Haploinsufficiency of Human APOE Reduces Amyloid Deposition in a Mouse Model of Amyloid- β Amyloidosis

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The $\epsilon 4$ allele of the apolipoprotein E (*APOE*) gene is the strongest genetic risk factor for Alzheimer's disease (AD). Evidence suggests that the effect of apoE isoforms on amyloid- β ($A\beta$) accumulation in the brain plays a critical role in AD pathogenesis. Like in humans, apoE4 expression in animal models that develop $A\beta$ amyloidosis results in greater $A\beta$ and amyloid deposition than with apoE3 expression. However, whether decreasing levels of apoE3 or apoE4 would promote or attenuate $A\beta$ -related pathology has not been directly addressed. To determine the effect of decreasing human apoE levels on $A\beta$ accumulation *in vivo*, we generated human *APOE* isoform haploinsufficient mouse models by crossing *APPPS1-21* mice with *APOE* isoform knock-in mice. By genetically manipulating *APOE* gene dosage, we demonstrate that decreasing human apoE levels, regardless of isoform status, results in significantly decreased amyloid plaque deposition and microglial activation. These differences in amyloid load between apoE3- and apoE4-expressing mice were not due to apoE4 protein being present at lower levels than apoE3. These data suggest that current therapeutic strategies to increase apoE levels without altering its lipidation state may actually worsen $A\beta$ amyloidosis, while increasing apoE degradation or inhibiting its synthesis may be a more effective treatment approach.

Introduction

Aggregation and accumulation of amyloid β ($A\beta$) in the brain is a key initiating factor in the pathogenesis of Alzheimer's disease (AD) (Holtzman et al., 2011). Familial AD-causing mutations in amyloid β precursor protein (*APP*), presenilin 1 (*PS1*), and *PS2* genes have provided strong support for the crucial role of $A\beta$ aggregation in AD pathogenesis (Holtzman et al., 2011). The strongest genetic risk factor for the most common form of late-onset AD is the $\epsilon 4$ allele of the apolipoprotein E (*APOE*) gene (Strittmatter et al., 1993). Although several mechanisms for the effect of apoE4 on AD pathogenesis have been proposed, the primary pathway appears to be the differential effects of apoE3 and apoE4 isoforms on $A\beta$ aggregation and clearance (Kim et al., 2009a; Castellano et al., 2011).

In addition to isoform status, it appears that the amount of apoE also plays a role in determining the extent of $A\beta$ accumulation in the brain. There has been intense debate as to whether potential AD therapeutics should increase or decrease apoE levels

(Ashford, 2002; Rebeck et al., 2002; Teter et al., 2002; Osherovich, 2009). Initial studies clearly indicated that the deletion of the endogenous murine *APOE* gene causes a dramatic decrease in fibrillar and total $A\beta$ deposition in *APP* transgenic mouse models (Bales et al., 1997). However, whether decreasing human apoE levels will have the same beneficial effect or not has not yet been answered. Some recent studies indirectly suggest that increasing, rather than decreasing, human apoE levels may be a good therapeutic approach. Therefore, understanding how modulation of human apoE levels will alter AD pathology must be addressed before the development of apoE-targeting drugs.

To directly test the effect of human *APOE* gene dosage on amyloid pathology, we generated $A\beta$ -depositing mouse models homozygous or hemizygous for the *APOE3* or *APOE4* gene. *APPPS1-21* mice with two copies of *APOE* (*APOE3/3* or *APOE4/4*) or one copy of *APOE* (*APOE3/-* or *APOE4/-*) were generated by breeding *APPPS1-21* mice with human *APOE* knock-in mice. Compared with the respective homozygous mice, *APOE3/-* and *APOE4/-* hemizygous mice had significantly decreased $A\beta$ accumulation, amyloid deposition, and microgliosis. Our results strongly suggest that the reduction of human apoE levels, regardless of the isoform present, will decrease $A\beta$ -related AD pathology.

Materials and Methods

Generation of human *APOE* isoform mice with APP^{swe}/PS1(L166P) mutant transgenes. To determine the gene dosage effect of human *APOE3* and *APOE4* on amyloid deposition, we used knock-in mouse models in which the endogenous murine *ApoE* gene is replaced with the *APOE3* or *APOE4* gene. Breeding pairs were gifts from Dr. Patrick Sullivan, Duke University, Durham, NC (Sullivan et al., 1997). *APPPS1-21* mice over-

Received July 24, 2011; revised Oct. 17, 2011; accepted Oct. 28, 2011.

Author contributions: J.K. and D.M.H. designed research; J.K., H.J., S.P., A.E.M.E., F.R.S., H.Y., J.M.B., M.B.F., and D.M.H. performed research; H.J. and D.M.H. contributed unpublished reagents/analytic tools; J.K., H.Y., J.M.B., M.B.F., and D.M.H. analyzed data; J.K., J.M.B., and D.M.H. wrote the paper.

This work was supported by NIH Grants AG13956 (D.M.H.) and P30NS069329 (J.K.), American Health Assistance Foundation grants (J.K., D.M.H.), NIH Neuroscience Blueprint Center Core Grant P30NS057105, and the Alafi Neuroimaging Laboratory Human. *APOE* knock-in and *APPPS1-23* mice breeding pairs were gifts from Dr. Patrick Sullivan (Duke University, Durham, NC) and Dr. Matthias Jucker (University of Tübingen, Tübingen, Germany), respectively.

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DOI:10.1523/JNEUROSCI.3773-11.2011

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express a human APP with a Swedish mutation (KM670/671NL) and mutant PS1 with the L166P mutation. Breeding pairs were obtained from Dr. Mathias Jucker, University of Tübingen, Tübingen, Germany (Radde et al., 2006). To replace the murine *ApoE* gene with human *APOE* isoforms, APPPS1-21 mice were bred with either *APOE3/3* or *APOE4/4* knock-in mice. APPPS1-21/*APOE3/3* mice and *APOE3/3* mice from the first generation were bred with each other to generate APPPS1-21/*APOE3/3* and *APOE3/3* mice. APPPS1-21/*APOE3/3* and *APOE3/3* mice were then bred to generate more APPPS1-21/*APOE3/3* mice. After successful generation of APPPS1-21/*APOE3/3* mice at the third generation, APPPS1-21/*APOE3/3* mice were bred with *ApoE* knock-out mice (Jackson Laboratory). APPPS1-21/*APOE3/3* mice from the fourth generation were bred with *APOE3/3* mice to generate APPPS1-21/*APOE3/3* and APPPS1-21/*APOE3/3* mice. Littermates generated at the fifth generation were used in our analyses. Similarly, APPPS1-21/*APOE4/4* and APPPS1-21/*APOE4/4* mice were generated by using the same mating strategy. All mice used in this study were maintained on a C57BL/6J background.

Quantitative real-time PCR. mRNAs were extracted from frozen cortical tissue using Trizol and reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR (qPCR) was performed in ABI 7500 using the default thermal cycling condition with Power SYBR (Applied Biosystems) with the following primers: forward primer, TTGCTGGTCACATTCCTGG; and reverse primer, AGGTAATCCCAAGCGACC. Mouse GAPDH endogenous control was used as a normalization reference. Relative mRNA levels were calculated by comparative Ct method. To confirm the specificity of qPCR reactions, dissociation curves were analyzed at the end of the qPCR assay.

Western blots. Cortical tissues were gently lysed in PBS and modified RIPA (1% NP-40, 1% sodium deoxycholate, 25 mM Tris-HCl, 150 mM NaCl) in the presence of 1 $\times$ protease inhibitor mixture (Roche). Tissue homogenates were centrifuged at 18,000 relative centrifugal force (rcf) for 30 min. Equal amounts of protein for each sample were run on 4–12% Bis-Tris XT gels (Bio-Rad) and transferred to PVDF membranes. Blots were probed with the following antibodies: apoE (Academy Biomedical); APP (ZYMED); PS1-NTF (EMD Chemicals); β -secretase 1 (BACE1) (Cell Signaling Technology); synaptophysin (or SYP) (Sigma); glutamate receptor (GluR) 2/3/4 (Cell Signaling Technology); NMDAR2b (Cell Signaling Technology); postsynaptic density protein 95 (PSD-95) (Millipore); and tubulin (Sigma). Tubulin-normalized band intensity was quantified using NIH ImageJ software.

Sandwich ELISA for A β and apoE. Cortical tissues were sequentially homogenized with PBS, modified RIPA, and 5 M guanidine HCl buffer. Tissue homogenates were centrifuged at 18,000 rcf for 30 min after each extraction. The levels of A β and apoE were measured by enzyme-linked immunosorbent assay (ELISA). For A β ELISA, HJ2 (anti-A β 35–40) and HJ7.4 (anti-A β 37–42) were used as capture antibodies, and HJ5.1-biotin (anti-A β 13–28) as the detection antibody. The anti-apoE monoclonal antibody WUE4 and another anti-apoE antibody (Calbiochem) were used for apoE ELISA.

Histology. Histology, staining, immunohistochemistry, and quantitative analysis were performed as described except that we used biotinylated mouse monoclonal antibody HJ3.4 (1:1000, targeted against amino acids 1–13 of the human A β sequence) to detect A β in tissue sections (Kim et al., 2009b).

Statistical analysis. To determine the statistical significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$), we first tested whether our datasets passed the equal variance test (Levene Median test) and normality test

Figure 1. Reduction of apoE levels in human *APOE* haploinsufficient mice. Cortex from *APOE* homozygous (*APOE3/3* and *APOE4/4*) and hemizygous (*APOE3/3*- and *APOE4/4*-) mice were used to measure apoE mRNA and protein levels. **A**, ApoE mRNA levels were measured by quantitative real-time PCR. **B**, Levels of PBS-soluble apoE were assessed by probing a membrane with anti-apoE antibody. **C**, Quantitative analyses of Western blots were performed with tubulin normalization ($n = 4$ per genotype). **D**, **E**, To validate the Western blot data, apoE levels were also measured by using an apoE-specific ELISA from *APOE3/3* and *APOE3/3*- mice (**D**) and *APOE4/4* and *APOE4/4*- mice (**E**) ($n = 9$ –18 per genotype). **F**, Levels of APP, APP CTFs, PS1 NTF, and BACE1 proteins were measured by Western blotting ($n = 4$ per genotype). Levels of these proteins after normalizing with tubulin signals were not significantly altered by *APOE* haploinsufficiency. All graphs represent values in mean $\pm$ SEM.

Figure 2. Decreased A β accumulation in human *APOE* haploinsufficient mice. Cortical tissues from 3-month-old *APOE* homozygous and hemizygous mice were sequentially homogenized with PBS and RIPA. Aggregated forms of A β in the RIPA-insoluble pellet were solubilized with 5 M guanidine HCl buffer. **A**, **B**, RIPA-insoluble A β 40 (**A**) and A β 42 levels (**B**) were measured from *APOE3/3* and *APOE3/3*- mice. **C**, **D**, Similarly, RIPA-insoluble A β 40 (**C**) and A β 42 levels (**D**) were measured from *APOE4/4* and *APOE4/4*- mice. ($n = 12$ –20 per genotype).

Figure 3. Haploinsufficiency of human *APOE* leads to reduction of A β and fibrillar A β plaque deposition. **A, B, D, E**, Brain sections from 3-month-old *APOE* homozygous (**A, D**) and hemizygous (**B, E**) mice were immunostained for amyloid with anti-A β antibody (HJ3.4-biotin). Scale bars, 400 μ m. **A–C**, The extent of A β deposition detected by HJ3.4-biotin antibody was quantified (**C**) from cortex of *APOE3/3* (**A**) and *APOE3/-* mice (**B**). **D–F**, The extent of A β deposition detected was quantified (**F**) from cortex of *APOE4/4* (**D**) and *APOE4/-* mice (**E**). **G, H, J, K**, Brain sections from 3-month-old *APOE* homozygous (**G, J**) and hemizygous (**H, K**) mice were stained with X-34 dye that recognizes only fibrillar plaques. Scale bar, 200 μ m. **G–I**, Fibrillar plaque load detected by X-34 dye was quantified (**I**) from cortex of *APOE3/3* homozygous (**G**) and *APOE3/-* mice (**H**). **J–L**, The extent of fibrillar plaque load was also analyzed (**L**) from cortex of *APOE4/4* (**J**) and *APOE4/-* mice (**K**). ($n = 12–20$ per genotype).

(Kolmogorov–Smirnov test) (SigmaStat 3.5.). After confirmation that the data did not violate the assumptions of parametric testing, a two-tailed Student's *t* test was used (GraphPad Prism 5). Variance in all graphs represents SEM.

Results

Generation of human *APOE* haploinsufficient mouse models

To directly examine the effect of decreasing human apoE levels on A β and amyloid deposition, we generated mouse models that have either one or two copies of the human *APOE3* or *APOE4* gene along with the *APP^{sw/PS1(L166P)}* transgene (*APPPS1-21* mice). *APOE* knock-in mouse models that express human apoE isoforms under the control of endogenous regulatory elements were used, eliminating any potentially confounding effects due to the presence of the endogenous murine *ApoE* gene.

First, we assessed the effect of *APOE* gene haploinsufficiency on apoE mRNA and protein levels in the cortex. As expected, apoE mRNA levels decreased by $\sim 50\%$ in the hemizygous *APOE3/-* and

APOE4/- mice (Fig. 1A). Western blot analysis indicated that apoE protein levels were also significantly decreased in the *APOE* hemizygous compared with *APOE* homozygous mice (Fig. 1B,C). Consistent with the Western blot data, a similar decrease in apoE levels was observed by apoE ELISA for both *APOE3* and *APOE4* isoform mice (Fig. 1D,E). There were no significant alterations in the levels of proteins involved in A β production (Fig. 1F).

Haploinsufficiency of human *APOE* dramatically inhibits A β accumulation

To determine the effect of *APOE* genotype and dosage on A β , we analyzed A β accumulation in *APOE* homozygous and hemizygous mice. Significant reductions in insoluble A β 40 and A β 42 levels were observed in *APOE3/-* mice, compared with *APOE3/3* mice (Fig. 2A,B). *APOE4/-* mice also had a dramatic decrease in insoluble A β 40 and A β 42 levels compared with *APOE4/4* mice (Fig. 2C,D). We also analyzed PBS-soluble A β levels in mice of all genotypes. Soluble A β levels were ~ 100 -fold less than PBS-insoluble A β levels. PBS soluble A β levels, except A β 42 levels between *APOE4/4* and *APOE4/-* mice, were not altered by *APOE* haploinsufficiency (*APOE3/3*: A β 40, 5.82 ± 0.31 pg/mg; A β 42, 0.77 ± 0.068 pg/mg; *APOE3/-*: A β 40, 5.27 ± 0.32 pg/mg; A β 42, 0.77 ± 0.099 pg/mg; *APOE4/4*: A β 40, 7.49 ± 0.88 pg/mg; A β 42, 1.39 ± 0.13 pg/mg; *APOE4/-*: A β 40, 7.90 ± 0.51 pg/mg; A β 42, 1.12 ± 0.058 pg/mg).

Reduction of A β and fibrillar A β plaque load in human *APOE* hemizygous mice

To determine the extent of A β deposition in *APOE* homozygous and hemizygous mice, brain sections from 3-month-old mice were immunostained with a biotinylated anti-A β antibody, HJ3.4 (Fig. 3A–F). Quantitative analyses of anti-A β immunostaining demonstrated that A β plaque load was significantly decreased in *APOE3/-* mice compared with *APOE3/3* mice (Fig. 3C). The level of A β deposition in both *APOE4/4* and *APOE4/-* mice was also analyzed in the same way (Fig. 3D,E). While *APOE4^{+/-}* and *APOE4^{+/+}* mice had significantly greater A β deposition than *APOE3^{+/-}* and *APOE3^{+/+}* mice respectively, *APOE4/-* mice had a dramatic reduction in A β plaque load, compared with *APOE4/4* mice (Fig. 3F). To further characterize the nature of the deposited A β plaques, brain sections were stained with X-34 dye that selectively detects only fibrillar, but not diffuse, A β deposits. Consistent with the anti-A β antibody staining, *APOE3/-* and *APOE4/-* hemizygous mice had significantly less X-34-positive fibrillar plaque load, compared with *APOE3/3* and *APOE4/4* homozygous mice (Fig. 3G–L). Together, our results clearly demonstrate that haploinsufficiency of *APOE* decreases both total A β and fibrillar amyloid deposition.

APOE dosage-dependent modulation of neuroinflammatory response

Aberrant inflammatory responses, such as activation of microglia, are common pathological features in the brains of AD patients. To identify activated microglia in the brain, mouse brain sections were stained with anti-CD45 antibody, a marker of microgliosis. Plaque-associated microglial activation was evident around A β deposits in APPPS1-21 mice with APOE isoforms (Fig. 4*A–D*). Quantitative analysis of microgliosis indicated an ~95% decrease in the CD45-positive activated microglial load in APOE3/– mice, compared with APOE3/3 littermates (Fig. 4*E*). A similar dramatic phenotype was observed between APOE4/4 and APOE4/– mice (Fig. 4*C,D*). There was an ~97% reduction in microgliosis in APOE4/– mice (Fig. 4*F*). These findings indicate that the reduction of A β and amyloid formation by APOE haploinsufficiency is closely associated with an attenuation of the microglial neuroinflammatory response. We also analyzed the levels of presynaptic and postsynaptic proteins. Analysis of synaptophysin, GluR2/3/4, NMDA receptor 2b, and PSD-95 levels indicated there was no significant alteration in these proteins between genotypes or APOE dosage levels (Fig. 4*G*).

Discussion

We tested whether decreasing human apoE levels will increase or decrease A β accumulation *in vivo*. Given the importance of this question, there have been numerous attempts to investigate the effects of modulating apoE levels on AD pathogenesis (Ashford, 2002; Rebeck et al., 2002; Teter et al., 2002). However, studies ranging from *in vitro* A β aggregation, *ex vivo* A β assays, and pharmacological approaches have provided conflicting results (Kim et al., 2009a). Most *in vivo* studies also provided limited information regarding the effect of human apoE modulation, since they indirectly inferred the effect of human apoE based on the manipulation of mouse apoE. To overcome some of the limitations of previous studies, we took a genetic approach by generating human APOE homozygous or hemizygous mice without the presence of the confounding endogenous mouse *ApoE* gene. A β -depositing mouse models with two copies (APOE3/3 or APOE4/4) or one copy (APOE3/– or APOE4/–) of each APOE isoform were successfully generated. APOE3/– and APOE4/– mice had significantly less A β accumulation, amyloid deposition, and microgliosis compared with the respective homozygous littermates. Thus, decreasing human apoE levels may be an attractive

Figure 4. Attenuation of microgliosis in human APOE haploinsufficient mice. *A–D*, Brain sections from 3-month-old male APOE homozygous (*A*, *C*) and male hemizygous (*B*, *D*) mice were immunostained with an antibody against activated microglial CD45. *E*, The percentage area covered by CD45 staining was quantified from the cortex of APOE3/3 and APOE3/– mice. *F*, The extent of microgliosis was also quantified from the cortex of APOE4/4 and APOE4/– mice. Scale bars: lower magnification, 200 μ m; higher magnification, 90 μ m. *n* = 6–12 male mice per genotype. *G*, Levels of synaptophysin, GluR2/3/4, NMDA receptor 2b, and PSD-95 levels were measured by Western blotting (*n* = 4 per genotype). Levels of these proteins after normalizing with tubulin signals were not significantly altered by APOE genotype or haploinsufficiency.

therapeutic strategy for attenuating A β deposition and its downstream effects in AD.

Given the critical role of A β in AD pathogenesis, the effect of apoE isoforms on A β deposition has been extensively investi-

gated. *APOE* isoform-dependent amyloid deposition has been consistently observed in humans and A β -depositing mouse models (Holtzman et al., 2000; Bales et al., 2009; Reiman et al., 2009; Morris et al., 2010). Consistent with previous studies with other *APP* mouse models, we also observed *APOE* isoform-dependent effects on A β deposition in the *APPS1-21* mouse model ($E4 > E3$). Since apoE levels differ between *APOE4/4* and *APOE3/3*, with levels of apoE4 being lower than apoE3, it is tempting to speculate that the difference in A β accumulation between the apoE isoforms is due to differences in apoE3 versus apoE4 levels. However, the structures of apoE3 and apoE4 differ significantly from each other (Mahley et al., 2006), and the observed difference in A β deposition cannot be simply attributed to apoE4 levels being lower than apoE3. For example, *APOE4/E4* mice have greater amyloid deposition than *APOE3/–* mice despite the fact that apoE4 levels are higher in the *APOE4/E4* than in the *APOE3/–* mice. *APOE* gene dosage-dependent amyloid deposition could be due to an effect of an alteration in A β clearance and/or aggregation. Several studies demonstrated that apoE isoforms can differentially affect A β clearance (Deane et al., 2008; Jiang et al., 2008; Castellano et al., 2011). ApoE isoforms may be slowing A β clearance in an apoE-isoform-dependent manner ($E4 > E3 > E2$). This idea is supported by the fact that mice lacking apoE have accelerated A β clearance from the brain relative to apoE-expressing mice (DeMattos et al., 2004). Another potentially important mechanism is direct facilitation of A β fibrillogenesis by an ApoE and A β interaction. While there are a few conflicting reports (Kim et al., 2009a), several groups have reported that all three apoE isoforms increase A β fibrillogenesis, with the effect being most exacerbated with ApoE4 and the least with ApoE2 (Ma et al., 1994; Wisniewski et al., 1994; Castano et al., 1995).

Given the isoform-dependent effect on AD pathology, one therapeutic approach is to convert apoE4 to an apoE3-like protein by using a small molecule (Mahley et al., 2006). Disrupting the apoE–A β interaction could be another attractive therapeutic approach to prevent apoE-mediated A β aggregation (Sadowski et al., 2004). Previous studies suggested that modulation of apoE levels and lipidation state, independent of *APOE* genotype, may be another potential strategy to decrease A β accumulation (Cao et al., 2006; Wahrle et al., 2008; Kim et al., 2009b). Interestingly, deletion of ATP-binding cassette, subfamily A1 (*Abca1*) and liver x receptor (*Lxr*) genes decreased apoE levels but increased or had no effect on A β deposition (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005; Wahrle et al., 2005; Zelcer et al., 2007). In addition, several studies have shown that activation of *Lxr* increases mouse apoE and decreases A β levels (Jiang et al., 2008). However, it is difficult to interpret the effect of altering apoE level in these studies, since modulation of *Abca1* and *Lxr* also influences the lipidation state of apoE. Whether reducing human apoE levels without affecting its lipidation state will promote or attenuate A β accumulation was not clear from previous studies. Our study demonstrates the beneficial effect of decreasing apoE levels on A β accumulation. Consistent with our finding, low levels of apoE were strongly associated with low cerebral A β load in humans (Darreh-Shori et al., 2011). *APOE* gene promoter studies also suggest that higher levels of apoE, regardless of isoform status, increase the risk of developing AD (Laws et al., 2003). *APOE* may also influence risk for AD by mechanisms that are not related to A β . We found no clear differences in presynaptic and postsynaptic proteins in *APPS1-21* mice of different *APOE* genotypes and doses. This, however, does not rule out the possibility the structural synaptic changes might be present that have been ob-

served by others (Dumanis et al., 2009). Our results in which apoE levels are altered by changing gene dosage suggest that strategies to decrease apoE levels in the brain should be further explored for the prevention/treatment of AD. Changing apoE levels in an adult in the context of two copies of *APOE* is different than having a lifelong genetic alteration of *APOE* dosage as in our experiments. It will be important to see in the former context whether altering apoE levels in an adult with two endogenous copies of *APOE* has similar or different effects as we report herein.

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