American Journal of Pathology, Vol. 155, No. 3, September 1999
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A Role for apoE in Regulating the Levels of α-1-Antichymotrypsin in the Aging Mouse Brain and in Alzheimer's Disease

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This study was designed to explore the possible functional relationships between apolipoprotein E (apoE) and the protease inhibitor α-1-antichymotrypsin in the aging mouse brain and in Alzheimer's disease. For this purpose, levels of EB22/5 (the mouse homologue to human α-1-antichymotrypsin) mRNA expression was studied in apoE-deficient mice. These mice showed an age-dependent increase of EB22/5 mRNA expression in the brain. Furthermore, overexpression of allele 3 of human APOE gene in transgenic mice (in an apoE-deficient background) resulted in normalization of levels of EB22/5 mRNA expression compatible with levels found in control mice. In contrast, overexpression of human APOE4 allele or down-regulation of the apoE receptor low density lipoprotein receptor-related protein by deletion of the receptor-associated protein was associated with elevated levels of EB22/5 similar to apoE-deficient mice. Consistent with the findings in murine models, human α-1-antichymotrypsin protein was increased in brain homogenates from patients with Alzheimer's disease, and levels of this serpin were the highest in patients with the APOE4 allele. In summary, the present study showed evidence supporting a role for apoE in regulating α-1-antichymotrypsin expression. This is relevant to Alzheimer's disease because these two molecules appear to be closely associated with the pathogenesis of this disorder. *(Am J Pathol 1999, 155:869–875)*

Apolipoprotein E (apoE) is a polymorphic protein that binds and transports cholesterol and other lipids.[1] Three alleles (APOE2, APOE3, and APOE4) on human chromosome 19 code for the different isoforms of apoE, and increased frequency of APOE 4 is associated with late onset familial[2] and sporadic[3] Alzheimer's disease (AD). Moreover, findings from some studies suggest that patients with AD and the APOE4 allele showed increased levels of amyloid deposition in the cortex.[4] Although apoE binds Aβ-peptide and promotes β-amyloid fibrillogenesis[5] *in vitro*, it is unclear whether the APOE4 genotype is associated with accelerated cognitive deterioration.[6,7] Thus, the physiopathological role(s) of apoE in AD remains to be clarified.

α-1-Antichymotrypsin (ACT) is one of the serpins that has been shown to be altered in AD.[8] ACT, as well as other serpins, are important components of amyloid deposits which represent one neuropathological hallmark of AD.[9] ACT binds Aβ-peptide and can affect the rate of amyloid fibril formation *in vitro*.[10–12] The above findings, together with the observation that mRNA expression of ACT is increased in astrocytes surrounding the neurodegenerative lesions of AD brains,[13,14] has led to the hypothesis that ACT may play a role in the pathogenesis of neurodegeneration associated with AD. ApoE and ACT share some functional characteristics, since they both colocalize in β-amyloid deposits, bind to Aβ-peptides, and affect fibril formation, which may be important in the pathogenesis of the disease. Recently, we found that ACT levels in AD brains correlated with activated astrocytes scattered in the neuropil and these scattered astrocytes were increased in AD patients with the APOE4 allele.[15] However, possible relationships between these two molecules in AD pathogenesis are still largely to be determined. In this context, the main objective of the present study was to better understand the functional relationship between apoE and ACT during aging of the brain and in AD. For this purpose, levels of EB22/5 (also

Supported by grants from the Italian Ministero dell'Universita e della Recercia Scientifica e Tecnologia and Associazione de Ricerca e Assistenze delle Demenze (to FL), National Institutes of Health Grants AG5131 and AG10869, The Broad Foundation, Alzheimer's Association, and EBEWE (to EM).

Accepted for publication May 24, 1999.

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Table 1. Neuropathological Features of Control and Alzheimer's Disease Patients with Different APOE Genotypes

Cases	*N*	APOE genotype	Age (years)	Blessed score	Area covered by amyloid (%)
Controls	4	3/3	76 ± 7	1 ± 0.3	
Alzheimer's disease	11	3/3	78 ± 3	24 ± 3	11.8 ± 2.2
	9	3/4	77 ± 3	23 ± 2	33.2 ± 6.9
	5	4/4	74 ± 4	22 ± 7	31.9 ± 5.6

known as contrapsin-2) which is the murine homologue to human ACT [16,17] were evaluated in the brains of young and aged apoE-deficient mice, transgenic mice overexpressing human apoE3 and apoE4, as well as in mice where the apoE receptor low density lipoprotein receptor-related protein (LRP) was down-regulated by deleting the 39-kd receptor-associated protein (RAP).[18] To further validate the results observed in the experimental animal models, levels of ACT were determined in the brains of control and AD cases bearing the APOE3 and/or APOE4 alleles. Our study supports a role for apoE in regulating ACT in aging and in AD.

Materials and Methods

Colony Breeding and Characterization

ApoE-deficient homozygous mice ($n = 3$, 3 months; $n = 5$, 6 months; $n = 5$, 8 months; $n = 4$, 9 months; $n = 2$, 15 months; $n = 2$, 24 months) were obtained by cross-breeding heterozygous mutants that were kindly provided by Dr. J. Breslow (Rockefeller University, New York, NY).[19] Characterization of homozygosity or heterozygosity for apoE-deficient mice was carried out by polymerase chain reaction (PCR) with DNA extracted from the tail, as previously described.[20] In addition, a total of 10 wild-type mice were used for the study ($n = 2$, 3 months; $n = 5$, 6 months; $n = 5$, 9 months; $n = 2$, 15 months; $n = 2$, 24 months).

ApoE3 heterozygous ($n = 5$, 12 months) and apoE4 heterozygous ($n = 5$, 12 months) transgenic mice were obtained by cross-breeding heterozygous transgenic mice in a homozygous apoE-deficient background. Briefly, mice were obtained by microinjecting genomic sequences for human APOE3 or APOE4 into single-cell embryos from apoE-deficient mice.[21] Confirmation of the presence of human APOE and absence of endogenous mouse APOE gene was done by PCR as previously described.[21]

RAP-deficient homozygous mice ($n = 5$, 12 months) were obtained by cross-breeding heterozygous mutants from Jackson Laboratories (Bar Harbor, ME). Briefly, as previously described[18] these mice were generated by recombination of the *neo* cassette with exon 1 of the RAP gene. Confirmation of the genotype was done by PCR as previously described.[22]

Murine Tissue Preparation

Anesthetized mice were perfused with cold saline and their brains removed. The left hemibrain was frozen in liquid nitrogen and the right hemibrain was immersion-fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. Poly(A)+ RNA was isolated as previously described[23] from frozen hemibrains. Fixed hemibrains were paraffin-embedded and serially sectioned (10 μm) for hematoxylin and eosin/cresyl violet staining or *in situ* hybridization.

Analysis of RNA Expression in Mouse Brain Samples

For the analysis of gene product expression, RNase protection assays (RPA) were performed as previously described.[23] Multiprobe sets were used that permitted simultaneous detection of various cytokine mRNAs, such as interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, or IL-6, interferon-γ, tumor necrosis factor (TNF)-α and TNF-β, or the mRNA expression of the following cellular response genes: EB22/5, glial fibrillary acid protein (GFAP), intercellular adhesion molecule-1 (ICAM-1), inducible nitric oxide synthase (iNOS), and the macrophage adhesion molecule-1 (Mac-1). The development and characterization of the cytokine[24] and cellular response[23] RPA probe sets were described previously.[23] A cloned fragment of the ribosomal protein RPL32 (L32) cDNA was used as a control of RNA loading.[23]

In situ hybridization was carried out using ^{35}S-labeled cRNA corresponding to EB22/5 sense and antisense riboprobes essentially as described previously.[23] The probe for EB22/5 was synthesized from the pGEM4 vector that contained a 1.8-kb *Eco*RI fragment of EB22/5.[16] Briefly, paraffin sections were treated with proteinase K, incubated in prehybridization solution and hybridized with riboprobes at 60°C. Sections were exposed to film and developed 3 days to 2 weeks later. These sections were then dipped in Kodak NTB-2 emulsion and developed 10 days later.

Characterization and Processing of Human Brain Specimens

Twenty-nine autopsy cases from the Alzheimer's Disease Research Center at UCSD were used for the present study. Of them, 25 were clinically and histopathologically diagnosed[25] as AD and 4 as controls (Table 1). For all cases, the postmortem delay was approximately 6 hours. APOE genotyping was performed by PCR as previously described[26] (Table 1). Paraffin sections from cortical and subcortical regions were stained with hematoxylin and eosin, thioflavin-S, and cresyl violet for routine histopatho-

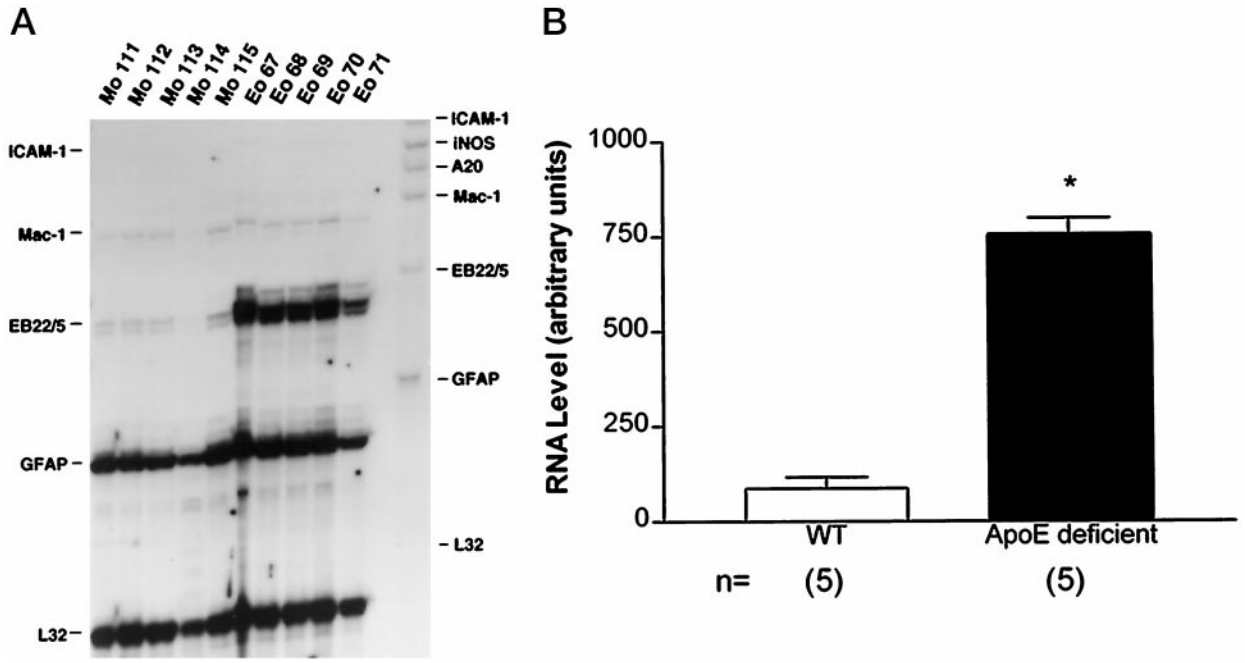

Figure 1. Levels of EB22/5 mRNA expression in apoE-deficient mice. A: RPA analysis of the expression level of different genes (ICAM-1, iNOS, A20, Mac-1, EB22/5, GFAP, and L32) in brains of 5 control (Mo 111–115) and 5 apoE-deficient (Eo 67–71) 8-month-old mice. Solution hybridization was done for 18 hours at 55°C in a volume of 10 μl. B: EB22/5 expression in brains of apoE-deficient compared to wild-type control (WT) mice was significantly increased (Student's *t*-test, $P < 0.05$) (*).

logical examination and morphometric analysis, as previously described.[27] Additional paraffin sections were immunostained with the monoclonal antibody against amyloid β-protein (clone 10D5, Athena Neurosciences, San Francisco, CA), as previously described[28] and analyzed with the Quantimet 570C to determine the percent area of the neuropil covered by amyloid (Table 1).

Determination of ACT Levels in Human Brain Homogenates

Brain homogenates of the mid frontal cortex were prepared as follows: 0.1 g of specimens were dissected from mid-frontal area of frozen tissues and homogenized in 0.9 ml of cold buffer (1 mmol/L HEPES, 5 mmol/L benzamide, 2 mmol/L 2-mercaptoethanol, 3 mmol/L EDTA, 3 mmol/L EDTA, 0.5 mmol/L $MgSO_4$, 0.05% sodium azide, pH 8.8) containing 0.01 mg/ml leupeptin. Homogenates were centrifuged (5000 $\times$ *g*) for 10 minutes at 4°C, and supernatants were collected and centrifuged again (100,000 $\times$ *g*) for 1 hour at 4°C. Protein determination was performed according to the method of Lowry[29] and samples were stored at −80°C.

Levels of human ACT, inhibitor of the complement C1 component (C1-I), and C-reactive protein (CRP) were measured by a radial immunodiffusion kit (NANORID; the Binding Site, Birmingham, UK) as previously described.[30] The lowest detectable level of ACT, C1-I, or CRP was 0.1 mg/l.

Statistical Analysis

Both murine and human samples were examined blind and the diagnosis and genotype were assigned only after the experiments were completed. Comparisons among groups of mouse or human samples were performed using one-way analysis of variance followed by post hoc comparisons using the Fisher test. All results were expressed as means ± SEM.

Results

Expression of EB22/5 Is Increased in Brains of apoE-Deficient Mice

Since the main objective of the present study was to determine whether apoE regulates the expression of ACT, levels of EB22/5 (murine homologue of human ACT) were determined by RPA in the brains of apoE-deficient mice. EB22/5 mRNA was detected at low levels in wild-type samples. In contrast apoE-deficient mice showed a sevenfold increase in EB22/5 mRNA levels (Figure 1, A and B).To determine whether the increased expression of EB22/5 mRNA in apoE-deficient mice was the result of either a direct interaction between apoE and ACT or a secondary response to brain injury, levels of GFAP, ICAM-1, iNOS, A20, and Mac-1 mRNA were determined. No significant difference in levels of expression for all these molecules associated with glial cell reactivity was observed between wild-type and apoE-deficient mice (Figure 1A). Additional RPA analysis for IL-1 and IL-6

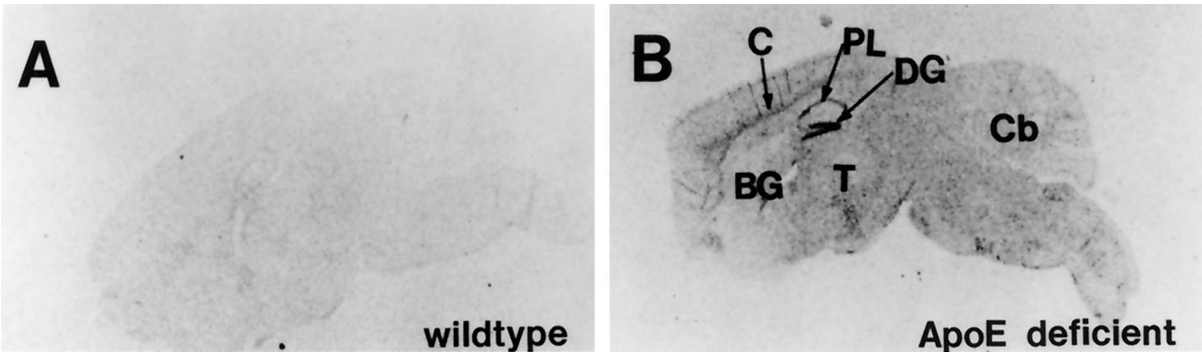

Figure 2. Analysis of EB22/5 gene expression by *in situ* hybridization. Levels of EB22/5 expression were increased throughout the brain in the apoE-deficient (A) compared to wild-type control (B) mice. DG, dentate gyrus; PL, pyramidal layer; C, cortex; Cb, cerebellum; T, thalamus; BG, basal ganglia.

mRNAs showed that these molecules were not detectable in either wild-type or apoE-deficient animals (data not shown).

Further analysis of EB22/5 mRNA expression and distribution in the brain was assessed by *in situ* hybridization (Figure 2). Consistent with RPA results this study showed that in apoE-deficient mice (Figure 2A) compared to wild-type control mice (Figure 2B) there was a dramatic increase in the levels of EB22/5 expression. EB22/5 was widely distributed throughout the brain with the highest levels detected in the neocortex and hippocampus (Figure 2). The patterns of hybridization in the dentate gyrus and pyramidal cell layer in the hippocampus indicates a neuronal expression for this gene (Figure 2).

Effect of Age on EB22/5 mRNA Expression in apoE-Deficient Mice

To determine whether the increase in EB22/5 levels if apoE-deficient mice was developmental or related to the aging process, levels of EB22/5 were determined in wild-type and apoE-deficient mice ranging in age from 3 to 24 months. At 3 months of age levels of EB22/5 were similar in wild-type and apoE-deficient mice (Figure 3). However, at 6 months of age there was a dramatic sixfold increase in EB22/5 mRNA expression in apoE-deficient mice, which remained elevated during their life spans (Figure 3).

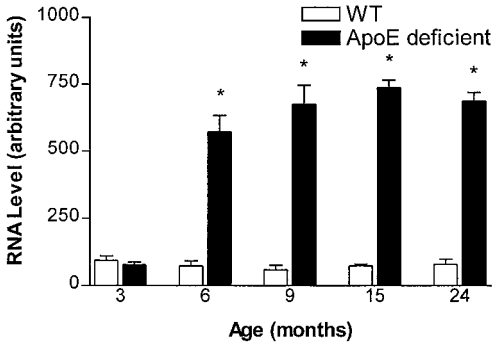

Figure 3. Age-dependent increase in EB22/5 expression in apoE-deficient mice by RPA. EB22/5 expression was not different at 3 months of age but was up-regulated beginning at 6 months of age in apoE-deficient mice compared to wild-type controls (WT). For each age time point $n = 3$, 3 months; $n = 5$, 6 months; $n = 5$, 8 months; $n = 4$, 9 months; $n = 2$, 15 months; $n = 2$, 24 months old mice. Statistical analysis was done by one-factor analysis of variance with post hoc Scheffé test, $P < 0.05$ (*).

EB22/5 mRNA Expression in apoE3 and apoE4 Transgenic and RAP-Deficient Mice

To determine whether the elevation in the apoE-deficient mice was more efficiently corrected by apoE3 *versus* apoE4, levels of EB22/5 mRNA expression were analyzed in the brains of apoE3- and apoE4- transgenic mice. This study showed that apoE3 was more effective in reestablishing the normal levels of EB22/5 expression in the endogenous apoE-deficient background, when compared to apoE4 transgenic mice (Figure 4). Levels of EB22/5 mRNA were still moderately elevated in apoE4 transgenic mice, when compared to both wild-type and apoE3 transgenic mice (Figure 4). Since previous studies have shown that the cellular effects of apoE are mediated by LRP,[31,32] we wanted to determine whether the effects of apoE on ACT were mediated by this receptor. For this purpose, levels of EB22/5 were determined in RAP-defi-

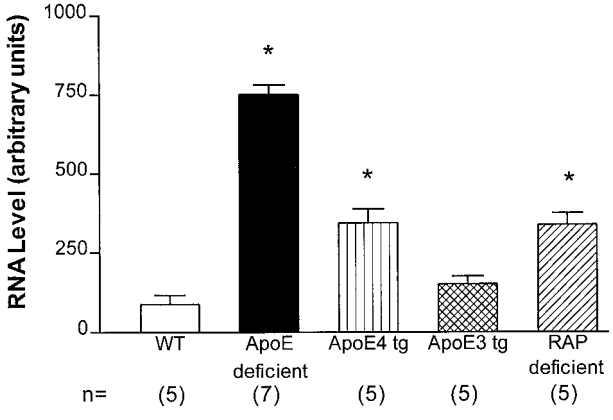

Figure 4. EB22/5 mRNA expression in apoE3 and apoE4 transgenic and RAP-deficient mice by RPA. ApoE3 was more effective in reestablishing the normal levels of EB22/5 expression in the endogenous apoE-deficient background when compared to apoE4 transgenic mice. Levels of EB22/5 mRNA were still moderately elevated in apoE4 transgenic mice when compared to both wild-type (WT) and apoE3 transgenic mice. Compared to the wild-type mice, levels of EB22/5 were elevated in the RAP-deficient mice. However, these levels were lower compared to the apoE-deficient mice, but comparable to levels observed in apoE4 transgenic mice. Statistical analysis was done by one-factor analysis of variance with post hoc Scheffé test, $P < 0.05$ (*).

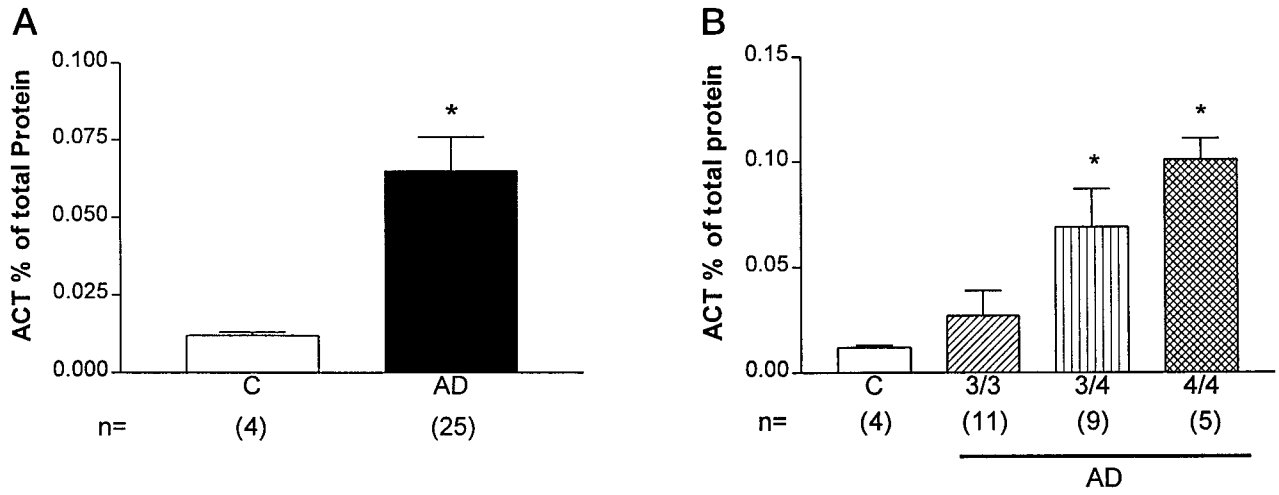

Figure 5. ACT expression in the brains of Alzheimer's disease (AD) patients. A: Compared to controls (C), AD cases showed a significant increase in ACT expression in the frontal cortex. B: Presence of allele 4 of APOE was associated with a significant increase in ACT levels in the frontal cortex compared to AD cases with allele 3 or controls. Statistical analysis was done by one-factor analysis of variance with post hoc Scheffé test, $P < 0.05$ (*).

cient mice. These mice were selected, as previous studies have shown, these mice are viable despite a 75% reduction in the levels of LRP.[18] Homozygous LRP-deficient mice cannot be used for these experiments because they are not viable.[18] Compared to the wild-type mice, levels of EB22/5 were elevated in the RAP-deficient mice (Figure 4). However, these levels were lower compared to the apoE-deficient mice, but comparable to levels observed in apoE4 transgenic mice (Figure 4). This supports the possibility that the effects of apoE on ACT might be mediated by LRP.

Determination of ACT Concentration in Human Brain Homogenates

Since the presence of the allele 4 of APOE is associated with risk for AD[33] and studies in apoE4 transgenic mice showed that apoE4 was less effective than apoE3 in reducing levels of EB22/5 expression in apoE-deficient mice (Figure 4), we wanted to determine whether a similar APOE allele effect could be observed in AD brains. For this purpose, levels of ACT were determined in brain cytosolic fractions from AD and non-demented controls with different APOE genotypes (Figure 5). Overall levels of ACT were significantly increased in AD compared to controls (Figure 5A). However, among AD cases, those with APOE 4/4 genotype showed the highest ACT levels when compared to controls (Figure 5B). Differences in ACT brain levels between AD patients with APOE 4/4 or APOE 3/4 and those with APOE 3/3 were also significant (Figure 5B). Differences of ACT levels between patients with APOE 4/4 and those with APOE 3/4 were not significant (Figure 5B). To further determine whether changes in ACT expression in AD cases were related to apoE effects or to a systemic reactive condition, levels of C1-I and CRP were determined. This analysis showed no significant differences between control and AD (not shown).

Discussion

The present study showed that apoE plays and important role in regulating the *in vivo* expression of ACT in the brain. One possible interpretation for this result is that under physiological conditions apoE might have a suppressive effect on the ACT gene or protein expression and this regulation might occur at the transcriptional or post-transcriptional level. Since we observed that apoE had significant effects on ACT mRNA expression it is reasonable to propose that apoE might negatively regulate ACT gene by blocking the expression of transcription factors necessary to trigger the expression of this gene. Recent studies have shown that regulation of the ACT gene might be mediated by two binding elements, namely STAT1 and STAT3 (signal transducer and activator of transcription).[34] In astrocytes, oncostatin M and IL-1β are potent stimulators of ACT mRNA expression via STAT1 and STAT3 induction. IL-6 is also a potent stimulator of ACT if the appropriate receptor is present in the cell surface.[34] This suggests that up-regulation of IL-1, IL-6, or oncostatin M in apoE-deficient mice could trigger the up-regulation of ACT. However, the RPA showed that at least levels of IL-1 and IL-6 were unaltered in the apoE-deficient mice compared to controls. We did not measure levels of oncostatin M for the present study and this analysis is currently underway. ApoE might also regulate the ACT gene via induction of STAT inhibitory factors. In this regard, recent studies have shown that factors such as SOCS-1 are capable of inhibiting IL6-induced receptor phosphorylation and STAT activation.[35] The SOCS family of factors may act in a classic negative feedback loop to regulate cytokine signal transduction.[35] It is possible that while apoE might induce (through a yet unknown pathway) the production of anti-STAT factors, the absence of apoE will be followed by STAT1- and STAT3- mediated activation of the ACT gene.

Alternatively, post-transcriptional effects of apoE on ACT might be related to alterations in cholesterol levels associated with the apoE-deficient state of the mice,[13] since apoE is a well known cholesterol carrier that is produced by astrocytes within the brain.[1] In apoE-deficient mice, there is a significant increase in plasma cholesterol levels,[13] and cholesterol content in the neuronal membrane is abnormal.[36] Similarly, ACT is known to bind cholesterol as well as other hydrophobic molecules.[37] Also, serpins have been shown to bind cholesterol and other lipids.[38] This could indicate that abnormal cholesterol levels might trigger ACT up-regulation as a compensatory response to bind and transport cholesterol in the apoE-deficient mice.

Cholesterol transport in the CNS might be necessary to maintain synaptic plasticity and neuronal repair after injury or during aging.[39–41] In some models, apoE deficits are associated with neurodegeneration, abnormal repair after injury and neurophysiological deficits,[20,42–45] However, there is some variability in the results observed with these paradigms in apoE-deficient mice.[42,46,47] Then, in models such as ours where neurodegeneration is observed, an alternative explanation for the increased EB22/5 in the apoE-deficient mice might be related with astroglial response to neuronal injury.

Since previous studies have shown that higher risk for AD is associated with the presence of the allele 4 of APOE[33] and in *vitro* and *in vivo* models apoE3 appears to be more bioactive than apoE,[44,48] we seek to determine whether one isoform was more effective than the other. In this respect, it is of interest that apoE4 was less effective than apoE3 at reestablishing the baseline levels of EB22/5 expression in apoE-deficient mice. This data are in accordance with the *in vivo* studies in human brain showing significant increase in ACT cases with APOE4 alleles and with other studies showing that apoE4 was less effective that apoE3 in promoting in *vitro* neurite extension.[49,50] Furthermore, overexpression of apoE4 has deleterious effects in the CNS of transgenic mice when compared to apoE3 or non-transgenic littermates.[51] This suggests that apoE4 might represent a metabolically abnormal isoform, since in humans apoE4 showed a faster catabolism then apoE3, induced a rapid catabolism of cholesterol-enriched particles and was associated with increased plasma and LDL cholesterol concentrations.[52]

Finally, to gain more knowledge as to the potential mechanisms involved in ACT regulation by apoE, we determined the levels of EB22/5 in RAP-deficient mice and showed a persistent elevation of EB22/5 in RAP-deficient mice. ApoE is a ligand for LRP[32] and RAP is a 39-kd molecule that regulates LRP transport to the cell membrane and functional properties of this receptor.[32] In fact, RAP-deficient mice showed a reduced expression of LRP[18] associated with altered neurotransmitter expression and behavioral deficits.[22] Furthermore, LRP has been recently shown to mediate the physiological effects of apoE.[49,53,54] Taken together, these studies suggest that the effects of apoE on ACT might be mediated via LRP. However, the precise intracellular events involved are unclear since LRP has not yet been linked to an intracellular signaling pathway.

In summary, the present study showed evidence supporting a role for apoE in regulating ACT expression via LRP, which is relevant to AD since these molecules appear to be closely associated with the pathogenesis of this neurodegenerative disorder.

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