Journal of Neurochemistry, 2003, **84**, 1442–1451 doi:10.1046/j.1471-4159.2003.01643.x

Apolipoprotein E enhances uptake of soluble but not aggregated amyloid-β protein into synaptic terminals

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Abstract

The cellular mechanism by which apolipoprotein E (apoE) affects the pathogenesis of Alzheimer's disease (AD) is not understood. We have examined the effect of apolipoprotein E on the internalization of exogenous amyloid-β 1–40 (Aβ40) into a rat brain crude synaptosomal preparation. Aβ40 peptide in soluble (within 1 h of dilution in buffer) or aggregated (aged 4 days before dilution in buffer) form was pre-incubated with lipidated apoE then added to synaptosomes; intraterminal amyloid-β labeling was quantified using flow cytometry following immunolabeling with the anti-Aβ (10G4) antibody. The number of Aβ-positive synaptosomes was increased (∼50%) by treatment with a soluble Aβ/apoE mixture compared with treatment with soluble Aβ40 alone. However, when the Aβ was aggregated, less sodium dodecyl sulfate (SDS)-stable Aβ/apoE complex was formed and the addition of apoE decreased the number of Aβ-positive terminals. The addition of the lipoprotein-receptor related protein (LRP) antagonist receptor-associated protein (RAP) inhibited the apoE-induced increase in synaptosomal Aβ, and controls treated with trypsin and heparinase confirm intraterminal localization of the majority of the soluble Aβ. The apoE-mediated increase in Aβ labeling was confirmed in intact cells by immunocytochemistry of dorsal root ganglion (DRG) neurons. These results suggest that complex formation with apoE enhances internalization of soluble Aβ uptake into terminals.

Keywords: Alzheimer's disease, amyloid, apolipoprotein E, flow cytometry, lipoprotein-receptor related protein, synaptosomes.

J. Neurochem. (2003) **84,** 1442–1451.

Much evidence supports the *in vitro* and *in vivo* neurotoxicity of amyloid-β, a peptide whose progressive self-aggregation in solution is well documented, and fibrillar amyloid-β protein (Aβ) results in increased cellular toxicity (Pike *et al*. 1991a, 1993; Lorenzo and Yankner 1994). The longer, more fibrillogenic 1–42 peptide is more abundant in Alzheimer's disease (AD) brain than in controls (Roher *et al*. 1993), and presenilin mutations that result in early onset AD increase production of 1–42 peptide (Scheuner *et al*. 1996). These findings have led to an amyloid cascade hypothesis in which seeding of insoluble Aβ42 represents the initiating event in plaque development (Selkoe 1999). However, amyloid plaques may be distant from sites of synaptic and neuron loss, and loss of synaptic terminals in AD brains demonstrates a higher correlation with loss of cognitive function than other pathological markers such as cell death or plaque formation (Masliah *et al.* 1994; Terry 1999). Likewise, transgenic mouse models have previously shown a weak correlation between amyloid load and memory/cognitive function (Terry 1999; Klein *et al*. 2001). For example, in transgenic mouse models, overexpressing mutant or wild-type hu-APP, synapse loss correlated with soluble Aβ level rather than amyloid-β precursor protein (APP) level or amyloid deposits, i.e. synapse loss occurred even when amyloid was not deposited (Mucke *et al*. 2000).

Recent evidence suggests that soluble Aβ forms, particularly protofibrils and small oligomers, are potent neurotoxins that may play an important role in early pathogenesis and synapse loss (Lambert *et al*. 1998). Observations in

Received August 22, 2002; revised manuscript received December 13, 2002; accepted December 16, 2002.

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Abbreviations used: AD, Alzheimer's disease; Aβ, amyloid-β protein; apoE, apolipoprotein E; Aβ40, amyloid-β 1–40; APP, amyloid-β precursor protein; FACS, fluorescence-activated cell sorting; HSPG, heparan sulfate proteoglycans; KRP, Kreb's–Ringer phosphate buffer; LRP, lipoprotein-receptor related protein; RAP, receptor-associated protein.

post-mortem human studies also support the notion that soluble Aβ forms may make a previously unrecognized contribution to early synaptotoxicity in AD (Klein *et al*. 2001). For example, the more soluble 1–40 Aβ peptide correlates better than the 1–42 species with disease severity (McLean *et al*. 1999), and with apolipoprotein E (apoE) 4 dosage in late onset AD (Ishii *et al*. 1997), and Aβ40 level is a stronger predictor of synaptic changes (Funato *et al*. 1998; Lue *et al*. 1999).

To date, inheritance of the apoE4 allele is the major genetic factor associated with the common late-onset form of AD (Corder *et al*. 1993), and possession of one or two apoE2 alleles seems to serve a protective function (Corder *et al*. 1994). Transgenic studies have shown that, in mice overexpressing human APP with zero, one or two apoE genes, amyloid deposits are markedly reduced in apoE-negative mice (Bales *et al*. 1999). The simplest explanation for a contribution of apoE4 to AD development is a direct interaction between apoE and Aβ protein, and one compelling hypothesis has been that apoE-bound Aβ is a ligand for the multifunctional low-density lipoprotein-related protein (LRP) receptor: uptake of Aβ/apoE complex by LRP receptor may then be a mechanism of Aβ clearance from the neuropil (Rebeck *et al*. 1995). With the objective of clarifying how Aβ internalization is affected by apoE, the studies reported here used a rat brain crude synaptosomal preparation as a model system for the study of Aβ accumulation in nerve terminals. We have examined the effects of exogenously applied apolipoprotein E in combination with soluble and aggregated forms of Aβ40 peptide, and quantified terminal-associated Aβ labeling using flow cytometry analysis.

Materials and methods

Materials

Recombinant human apoE3 was purchased from Panvera (Madison, WI, USA), and β-amyloid (1–40) was purchased from Anaspec, Inc. (San Jose, CA, USA). Phycoerythrin-conjugated anti-mouse IgG was purchased from Molecular Probes (San Diego, CA, USA). Receptor-associated protein (RAP) was kindly provided by G. Bu (University of Washington University School of Medicine, St Louis, MO, USA). Polystyrene microsphere size standards were purchased from Polysciences, Inc. (Warrington, PA, USA). Dimyristoylphosphatidylcholine (DMPC), heparinase, and other reagents were purchased from Sigma (St Louis, MO, USA).

P-2 preparation

The P-2 (crude synaptosome) fraction was prepared as described previously (Weiler *et al*. 1981); briefly, adult male Sprague Dawley rats were decapitated and brains (minus cerebellum) were rapidly removed and placed in 10 volumes of ice cold 0.32 M sucrose in 10 mM Tris buffer (pH 7.4) with protease inhibitors (2 mM EGTA, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/mL leupeptin, 4 μg/mL pepstatin, 5 μg/mL aprotinin, 20 μg/mL trypsin I). The tissue was homogenized in a Teflon/glass homogenizer (clearance 0.1–0.15 mm) by eight gentle up and down strokes at 800 rpm (Setting 1.5; Tri-R Instruments Model K-41, Rockville Center, NY, USA). The homogenate was spun at 1000 *g* for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 10 000 *g* for 20 min to obtain the crude synaptosomal pellet. The final pellet was washed with 2 mLs of Krebs–Ringer phosphate buffer [KRP; 118 mM NaCl, 5 mM KCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 16 mM sodium phosphate buffer (KRP; pH 7.4), and 10 mM glucose] and centrifuged (4 min, 2000 *g*, 4°C) before resuspension in KRP for incubations.

Preparation of amyloid-β/apoE complex

For all experiments, apoE was first relipidated with dimyristoylphosphatidylcholine (DMPC) using a procedure modified from Innerarity *et al*. (1986). Briefly, hydrated DMPC was sonicated for 1 h then filtered (0.2 μm). ApoE was solubilized in 4 mM HEPES, added to sonicated DMPC, and then cycled through the transition temperature three times by warming to 30°C and cooling to 15°C in a thermocycler (Perkin Elmer GeneAmp, Foster City, CA, USA). This lipidated apoE mixture was immediately added to Aβ solutions and incubated at 37°C for 30 min to allow complex formation prior to addition to synaptosomal preparations.

Soluble Aβ

Lyophilized peptides were solubilized in deionized water (230 μM) and stored in aliquots at −80°C until dilution in KRP buffer immediately prior to experiments.

Aged Aβ

Peptide aliquots were diluted 1 : 1 in 2 × phosphate-buffered saline (PBS) and incubated in a capped vial at 37°C for 4 days before use (Pike *et al*. 1993).

Treatment and immunolabeling of P-2 fraction

Aliquots of P-2 homogenate were incubated with Aβ or Aβ/apoE (5.6 μM and 1 μM, respectively) in KRP buffer for the time indicated in a 32°C water bath with intermittent agitation. Treated P-2 aliquots were immunolabeled for flow cytometry analysis according to a method for staining of intracellular antigens (Schmid *et al*. 1991). Pellets were fixed in 0.25% buffered paraformaldehyde (1 h, 4°C) and permeabilized in 0.2% Tween20/PBS (15 min, 37°C). The monoclonal antibody 10G4 (Yang *et al.* 1994) (raised against Aβ1–40 and mapping to residues 5–13), was diluted 1 : 1000 in 2% fetal bovine serum (FBS)/PBS. Following a 30-min incubation with primary antibody at 4°C, pellets were washed two times with 1 mL 0.2% Tween20/PBS, then incubated (20 min, 4°C) with secondary antibody (FITC-conjugated anti-mouse IgG) followed by two additional washes before resuspension in KRP buffer for flow cytometry analysis.

Flow cytometry

Flow cytometry was performed with a FACScan (Becton-Dickinson, Mountain View, CA, USA). Sample flow rate was approximately 3000 events per second; 20 000–50 000 events were collected for analysis. A threshold was set on forward light scatter (channel 42) to exclude debris. Drawing an analysis gate on the largest particles

(greater than 1 μm based on size standards) excludes most contaminants and results in focus on the neuronal (SNAP-25-positive) elements in the homogenate, therefore a data analysis gate was drawn to include only the largest particles for each experiment, which are greater than 95% neuronal (SNAP-25-positive; Gylys *et al*. 2000, 2002). Analysis was performed using FCS Express software (DeNovo software, Ont., Canada). Student's *t*-test for independent observations and two-way ANOVA procedures were performed using the Vassarstat statistical computation web site.

Western analysis

Aliquots of the Aβ/apoE complex mixture (1.08 μg Aβ; 1.57 μg apoE) were boiled for 5 min in non-reducing Laemmli buffer [4% sodium dodecyl sulfate (SDS), no β-mercaptoethanol], and loaded onto a 10–20% SDS/Tricine gel. The separated proteins were transferred to Immobilon-P membranes, and probed with the 10G4 antibody to Aβ in an overnight incubation (1 : 1000 dilution). Proteins on western blots were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Corp., Piscataway, NJ, USA).

Cell culture

Dorsal root ganglion (DRG) were cultured from C57BLK mice pups, p 0–1. The ganglia were quickly dissected out, enzymatically digested in trypsin, triturated to create a dissociated cell suspension and then plated onto coverslips coated with poly ornithine and laminin. The cultures were grown in Neurobasal media supplemented with B25, glutamine, penstrep, and fluoro-deoxyuridine to prevent glial proliferation; nerve growth factor was included for the initial 3 days then withheld upon subsequent feedings. On day 7 *in vitro* (DIV), the cultures were treated with soluble or aggregated Aβ peptides, with and without lipidated apoE, for 1 h as described above for synaptosomes. Cells were immunolabeled for Aβ with the 10G4 antibody as described above with the following exceptions: the fixation step utilized 4% PBS-buffered paraformaldehyde, and 0.1% Triton X-100 was used for permeabilization. Cells were incubated overnight with primary antibody, and for 2 h with FITC-conjugated anti-mouse IgG.

Results

Aβ/apoE complex formation is increased with soluble compared with aggregated Aβ40 peptide

In order to examine how Aβ aggregation state affects interactions with apoE, Aβ/apoE mixtures were incubated (30 min, 37°C) to allow complex formation using conditions based on the characterization of amyloid-β/apoE interactions by LaDu and colleagues (LaDu *et al*. 1994, 1997). Soluble Aβ was diluted from frozen aliquots immediately prior to experiments; aggregated Aβ was pre-aggregated in a capped vial for 4 days before complex formation with apoE. Electron microscopy and thioflavin-T staining have demonstrated that visible protofibrils and β-sheet structure are not detected in freshly solublized Aβ40 peptide (Michikawa *et al*. 2001). The biochemical properties and toxicity of aggregated Aβ40 under our conditions have also been

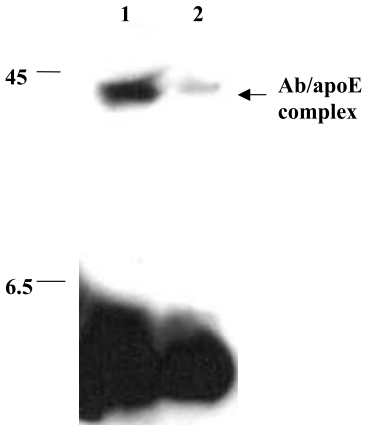

Fig. 1 SDS-stable complex formation between apoE3 and Aβ40. Western blot of binding reaction containing apoE (1 μM) and either soluble (lane 1) or aggregated (lane 2) Aβ40 peptide (5.6 μM). Lipidated apoE was incubated with Aβ for 30 min (37°C) prior to loading onto a non-reducing 10–20% SDS/tricine gel. The blot was probed with the 10G4 antibody against the Aβ peptide.

documented (Pike *et al*. 1991a,b; Burdick *et al*. 1992; Pike *et al*. 1993). When Aβ/apoE mixtures (5.6 and 1 μM, respectively) were analyzed on non-reducing SDS gels, monomeric Aβ was observed at 4 kDa, and a ~43 kDa band consistent with the SDS-stable Aβ/apoE complex observed in previous work was detected with the 10G4 antibody against Aβ (Fig. 1; LaDu *et al*. 1994). In longer exposures, a small amount of Aβ immunoreactivity was observed at the expected position for a Aβ/apoE dimer complex (data not shown). Despite equal loading, an apparent decrease in the amount of monomeric Aβ in the aggregated condition is visible in Fig. 1. Consistent with previous evidence that apoE accelerates Aβ fibril formation, this discrepancy likely results from insoluble high-molecular weight aggregates in the Aβ/apoE mixture that fail to enter the gel and transfer to the filter (Ma *et al*. 1994; Wisniewski *et al*. 1994; Golabek *et al*. 1996). When Aβ peptide was pre-aggregated for 4 days in PBS prior to complex formation with apoE (lane 2), the amount of complex was decreased (~nine-fold) compared with the amount formed with freshly solubilized Aβ40 (lane 1). These results indicate that Aβ pre-aggregation reduces the formation of SDS-stable Aβ/apoE complex.

Treatment of synaptosomes with the soluble Aβ/apoE combination increases the number of Aβ-positive terminals

In order to examine the hypothesis that the Aβ/apoE complex results in differential uptake of Aβ into neurons, we compared the amount of Aβ in neuronal terminals following *in vitro* treatment of synaptosomes with Aβ alone or with Aβ/apoE mixture. In all experiments, Aβ was examined in both soluble and pre-aggregated form; Aβ/apoE mixtures were incubated for 30 min immediately prior to synaptosome

treatments to allow complex formation. After staining with anti-Aβ antibody using a protocol for staining of intracellular antigens, the number of positive particles was quantified using flow cytometry. Figure 2 shows representative density plots of Aβ fluorescence versus forward scatter, which is proportional to size. The rectangular data analysis gate is drawn to include only synaptosomes that are specifically immunolabeled with the anti-Aβ antibody. The distribution of synaptosomes with non-specific labeling (mIgG; Fig. 2a) is similar to that of untreated (time 0) synaptosomes positive for Aβ labeling (Fig. 2b). The number of Aβ-positive particles is increased following incubation with the Aβ/apoE mixture (Fig. 2c) compared with synaptosomes incubated with Aβ alone (Fig. 2d). In contrast to the results with soluble Aβ, when the Aβ is pre-aggregated, addition of apoE to the mixture does not result in increased Aβ labeling compared with the aggregated Aβ alone (Fig. 2f). Because smaller Aβ aggregates would be expected to be in the same size range as synaptosomes, we immunolabeled pre-aggregated Aβ; Fig. 2(g) illustrates that unbound aggregates do not contribute to the fluorescence measured when tissue is treated with aggregated Aβ (Figs 2e and f). Polystyrene microspheres (Fig. 2h) analyzed with identical cytometer settings show that most synaptosomes are from 0.5 to 1 μm in size, consistent with previous flow cytometry (Wolf and Kapatos 1989) and electron microscopy studies (Nagy and Delgado-Escueta 1984; Dunkley *et al*. 1986). Previous characterization of our flow cytometry assay has shown that virtually all of the large particles (∼1–4 μm) in the crude preparation represent intact synaptosomes that stain with a neuronal marker (Gylys *et al*. 2000).

The magnitude and time course of apoE effects on terminal Aβ labeling is illustrated graphically in Fig. 3 for both soluble (Fig. 3a) and aggregated (Fig. 3b) Aβ1–40. The number of Aβ-positive particles was taken from data analysis gate R2 described above; to avoid potential inclusion of free mitochondria or membrane fragments, particles less than 0.75 μm were eliminated by gating analysis. In all conditions, the percentage of Aβ-positive synaptosomes is increased to near maximum levels after a 10-min incubation, revealing rapid kinetics in this preparation for increased intraterminal labeling following addition of exogenous Aβ. Figure 3 also reveals that the percentage of Aβ-positive particles is higher at all time points for aggregated compared with soluble Aβ. The soluble Aβ/apoE mixture significantly increased the number of Aβ-positive synaptosomes compared with soluble Aβ alone ($F = 11.66$; d.f. 1,14; $p < 0.005$; two-way ANOVA): after 10 min, the number of amyloid-positive particles increased from 19 (± 2.9) to 29% (± 3.0). The maximum increase, from 20 (± 0.3) to 38% (± 4.6) was observed after 3 h. Figure 3(b) shows that when Aβ was in aggregated form, the Aβ/apoE mixture decreased the number of Aβ-positive terminals compared with aggregated Aβ alone ($F = 14.84$; d.f. 1,10; $p < 0.005$; two-way ANOVA).

The apoE-mediated increase in terminal labeling of soluble Aβ is blocked by RAP

To test the hypothesis that the low-density LRP mediates binding and uptake of the Aβ/apoE complex, we tested the effect of RAP, an LRP antagonist, on synaptosomal Aβ labeling. Figure 4a illustrates that the addition of RAP (1 μM) decreased the number of Aβ-positive particles from 21.02 (± 5.5) to 8.43 (± 1.62) for the soluble Aβ form when apoE was present, indicating receptor-mediated uptake of the Aβ/apoE complex ($p < 0.05$, student's *t*-test). RAP did not affect the number of amyloid-positive terminals for soluble Aβ alone, and did not result in significant decreases in labeling of aggregated Aβ (Fig. 4b). However, the trend toward a decrease observed when RAP was added to aggregated Aβ alone (Fig. 4b), though not statistically significant ($p = 0.20$, student's *t*-test), suggests some blockade of non-specific uptake by RAP. RAP-mediated blockade of aggregated Aβ may also indicate the presence of a minor pool of monomer in the aggregated peptide that is able to bind apoE and be taken up by the LRP mechanism. It should be noted that RAP is an antagonist for all ligands of the LRP receptor which include alpha-2 macroglobulin and plasminogen activators in addition to other lipoproteins (Herz *et al*. 1991; Rebeck *et al*. 1995).

Previous reports have suggested that amyloid peptides may interact with heparan sulfate proteoglycans (HSPG) on the cell surface (Ji *et al*. 1997, 1998). Other studies have shown that Aβ1–40 peptide associated with PC12 cells can be removed by trypsin incubation (Burdick *et al*. 1997). Because both cell surface and intracellular Aβ antigen are detected with the staining procedure used in the present experiments, we examined the effect of trypsin and heparinase on synaptosomal Aβ labeling. Aβ-treated synaptosomes were incubated with trypsin (1 : 1000) or heparinase (10 U/mL) prior to fixation, permeabilization, and staining. Following treatment with trypsin, 90% of the Aβ remains in synaptosomes treated with soluble peptide, and 60% of the amyloid remains in aggregated Aβ-treated samples. These results suggest that a portion of aggregated Aβ is bound but not internalized, but that the majority of soluble Aβ measured by flow cytometry is intraterminal. Figure 4(c) shows that heparinase does not significantly reduce the percentage of Aβ-positive synaptosomes following treatment with either soluble or aggregated peptide, indicating that HSPG may not play an essential role in the association of either form of Aβ with terminals.

ApoE enhances immunolabeling of soluble Aβ in cultured DRG neurons

To confirm that our preparation of relipidated apoE can increase receptor-mediated Aβ uptake into neurons and their terminals, dorsal root ganglion cultures were treated with soluble and aggregated Aβ alone and in combination with relipidated apoE; Aβ was visualized using an intracellular staining protocol (Fig. 5). Aβ-treated cultures were compared

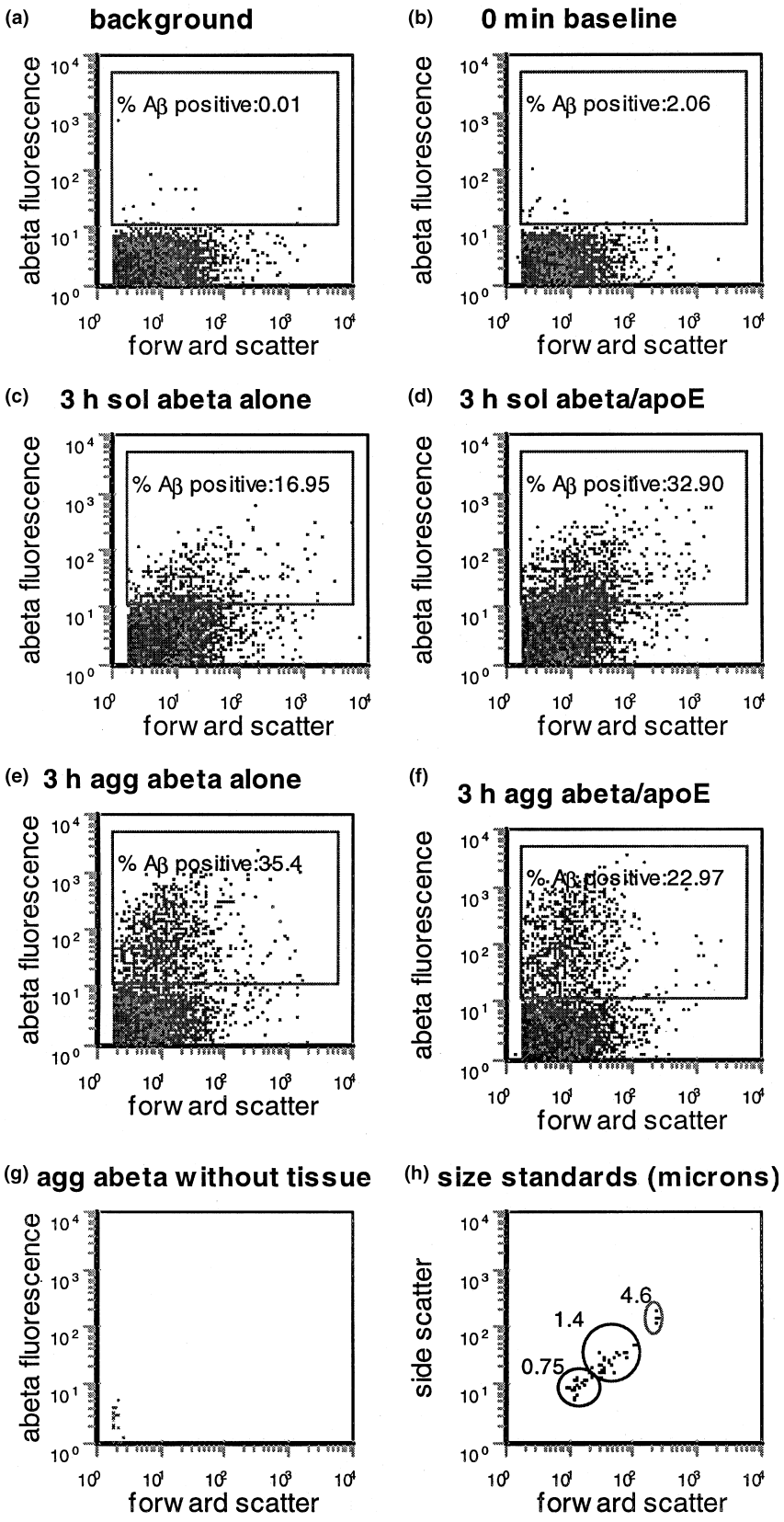

Fig. 2 Flow cytometry assay for Aβ40 levels in nerve terminals. Representative density plots of Aβ fluorescence versus forward scatter (which is proportional to size). Crude synaptosomes were incubated as described in Materials and methods with the Aβ/apoE mixture or with Aβ alone at 32°C prior to immunolabeling with an antibody against Aβ. The rectangular analysis gate is drawn to exclude non-specific labeling determined by staining with isotype-specific (mouse IgG) control antibody; 10 000 events are plotted. Synaptosomes that are non-specifically labeled are located below the gate. The distribution of background-stained synaptosomes (a) is similar to that of untreated (time 0) synaptosomes stained with the 10G4 antibody (b). The percentage of Aβ positive particles is shown for synaptosomes treated with: soluble Aβ alone (c), with an Aβ/apoE mixture for 3 h (d), and with pre-aggregated Aβ alone (e) and with an aggregated Aβ/apoE mixture for 3 h (d). The particle distribution with identical instrument settings is shown for for immunolabeled Aβ aggregates without tissue (g), and for polystyrene microspheres (h).

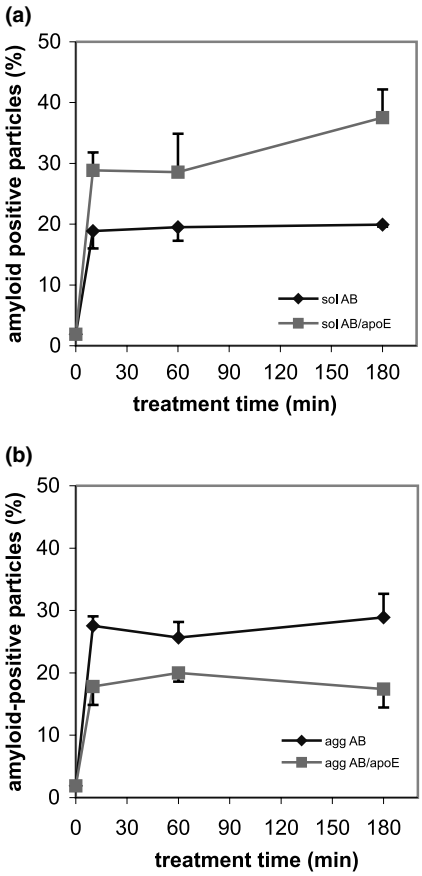

Fig. 3 ApoE3 enhances synaptosomal levels of soluble Aβ. Time course for apoE effects on terminal Aβ levels for soluble (a) and aggregated (b) Aβ40 peptide. Crude synaptosomes were treated as described with the Aβ/apoE mixture or with Aβ alone; the number of amyloid-positive particles is taken from data analysis gate R2 shown in Fig. 1. Data are from at least four independent experiments; apoE effect is significant at the 0.005 level by two-way ANOVA.

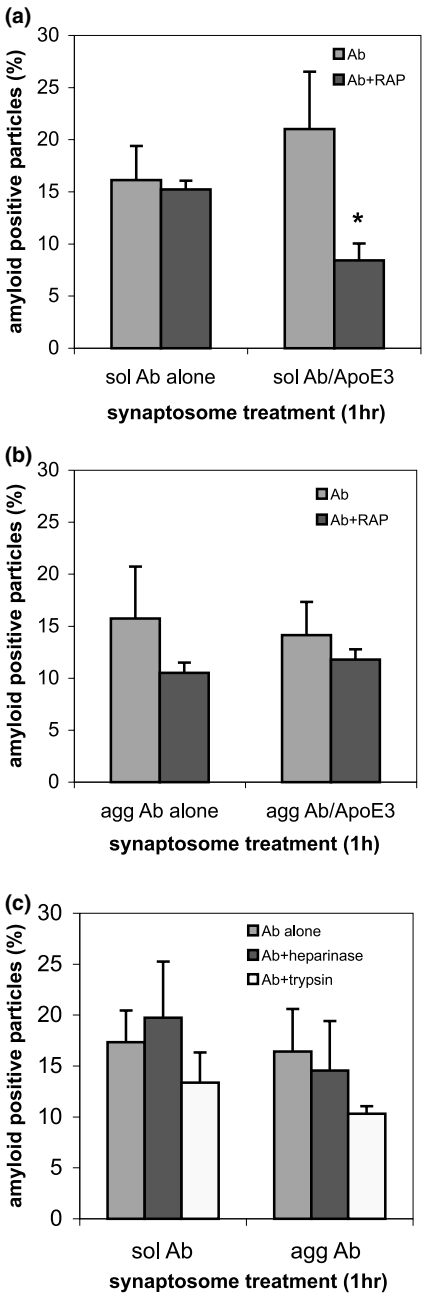

Fig. 4 RAP blocks the apoE-mediated increase in terminal levels of soluble Aβ. Effect of the addition of RAP (1 μM) to Aβ/apoE mixture prior to incubation with synaptosomes for soluble (a) and aggregated (b) Aβ40 peptide. (c) Heparinase and trypsin effects on terminal Aβ levels following treatment with soluble and aggregated Aβ alone. Synaptosomes were first incubated with Aβ for 1 h, then with heparinase (10 U/mL) or trypsin (1 : 1000) prior to labeling and flow cytometry analysis of Aβ-positive particles as described; the number of amyloid-positive particles is taken from data analysis gate R2 shown in Fig. 1. Data are from at least four independent experiments; $^*p \leq 0.05$ compared with Aβ/apoE treatment without RAP.

with cultures treated with Aβ/apoE complex. Cells treated with soluble Aβ alone demonstrate minimal staining (Fig. 5b), but those treated with the soluble Aβ/apoE mixture display intense punctate labeling along the neurites consistent with terminal staining and in the perinuclear region within the soma (Fig. 5c) that is attenuated by the addition of RAP (Fig. 5d). Aggregates varying in size from <1 to approximately 10 μm are visible on the glial monolayer in cells treated with aggregated Aβ alone (Fig. 5e). When cells were treated with aggregated Aβ/apoE mixture, there appear to be more neuron-associated aggregates compared with cells treated with aggregated Aβ alone (Fig. 5f), and the aggregates appear to be larger. When apoE is present, faint aggregates 1 μm or less (arrowhead, Fig. 5f), may represent a low level of internalization of small Aβ aggregates; the larger aggregates appear extracellular. These results in intact cells parallel our data in terminals showing that Aβ/apoE

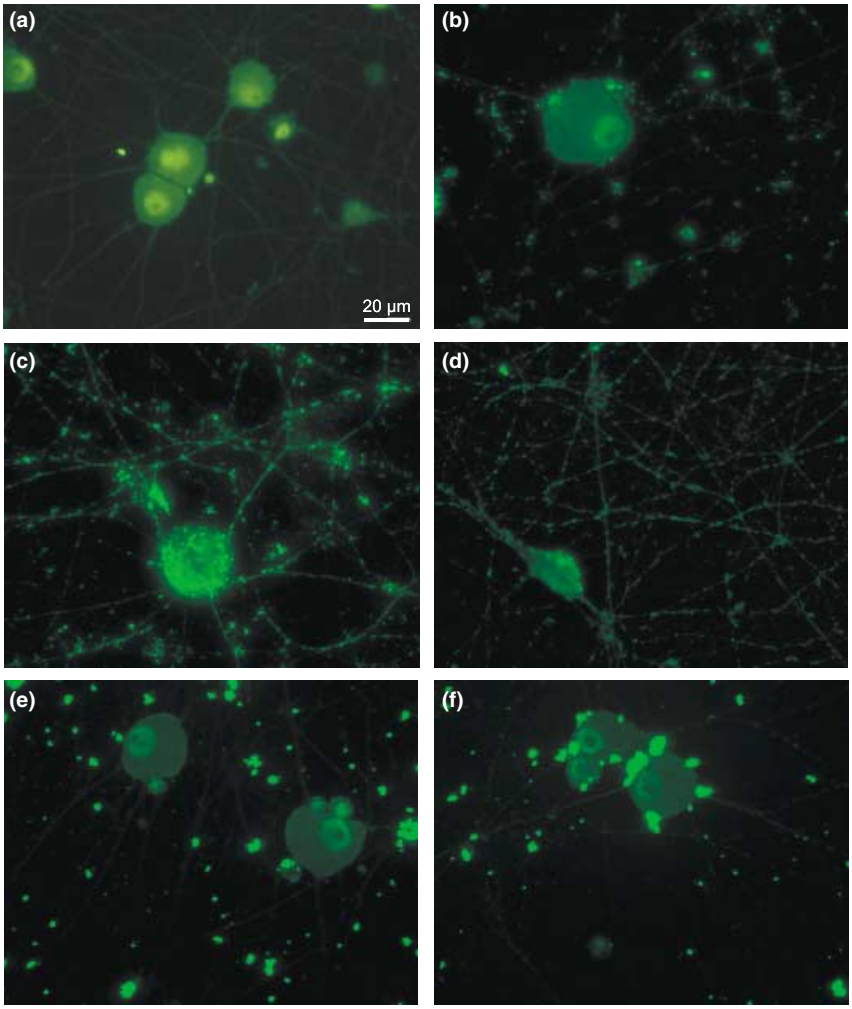

Fig. 5 ApoE3 enhances immunolabeling of soluble Aβ in cultured DRG neurons. Primary DRG cultures were treated with Aβ and Aβ/apoE mixtures (5.6 and 1 μM, respectively) for 1 h before immunolabeling with the 10G4 antibody using a protocol for intracellular antigens. Untreated DRG culture is shown in (a), (b–f) DRG treated with: soluble Aβ40 alone (b), Aβ/apoE3 mixture (c), Aβ/apoE3 mixture plus RAP (1 μM) (d), aggregated Aβ alone (e), and aggregated Aβ/apoE3 mixture (f).

complex increases soluble amyloid-β levels, but it should be noted that the large amyloid aggregates visualized in DRG neurons (Figs 5e and f) are excluded based on their size from the synaptosome studies by the flow cytometry analysis (see Fig. 2).

Discussion

Increasing evidence suggests that soluble and oligomeric Aβ forms may contribute significantly to early amyloid toxicity in AD, and that synapses are a focal site for early damage. The present experiments examine the initial binding/internalization response of nerve terminals to exogenously applied Aβ and compare accumulation of Aβ in terminals following soluble versus aggregated Aβ peptide treatment and treatment with a mixture of Aβ/apoE. The effect of apoE on Aβ labeling is quantified in a virtually pure synaptic terminal population from adult mammalian brain. Few studies have examined cellular effects of Aβ/apoE binding interactions, and the present results demonstrate that apoE increases terminal Aβ level in a RAP-dependent manner.

Both 1–40 and 1–42 species of the amyloid-β protein are found in AD plaques, but only the Aβ40 peptide correlates with apoE4 dosage and disease severity. Based on these and other results, McLean *et al*. (1999) have suggested that a large relatively static insoluble pool of Aβ may be derived from a smaller soluble pool that is constantly turning over. These authors suggest lowering the level of the soluble pool, which may be both intracellular and extracellular, as a therapeutic target. Moreover, high levels of Aβ in APP transgenic mice resulted in loss of synaptophysin-positive terminals in the absence of plaques (Mucke *et al*. 2000), suggesting that plaque-independent Aβ toxicity contributes to early synaptic deficits. Considered with results showing that behavioral impairments, synaptic loss and transmission deficits precede plaque formation in some transgenic AD models (Chapman *et al*. 1999), these results emphasize the need to understand mechanisms underlying initial cellular responses to both soluble and aggregated forms of Aβ peptides.

In vitro studies have shown that apoE binds avidly to Aβ peptides, forming SDS-stable complexes (Strittmatter *et al*. 1993a,b; Wisniewski *et al*. 1993; LaDu *et al*. 1994, 1997). Only a small proportion of the total Aβ peptide in the binding reaction complexes with apoE (Ladu *et al*. 1994), but the stoichiometry of the interaction is not understood, in part because denaturing sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) analysis may eliminate physiologically relevant classes of Aβ/apoE complex. For example, one study of native complex formation identified Aβ bound to apoE tetramer as one of several species of high molecular weight complexes (Chan *et al*. 1996). However, the potential importance of SDS-stable complex formation is highlighted by the observation that immunoprecipitaed Aβ from plaque-free control brains is bound to apoE in an SDS-stable complex (Russo *et al*. 1998). The level of Aβ/apoE complex was higher in control brains than AD brains, leading these authors to speculate that apoE sequesters Aβ and prevents aggregation. The observation that apoE increases the binding of Aβ40-lipid particles to primary cultures raises the additional possibility that that cholesterol and phosphatidylcholine may be a part of the complex that is ultimately internalized (Michikawa *et al*. 2001). The impaired complex formation by aggregated Aβ that we observe seems likely to result from the loss of apoE-binding sites to self-aggregation. This explanation is supported by previous work showing that adjacent sections of the amyloid peptide are involved in apoE binding and self-aggregation: hydrophobic residues at the C-terminus (25–35) underlie self-aggregation and neurotoxicity (Pike *et al*. 1993), and residues 13–28 are important for the binding of apoE and Aβ (Chan *et al*. 1996).

LRP is a multifunctional receptor that binds a number of complex and structurally unrelated ligands (Gliemann 1998) Several of these ligands (including α2 macroglobulin and lactoferrin) have been localized to amyloid plaques in AD, and the apoE4 isotype has been shown to form less SDS-stable complex than apoE3 (LaDu *et al*. 1994). These and other observations have prompted the hypothesis that normal clearance of apoE-bound Aβ via LRP internalization is impaired in AD (LaDu *et al*. 1994; Rebeck *et al*. 1995; LaDu *et al*. 1997). In the model outlined by Rebeck *et al*. (1995), LRP ligands that fail to be internalized and degraded by the usual clearance pathways become associated with the senile plaque. These authors speculated that, for apoE, the receptor-binding domain is available for binding to LRP, resulting in binding to the cell surface without internalization. Our results showing enhanced uptake of soluble but not aggregated Aβ are predicted by this model, as is the observation that apoE promotes cellular association of Aβ aggregates with DRG cells (Figs 5e and f). The present results indicate that lower levels of ligand-bound complex rather than impaired receptor function may be the cause of impaired internalization. Extracellular accumulation of aggregated Aβ resulting from either receptor dysfunction or reduced complex formation might serve as the seed for extracellular plaques.

Discrepencies exist in the literature regarding the cellular location of exogenously applied Aβ peptide, and previous work has shown clear cell-specific and peptide-specific differences. For example, oligomeric 1–42 peptide, a potent neurotoxin, is removed from cultured cells by trypsin treatment (Lambert *et al*. 1998); these authors suggest that toxicity does not follow internalization, but follows surface binding followed by activation of Fyn, a tyrosine kinase in the Src family. This is in contrast to PC12 cells, where both 1–40 and 1–42 Aβ peptides were internalized; the 1–40 peptide was eliminated by the cells with a half-life of 1 h, while most of the 1–42 peptide accumulated intracellularly (Knauer *et al*. 1992; Burdick *et al*. 1997). Internalization of Aβ40 peptide in the presence of apoE has not been examined, although studies in Chinese hamster ovary cells and in primary hippocampal neurons support the hypothesis that apoE increases cellular association of Aβ40 peptide (Beffert *et al*. 1998; Yang *et al*. 1999). The significant proportion of both aggregated and soluble Aβ labeling that remains in the presence of RAP in synaptosomes, considered together with the avid surface absorption of Aβ peptides (Burdick *et al*. 1997), and the non-specific internalization of amyloid-β aggregates by microglia (Paresce *et al*. 1996), indicates that both receptor-mediated and non-specific internalization occur in nerve terminals. Therefore the trypsin-insensitive Aβ labeling we observe in synaptosomes may represent surface-bound aggregates that are in the process of non-specific internalization by constitutive membrance endocytosis. Without apoE, there are more Aβ-positive terminals for aggregated compared with soluble Aβ; apoE raises the percentage of Aβ-positive terminals obtained with soluble Aβ to approximately the level obtained with aggregated Aβ alone (∼30% positive). Saturation of a non-specific mechanism for aggregated Aβ might provide an explanation for the absence of an apoE-induced enhancement of aggregated Aβ uptake in the present experiments. The size difference between synaptosomes and DRG neurons would be expected to yield differences with respect to internalization of aggregates: the larger aggregates are 5–10 μm, larger than most synaptosomes, which range from several tenths to 4 μm (Gylys *et al*. 2000) and particles larger than 5 μm are excluded from the flow cytometry analysis. A higher endocytotic rate in the *in vitro* preparation might also be expected, given previous work showing increased oxidative activity in synaptosomes compared with cells (McKenna *et al*. 1993).

In summary, our results demonstrate that addition of apoE increases terminal Aβ labeling for soluble but not aggregated Aβ1–40 peptide. The blockade of this increase by RAP indicates receptor-mediated internalization of a soluble Aβ/apoE complex, and the model system and flow cytometry method described offer a quantitative and rapid assay for future studies to examine the neuronal effects of additional amyloid peptide species and the three human apoE isoforms.

The present results highlight the importance of the aggregation state of amyloid-β, and suggest that peptide that becomes self-aggregated before internalization remains in the extracellular space, and may be a source of initial amyloid deposition.

Acknowledgements

This work was supported by NIA AG13741 and G16570 to GMC and by NIH CA-16042 and AI-28697 to the Jonsson Cancer Center at UCLA.

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