beta \) secretion. In human AD, the APOE \( e4 \) allele is associated with increased brain levels and deposition of A\( \beta \)40 (Gearing et al. 1996; Ishii et al. 1997; Mann et al. 1997; McNamara et al. 1998). Lower levels of apoE would thus predispose to increased A\( \beta \)40 production and deposition. In plasma and serum, the APOE genotype affects total apoE protein levels, with the APOE \( e4 \) allele associated with lower apoE levels (Schiele et al. 2000); lower apoE levels have also been reported in AD CSF (Blennow et al. 1994; Skoog et al. 1997; Hesse et al. 2000). Hence, the isoform-specific effect of apoE on the risk of AD may be related to its levels of expression and modulation of APP/\( \gamma \)-secretase interactions, in addition to other isoform-specific effects on \( \alpha \) fibrillation and \( \alpha \) clearance within the brain.

We have shown that apoE results in a dose-dependent reduction of A\( \beta \) secretion in multiple cell lines by an LDL receptor family-independent mechanism. The concurrent accumulation of APP-CTFs and reduction of AICD signaling indicate that the apoE effect involves \( \gamma \)-secretase, although a direct pharmacological induction of \( \gamma \)-secretase enzymatic activity is unlikely given the preservation of Notch signaling. Clarifying the interaction of apoE and \( \gamma \)-secretase-mediated cleavage of APP could help to develop a therapeutic approach to \( \gamma \)-secretase modulation.

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