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ATTENUATION OF THE NEUROTOXIC EFFECT OF Aβ AMYLOID PEPTIDE BY APOLIPOPROTEIN E

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Alzheimer's disease patients have increased frequency of apolipoprotein E allele ε4, suggesting apoE4 is a risk factor determining disease. ApoE binds Aβ amyloid peptide with great avidity in vitro and in the neuritic plaque. Potentially, binding of Aβ to apolipoprotein E could increase Aβ neurotoxicity. However, in hippocampal cultures, 0.1 μM apolipoprotein E eliminated the neurotoxicity of 10 μM Aβ. Neuronal rescue was dose-dependent and occurred even after 48 hours exposure to Aβ, but was overwhelmed by excess Aβ. Thus, interaction between these proteins does not directly increase Aβ neurotoxicity, and the role of ApoE in Alzheimer's disease remains to be elucidated.

Alzheimer's disease (AD) is characterized pathologically by diffuse neuronal loss accompanied by abundant intracellular neurofibrillary tangles, extracellular senile plaques, and cerebrovascular amyloidosis. Recent attention has focused on the senile plaques (SP) which occur abundantly in the AD cortex and hippocampus and are typically composed of a compact amyloid core surrounded by dystrophic neurites. Aβ protein, a 40-43 amino acid residue protein derived from a larger precursor, the amyloid precursor protein (APP) (1), is the primary protein component of both SP and cerebrovascular amyloid (2,3,4). Aβ protein aggregates are also found in AD as non-amyloid protein deposits in various brain regions including hippocampus, cortex and cerebellum (5,6,7,8). Aβ is toxic to hippocampal neurons in vitro (9,10) after a transient trophic period (11,12), possibly via the activation of apoptotic mechanisms (13).

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Apolipoprotein E (apoE) is a 34 kilodalton protein which participates in serum cholesterol transport (14,15) and is also found in cerebrospinal fluid, where it constitutes a distinct, brain-derived pool (16,17). ApoE levels in brain are increased following entorhinal cortex lesion (18,19). ApoE is associated with peripheral nervous system regeneration (20,21,22,23,24) and participates in the regulation of neuritic outgrowth from dorsal root ganglion cells *in vitro* (25). Recently, ApoE has been immunohistochemically identified as a component of AD amyloid (26,27,28, but see also 29); increased levels of apoE mRNA have been demonstrated in AD brain (30); and one of the apoE alleles, c4 (which codes the E4 isoform of apoE), has been associated with both late-onset familial and sporadic AD (28,31,32).

Interestingly, apoE binds with high avidity to Aβ protein (28,32). This binding occurs with similar affinity but different kinetics for the common E3 and the AD-associated E4 isoforms (32). Furthermore, both congophilic staining of amyloid and immunohistochemically identified Aβ are increased in the plaques and cerebral blood vessels of individuals homozygous for the c4 allele (33). Thus, the high affinity of apoE for Aβ may play a role in the amyloidogenesis of AD. However, whether apoE/Aβ binding is relevant to the neurotoxicity of Aβ or to Alzheimer's disease is unknown.

METHODS

Preparation of Hippocampal Cultures

Embryonic day 18/19 Sprague-Dawley rats were decapitated and their hippocampi dissected in Hanks Balanced Salt Solution with 4.2mM bicarbonate, 1mM pyruvate, 20mM HEPES and 3mg/ml bovine serum albumin (BSA). Hippocampi were rinsed once in 3ml B18 medium (34) minus glutathione (defined medium--DM) and were dissociated in 1ml of the same medium. 2ml of Dulbecco's modified Eagle's medium with 26.2mM bicarbonate, 1mM pyruvate, 20mM HEPES, 100μg/ml penicillin, 100 units/ml streptomycin, and 10% heat-inactivated fetal calf serum was added to the cell suspension, which was then allowed to stand for 3 minutes to allow any cell clumps to settle. Approximately 2.5ml of the supernatant was removed and centrifuged at 200xg for 3 minutes. Cell pellets were resuspended in 2ml DM and the number of viable cells determined by Trypan blue exclusion via hemocytometer counts. Final cell density in 96-well poly-L-lysine coated plates was 8,000 cells/cm^2.

Cultures prepared as described above were $\geq$94% NFP positive and contained $<$2% GFAP positive cells at d3 *in vitro*.

Preincubation of B40 and ApoE

β-Amyloid (1-40) (Bachem; Lots WJ209 and ZI960) was solubilized at 2 mg/ml in vehicle (sterile 0.01% trifluoroacetic acid) and stored at 4°C for for up to three weeks.

Purified, delipidated rabbit apoE (35) was solubilized in Dulbecco's phosphate buffered saline (DPBS). Following protein determination (microtiter BCA assay; Pierce) bovine serum albumin (BSA) was added as a carrier protein (9 mg BSA:1 mg apoE). Each lot of apoE was purified from the plasma of a different rabbit. ApoE vehicle was prepared individually for each lot of apoE so that both the amount of BSA and the volume of DPBS added to vehicle-treated cultures was identical to that in apoE-treated cultures.

For preincubation experiments, 20 μM B40 and 0.2 μM apoE were incubated for three days at 4°C in Dulbecco's phosphate buffered saline (DPBS), then diluted 1:1 with 2X DM. Hippocampal cells were plated at a final density of 8,000 cells/cm^2.

For comparison of results with and without preincubation, 60 μM B40 and 4 μM apoE were either preincubated together for 3 days at 4°C as above or were added along with DPBS to 2x DM immediately prior to cell plating. Final concentrations were 30 μM B40, 2 μM apoE.

Culture Fixation and Counting

Cultures were fixed by the gentle 1:1 addition of either 4% glutaraldehyde or 8% paraformaldehyde. The number of viable cells at the time of fixation was determined by cell counting using morphological criteria previously described (12). Survival was calculated as a percentage of vehicle-control survival at the same timepoint. Values given are mean +/- standard error of measurement (SEM). Significance was determined by Analysis of Variance (ANOVA) with post hoc Fisher Positive Least Squares Difference (Fisher PLSD).

RESULTS

In the present study, we determined whether apoE alters the neurotoxicity of Aβ. Primary cultures from E-18/19 rat hippocampus were treated at the time of seeding (day 0) with 10 μM B40, a synthetic Aβ homolog which had been preincubated for 3 days under sterile conditions at 4° centigrade with 0.1 μM rabbit apoE, a protein which exhibits 80% sequence identity to human apoE3 (35). Sister cultures were treated with B40 and vehicle (B40/veh), vehicle and apoE (veh/apoE), or vehicle alone (veh/veh) preincubated under identical conditions. The apoE concentration used (1 μM; 4 μg/ml) was within the range of apoE concentrations found in normal rat CSF (3.7-4.3 μg/ml) (16). Three days after treatment with the preincubated proteins, neuronal survival was quantitated (Figure 1). In cultures treated with B40/veh, roughly 60% of the neurons survived (57$\pm$2%) compared to veh/veh control cultures. However, in cultures treated with B40/apoE, the neurotoxicity of B40 was virtually eliminated, with more than 90% of the neurons surviving (92$\pm$4%). Treatment with veh/apoE did not alter neuronal survival (101$\pm$4%).

In some of these experiments, cultures treated with preincubated B40 and apoE were compared to cultures treated with B40 and apoE that had not been preincubated. Results were

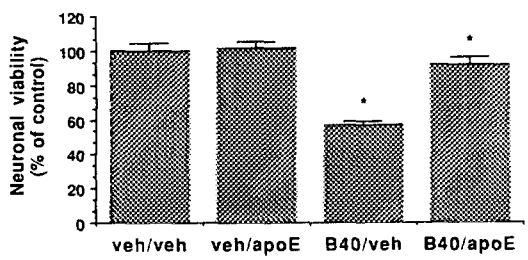

Figure 1. The neurotoxicity of Aβ is attenuated by apoE. 10 μM B40 and 0.1 μM apoE were incubated for three days at 4°C. Hippocampal cells were plated in medium containing preincubated proteins or vehicle. After three days, surviving neurons were counted and viability calculated as a percentage of control viability [* = $p \leq 0.05$, Fisher PLSD]. Values are mean $\pm$ SEM; n = 12. ANOVA: $F(3,44) = 30.066$, $p = 0.0001$.

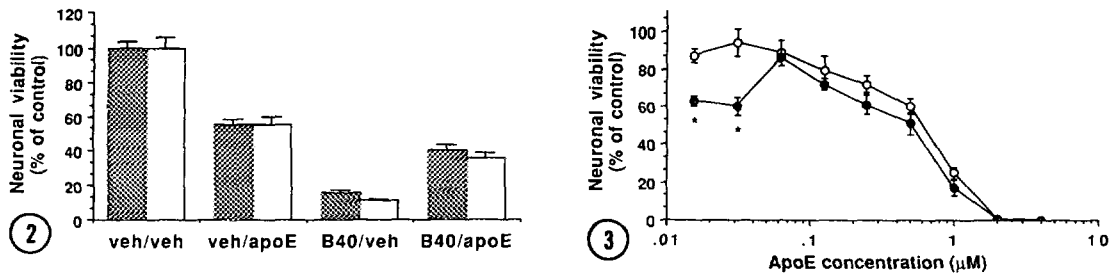

Figure 2. ApoE attenuates Aβ toxicity after three days in culture independent of whether the proteins have been preincubated prior to cell plating. Neurons were plated in medium containing 30 μM B40 and 2 μM apoE which had been either freshly combined or preincubated. At this concentration of apoE, neurons treated with apoE alone exhibited decreased viability after 3 days. Later experiments showed that supra-physiological concentrations of apoE decreased cell adhesion in vitro. Thus B40/apoE viability represents the combined effects of decreased cell adhesion, B40 neurotoxicity, and neuronal rescue by apoE. Values are mean ± SEM; n = 6. Cross-hatched bars, preincubated; white bars, not preincubated.

Figure 3. Neuronal viability in apoE with or without Aβ. Neuronal viability was quantitated after three days *in vitro* and expressed as a percentage of 0 μM apoE/0 μM B40 viability. The B40 toxicity apparent at low apoE concentrations was inhibited by physiological concentrations of apoE. However, decreased neuronal viability observed at higher apoE concentrations, irrespective of the presence or absence of B40, represents decreased cell adhesion. *Viability significantly less than in the absence of B40, $p \leq 0.05$, Fisher PLSD. Values are mean ± SEM; n = 9-12. ANOVA: $F(19,225) = 56.713$, $p = 0.0001$. Open circles, without B40; closed circles, with 10 μM B40.

similar (Figure 2) and all further experiments were performed without preincubation. Two different lots of apoE were used in these experiments. Both gave similar results (data combined in Figures 1, 2 and 3).

To determine if the inhibition of B40 neurotoxicity by apoE was dose-dependent, we added from zero to 4 μM apoE to cultures with and without 10 μM B40 (Figure 3; 0 μM apoE not shown). Very low concentrations of apoE did not alter neuronal viability in the presence of B40 (63±8%, 63±3% and 60±5% of control survival for 0, 0.016 and 0.031 μM apoE respectively). However, in the presence of 0.062 μM and 0.125 μM apoE, 10-20% more neurons remained alive than in B40 alone (86±4% and 72±3% respectively). Unexpectedly, at higher apoE concentrations neuronal viability was not increased (61±5% to 0±0% from 0.25 μM to 4 μM, respectively). Indeed, at apoE concentrations of 0.5 μM or greater, neuronal viability was decreased relative to viability in the presence of 10 μM B40 alone (51±6%, 17±4%, 0.4±0.2%, 0±0% for 0.5, 1, 2, and 4 μM apoE respectively).

Since neuronal viability was decreased at high concentrations of apoE, the effect of apoE alone on neurons was further examined. Handelmann et al. (25) have reported that apoE decreased the attachment of dorsal root ganglion neurons to substrate. Therefore we quantified neuronal plating one hour after seeding in the presence of apoE (0.25, 0.5, 1, 2, and 4 μM). We found that apoE concentrations of 0.5 μM and up decreased the plating efficiency of neurons

(84±11%, 86±10%, 61±10%, and 28±4% relative to vehicle-treated controls for 0.5, 1, 2, and 4 μM apoE respectively, 1 hr after plating).

Furthermore, no alteration in neuronal viability was observed when apoE at concentrations up to 4 μM was added to cultures at day 1 (dl), after the neurons had firmly attached to the plate (data not shown). Thus, the cell loss observed at high apoE concentrations was not due to direct toxicity of apoE, but probably reflected decreased attachment of the anchorage-dependent hippocampal neurons.

Having found that the interaction between apoE and B40 was dose-dependent over a range of apoE concentrations similar to that found in CSF, we then tested whether the interaction of apoE and B40 was saturable, as would be expected of complex formation. ApoE concentration was held constant at 0.1 μM and the concentration of B40 was doubled (to 20 μM). The increased concentration of B40 overwhelmed the protective effect of apoE (Figure 4).

Last, we investigated the temporal window for neuronal rescue by apoE. Cultures were exposed to B40 (20 μM) from d0-d3; neuronal viability was quantified on d3. ApoE (0.1 μM) was added at d1 or d2. In cultures not treated with apoE, only about 20% of the neurons survived. However, in cultures treated with apoE significant neuronal rescue occurred (Figure 5). After 24 hours exposure to B40 alone, incubation with apoE increased neuronal

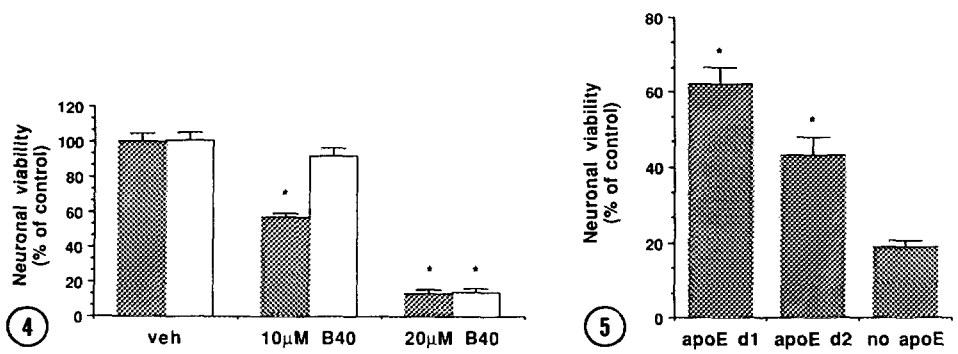

Figure 4. The capacity of apoE to interfere with βA toxicity can be saturated and overwhelmed by increased amounts of βA. In cultures treated with 10 μM B40, 0.1 μM apoE rescues neurons, but in cultures treated with 20 μM B40, 0.1 μM apoE is not sufficient to increase neuronal viability after 3 days in vitro. (However, higher apoE concentrations successfully inhibit even the toxic effect of 30 μM B40; see Figure 3.) Values are mean ± SEM; $n = 12$. * Values significantly less than veh/veh control, $p \leq 0.05$, Fisher PLSD. ANOVA: $F(5,66) = 151.672$, $p = 0.0001$. Cross hatched bars, cultures without apoE; white bars, cultures with apoE.

Figure 5. Delayed treatment with apoE rescues neurons from Aβ toxicity. Cultures exposed to 20 μM B40 from d0-d3 exhibited increased neuronal viability after 3 days in vitro even when apoE treatment was delayed until d1 or d2. *Significantly different than no apoE, $p \leq 0.05$, Fisher PLSD. Values are mean ± SEM; $n = 18$. ANOVA: $F(8,153)=22.404$, $p = 0.0001$.

survival to 70% (70±6%). Even after 48 hours of exposure to B40 alone, addition of apoE almost doubled neuronal viability relative to cultures grown in the absence of apoE (35±4% viability with apoE; 19±2% viability without apoE). In cultures incubated with 10 μM B40, apoE added on d1 and d2 were equally effective (data not shown).

DISCUSSION

Our data indicate that apoE rescues neurons from Aβ toxicity, suggesting the possibility that at normal CSF concentrations apoE may have a role in neuroprotection. The observation that apoE mRNA is increased in the hippocampus following entorhinal cortex lesion with a time course similar to that of alpha 1 tubulin, a protein associated with neuronal regeneration and/or sprouting (18,19) is also consistent with a possible neuroprotective role for apoE. Furthermore, increased apoE has been associated with axonal repair following sciatic nerve injury (20). In that instance it has been suggested that apoE mediates regeneration by increasing the availability of recycled membrane lipid to the regenerating axons. Our data demonstrate a neuroprotective activity of apoE most likely via its binding of the potentially neurotoxic Aβ fragment and probably unrelated to its role in lipid transport.

The notion that the apoE4 isoform specifically increases toxicity of Aβ is unlikely in the light of our experiments, although the apoE4 isoform itself was not tested. The sequence of apoE4 differs from that of apoE3 only in the substitution of an arginine for the cysteine at position 112, with both isoforms binding equally well to the apoE receptor (B/E receptor; LDL receptor) (14). Furthermore, a 2-hour incubation is sufficient for both apoE3 and apoE4 to form SDS insoluble complexes with B40 (32). Thus another explanation for the association of the apoE ε4 allele with AD must be sought.

Our data suggest that apoE may normally play a neuroprotective role in the brain and raise the possibilities that binding to Aβ may physically prevent apoE from fulfilling its normal function. Since apoE4 binds to Aβ much more rapidly than does apoE3, binding in minutes rather than in hours (32), it is conceivable that in the presence of Aβ, ApoE4 might have a shorter functional lifespan than apoE3 isoform. In addition to decreasing the functional neuroprotective lifespan of apoE4, the rapid formation and precipitation of E4/Aβ complexes could shunt Aβ away from normal degradative and/or regulatory mechanisms resulting in upregulated Aβ production. Increased Aβ product could result in greater Aβ deposition and accelerated amyloidogenesis. Such a scenario is consistent with the observations of Schmechel et al. that Aβ plagues are increased in both size and number in the brains of AD individuals homozygous for the ε4 allele (as compared to ε3 homozygotes; 33). Additionally, with its normal neuroprotective mechanisms compromised, the cell would be increasingly vulnerable to

secondary insults. Alternatively, the role of apoE4 in Alzheimer's disease may be independent of its interaction with Aβ peptide.

In summary, the ability of apoE to rescue neurons from Aβ toxicity, which we have reported here, indicates a possible neuroprotective role for apoE in early AD via its binding of the Aβ protein and offers one potential explanation for the association of the apoE4 isoform with AD.

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REFERENCES

1. Kang, J., et al. (1987) Nature 325, 733-736.
2. Glenner, G.G., and Wong, C.W. (1984) Biochem. Biophys. Res. Commun. 120, 885-890.
3. Glenner, G.G., and Wong, C.W. (1984) Biochem. Biophys. Res. Commun. 122, 1131-1135.
4. Masters, C.L., et al. (1985) Proc. Natl. Acad. Sci. USA 82, 4245-4249.
5. Iseki, E., Matsushita, M., Kondo, H., Ishii, T., and Amano, N. (1989) Acta Neuropathol. 78, 131-136.
6. Joachim, C.L., Morris, J.H., and Selkoe, D.J. (1989) Am. J. Pathol. 135, 309-319.
7. Tagliavini, F., Giaccone, G., Frangione, B., and Bugiani, O. (1988) Neurosci. Lett. 93, 191-196.
8. Verga, L., et al. (1989) Neurosci. Lett. 105, 294-299.
9. Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.J., and Cotman, C.W. (1993) J. Neurosci. 13, 1676-1687.
10. Yankner, B.A., Duffy, L.K., and Kirschner, D.A. (1990) Science 250, 279-282.
11. Whitson, J.S., Glabe, C.G., Shintani, E., Abcar, A., and Cotman, C.W. (1990) Neurosci. Lett. 110, 319-324.
12. Whitson, J.S., Selkoe, D.J., and Cotman, C.W. (1989) Science 243, 1488-1490.
13. Loo, D.T., et al. (1993) Proc. Natl. Acad. Sci. USA 90, 7951-7955.
14. Mahley, R.W. (1988) Science 240, 622-630.
15. Mahley, R.W., Innerarity, T.L., Rall, Jr., S.C., and Weisgraber, K.H. (1984) J. Lipid Res. 25, 1277-1294.
16. Chiba, H., Mitamura, T., Fujisawa, S-I., Ogata, A., Aimoto, Y., Tashiro, K., and Kobayashi, K. (1991) Neurosci. Lett. 133, 207-210.
17. Linton, M.F., et al. (1991) J. Clin. Invest. 88, 270-281.
18. Poirier, J., Hess, M., May, P.C., and Finch, C.E. (1991) Mol. Brain Res. 9, 191-195.
19. Poirier, J., Hess, M., May, P.C., and Finch, C.E. (1991) Mol. Brain Res. 11, 97-106.
20. Boyles, J.K., Notterpek, L.M., and Anderson, L.J. (1990) J. Biol. Chem. 265, 17805-17815.
21. Boyles, J.K., et al. (1989) J. Clin. Invest. 83, 1015-1031.
22. Ignatius, M.J., et al. (1986) Proc. Natl. Acad. Sci. USA. 83, 1125-1129.
23. Ignatius, M.J., Shooter, E.M., Pitas, R.E., and Mahley, R.W. (1987) Science 236, 959-962.
24. LeBlanc, A.C., and Poduslo, J.F. (1990) J. Neurosci. Res. 25, 162-171.
25. Handelmann, G.E., Boyles, J.K., Weisgraber, K.H., Mahley, R.W., and Pitas, R.E. (1992) J. Lipid Res. 33, 1677-1688.
26. Namba, Y., Tsuchiya, H., and Ikeda, K. (1992) Neurosci. Lett. 134, 264-266.

27. Wisniewski, T., and Frangione, B. (1992) Neurosci. Lett. 135, 235-238.
28. Strittmatter, W.J., et al. (1993) Proc. Natl. Acad. Sci. USA. 90, 1977-1981.
29. Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., and Ikeda, K. (1991) Brain Res. 541, 163-166.
30. Diedrich, J.F., Minnigan, H., Carp, R.I., Whitaker, J.N., Race, R., Frey II, W., and Haase, A.T. (1991) J. Virol. 65, 4759-4768.
31. Corder, E.H., et al. (1993) Science 261, 921-923. Saunders, A.M., et al., Neurology, 43, 1467-1472.
32. Strittmatter, W.J., et al. (1993) Proc. Natl. Acad. Sci. USA. 90, 8098-8102.
33. Schmechel, D.E., et al. (1993) Proc. Natl. Acad. Sci. USA. 90, 9649-9653.
34. Brewer, G.J. and Cotman, C.W. (1989) Brain Res. 494, 65-74.
35. Lee, B-R., Miller, J.M., Yang, C-Y., Ramdas, L., Yang, M., Morrisett, J.D., Mims, M.P. (1991) J. Lipid Res. 32, 165-171.