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Research Article

Apolipoprotein E genotype and alpha-tocopherol modulate amyloid precursor protein metabolism and cell cycle regulation

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Apolipoprotein E4 (apoE4) genotype is associated with an increased risk for Alzheimer's disease (AD). This is thought to be in part attributable to an impact of apoE genotype on the processing of the transmembrane amyloid precursor protein (APP) thereby contributing to amyloid beta peptide formation in apoE4 carriers, which is a primary patho-physiological feature of AD. As apoE and alpha-tocopherol (α-toc) have been shown to modulate membrane bilayer properties and hippocampal gene expression, we studied the effect of apoE genotype on APP metabolism and cell cycle regulation in response to dietary α-toc. ApoE3 and apoE4 transgenic mice were fed a diet low (VE) or high (+VE) in vitamin E (3 and 235 mg α-toc/kg diet, respectively) for 12 weeks. Cholesterol levels and membrane fluidity were not different in synaptosomal plasma membranes isolated from brains of apoE3 and apoE4 mice (–VE and +VE). Non-amyloidogenic alpha-secretase mRNA concentration and activity were significantly higher in brains of apoE3 relative to apoE4 mice irrespective of the dietary α-toc supply, while amyloidogenic beta-secretase and gamma-secretase remained unchanged. Relative mRNA concentration of cell cycle related proteins were modulated differentially by dietary α-toc supplementation in apoE3 and apoE4 mice, suggesting genotype-dependent signalling effects on cell cycle regulation.

Keywords: Alpha-tocopherol / Amyloid precursor protein / Apolipoprotein E genotype / Cell cycle / Mice

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1 Introduction

Apolipoprotein E (apoE) is a major component of circulating lipoproteins with identified roles in lipoprotein metabolism in the circulation and the central nervous system. In the brain where it is mainly produced by astrocytes and to a lesser extent by microglia and neurons [1], apoE is generally involved in the regulation of cholesterol synthesis and distribution and thus, maintenance of synaptic function. Among the three common isoforms of the apoE protein (apoE2, E3, E4), that arise from a single-nucleotide polymorphism of the apoE gene, apoE4 serves as a strong independent risk factor for developing Alzheimer's disease (AD) irrespective of the age of disease onset [2, 3]. The *apoE*4 allele frequency is shifted from 14% in a non-diseased Caucasian population to 43% in AD patients [4, 5]. AD is characterised by massive neuronal loss particular in the hippocampus and cortex. Despite intensive research, the underlying pathology of neurodegeneration has not yet been clarified, although alterations in brain oxidative status, inflammatory profile and in cell signalling pathways have been implicated [6]. Among the different preventive and therapeutic strategies dietary α-tocopherol (α-toc) supplementation has been shown in some studies to exert positive effects in the brain [7, 8]. However, in other studies α-toc failed to ameliorate clinical AD symptoms [9, 10].

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Abbreviations: **AD,** Alzheimer's disease; **ADAM10**, a disintegrine and metalloproteinase 10; **α-toc**, alpha-tocopherol; **APP**, amyloid precursor protein; **apoE**, apolipoprotein E; **ARF**, alternate reading frame; **BACE-1**, beta-site APP cleaving enzyme-1; **DPH**, 1,6-diphenylhexa-1,3,5-triene; **f**, forward; **r**, reverse; **SPM**, synaptosomal plasma membrane; **TMA-DPH**, 1-[4′-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene; **VE**, vitamin E

Although apoE4 is strongly linked to AD pathology the underlying cellular and molecular mechanisms are still unclear. Besides the proposed differences in antioxidant activity, apoE2 > apoE3 > apoE4 [11], the ability to accomplish main tasks in the brain like neuronal repair is suggested to differ among the three apoE isoforms (reviewed in [12]). The apoE4 isoform has been shown to preferentially facilitate features of AD pathology such as amyloid plaque formation [13, 14] by promoting production and deposition of amyloid beta (Aβ) peptides *in vitro* and *in vivo* [15, 16]. The Aβ peptides are proteolytic cleavage products of the neuronal transmembrane amyloid precursor protein (APP) that undergoes two alternative processing pathways, the amyloidogenic (cleavage by β-secretase prior to γ-secretase) and non-amyloidogenic (cleavage by α-secretase prior to γ-secretase) pathways [17]. Shifting the balance between α- and β-secretase activity in favour of non-amyloidogenic α-secretase has been a focus of AD therapeutic strategies [18].

In the current study, we used an apoE transgenic mouse model to study the impact of the apoE genotype in the absence of AD hallmarks to identify early features that contribute to the aetiology of AD. Therefore, we examined whether apoE genotype, dietary α-toc and their interactions may have an impact on membrane characteristics such as cholesterol levels and membrane fluidity, and APP processing, in brain areas that are of particular relevance in AD. Furthermore, as hippocampal gene expression is altered in AD patients and is affected by the apoE genotype [19], and dietary α-toc deficiency has been shown to modulate hippocampal gene expression [20], our study also examined the transcriptional regulation of AD relevant genes in apoE3 and apoE4 transgenic mice that were fed diets with either high (+VE) or low (–VE) α-toc content.

2 Materials and methods

2.1 Animals

Twenty-four female apoE3 or apoE4 gene targeted replacement mice (Taconic, Denmark) aged 6–8 weeks were randomly assigned to two diet groups of six animals each. They were housed in macrolon cages according to the German regulations of animal welfare and had free access to tap water and experimental diets. The semi-synthetic diets (Ssniff; Soest, Germany) were based on casein and cornstarch with 10% fat from tocopherol stripped corn oil. One of the diets was deficient in vitamin E (VE) with an analysed α-toc content of 3 mg/kg. 200 mg/kg of all-rac-α-tocopheryl acetate was added to the –VE diet to provide the α-toc containing diet (+VE) with a final α-toc content of 235 mg/kg. After 12 weeks the mice were killed by cervical dislocation and decapitated. The brain was removed and dissected on ice. The hippocampus was directly suspended in RNA*later*™ RNA stabilisation reagent (Qiagen, Hilden, Germany), incubated overnight at room temperature and stored at –24°C until RNA isolation. The cortex was immediately snap frozen in liquid nitrogen and stored at –80°C until analysis.

2.2 Determination of the α-toc concentration in cortex homogenates

Approximately 25 mg of cortex tissue was homogenized in 750 µL of cold phosphate buffer (50 mmol/L Na_2HPO_4, 0.5 mmol/L EDTA, 0.5% ascorbic acid) and mixed with 1200 µL of ethanol/1% ascorbate. Homogenates were vortexed for 15 s and 1500 µL of hexane was added. Following vortexing, the sample was centrifuged at 1700 × *g* at 10°C for 10 min. Subsequently, 1200 µL of the hexane phase were collected and dried under N_2 and the samples were resuspended in methanol. For the HPLC analysis, the mobile phase was methanol:water (98:2) isocratically delivered at a flow rate of 1.2 mL/min. The α-toc concentration was analysed using a Jasco HPLC system with fluorometric detection (Groß-Umstadt, Germany) on a Waters Spherisorb ODS-2 3-µm column (100 × 4.6 mm) with the detector set to an excitation wavelength of 290 nm and emission wavelength of 325 nm. The concentration was calculated by using an external α-toc (Calbiochem; Darmstadt, Germany) standard curve.

2.3 Preparation of cortical synoptosomal plasma membranes

One cortex hemisphere was minced in 7 mL of chilled buffer (10 mmol/L HEPES, 1 mmol/L EDTA, 0.32 mol/L sucrose; pH 7.4), homogenized with a glass/Teflon Potter homogenizer and centrifuged at 585 × *g* at 4°C for 10 min in a JA-20 rotor using a Beckman J2-21 centrifuge (Beckman; Krefeld, Germany). One mL of the supernatant (7 mL) was collected and centrifuged at 20 000 × *g* at 4°C for 15 min. The pellet was resuspended in 0.5 mL of PBS for measurement of secretase activities or 0.5 mL of PBS with complete1 protease inhibitor cocktail from Roche Diagnostics (Mannheim, Germany) for Western blotting. The remaining cortex homogenate supernatant was used to obtain synaptosomal plasma membranes (SPM) according to the method of Eckert and co-workers [21]. Synaptosomes were isolated using discontinuous Ficoll-sucrose gradients. The homogenate was centrifuged at 17 400 × *g* at 4°C for 20 min and the resulting pellet was suspended in 1500 µL of sucrose buffer (0.32 mol/L) and layered over a 7.5 and 14% Ficoll solutions (w/v: Ficoll/sucrose buffer) containing 0.25 mmol/L EDTA. The gradients were centrifuged in a SW28 rotor at 87 300 × *g* at 4°C for 60 min using a Beckman L8-70M ultracentrifuge. Material at the 7.5/14% interface was carefully removed with a syringe and the syringe was washed with 2 mL sucrose buffer (0.32 mol/L). Suspension was centrifuged at 17 400 × *g* at 4°C for 20 min

using a JA-20 rotor in a Beckman J2-21 centrifuge. The synaptosomal pellet was resuspended in 5 mL sucrose buffer (without EDTA) and centrifuged again as described above to ensure the removal of any remaining Ficoll. Aliquots were stored at –40°C until analysis.

2.4 Determination of membrane fluidity in SPM

Membrane fluidity was determined in SPM with different fluorescent dyes, 1,6-diphenylhexa-1,3,5-triene (DPH) and 1-[4′-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH), according to Eckert and co-workers [22]. Membrane fluidity in the hydrocarbon core of the membrane bilayer was determined using fluorescence anisotropy measurements of DPH and in the region of the hydrophilic heads of phospholipids with the more amphiphilic TMA-DPH. Fluorescence anisotropy of both dyes is inversely correlated with membrane fluidity. SPM were suspended in a 5 mmol/L TRIS-HCl-buffer (pH 7.4) to give a protein concentration of 300 μg/mL as determined according to Lowry *et al*. [23]. Each fluidity measurement reaction contained 5% membrane suspension, 50% fluorescent dye (40 μmol/L DPH or 17 μmol/L TMA-DPH) and 45% TRIS-HCl-buffer. After incubation at 37°C for 30 min (DPH) and 20 min (TMA-DPH), respectively, steady-state anisotropy was detected in an Aminco Bowman series 2 spectrofluorometer (SLM Aminco; Urbana, IL, USA) using excitation and emission wavelengths of 360 and 450 nm.

2.5 Determination of free cholesterol in SPM

Measurement of unesterified cholesterol in membrane preparations was performed based on the CHOD-PAP method (cholesteroloxidase-peroxidase-aminophenanzonphenol method) with a modified protocol from Auerbach *et al*. [24]. Absorbance measurements were performed using a microplate reader (Digiscan, Asys Hightech; Eugendorf, Austria). Results were related to the protein content.

2.6 RNA isolation and real-time qRT-PCR

Total RNA was extracted according to the RNeasy® Lipid Tissue Protocol (Qiagen). DNA digestion was done with RNase-Free DNase Set (Qiagen). The concentration of isolated RNA was determined by measuring the absorbance at 260 nm and the purity was determined by the ratio of 260/280 nm in a spectrophotometer (Beckmann Instruments; Munich, Germany). RNA aliquots were stored at –80°C until PCR analysis. Primer sequences for real-time RT-PCR experiments were designed with primer3 software. For cyclin mRNA expression analyses major isoforms were chosen. Primer pairs (Table 1) were obtained from MWG (Ebersberg, Germany). One-step quantitative RT-PCR was carried out with the QuantiTec®SYBR®Green RT-PCR kit (Qiagen). Each PCR reaction (final volume 20.0 μL) contained 0.45 μL of the respective forward and reverse primer, 22.5 μL of QuantiTect® SYBR® Green RT-PCR Master Mix, 0.45 μL QuantiTect RT-Mix, 18.0 μL of RNA dilution and 3.15 μL of water. Real-time PCR amplification was performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Relative mRNA levels of genes were quantified as the ratio between the amount of target gene and the amount of a housekeeping gene (β-actin).

2.7 Western blot experiments in cortex homogenates

The total protein content of apoE4 cortex homogenates was determined according to Lowry *et al*. [23]. The samples were mixed with NuPAGE™ LDS buffer and NuPAGE™ Sample Reducing Agent (both from Invitrogen) and loaded (10 – 15 μg protein) on electrophoreses NuPAGE™ 4 – 12% Bis – Tris Gels (Invitrogen). Protein samples were then transferred to PVDF membranes (Amersham Biosciences; Piscataway, NJ, USA), incubated with the primary p53 antibody (PAb421, Calbiochem) and a secondary antibody (Calbiochem) conjugated to horseradish peroxidase, and processed for visualization by ECL™ Reagent (Amersham Biosciences). The primary antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374, BD Bioscience; Erembodegem, Belgium) served as endogenous control. Relative intensities of the bands were quantified by densitometry (Scion Image software).

2.8 Determination of α-, β- and γ-secretase activity in cortex homogenates

Activities of secretases in cortex homogenates were assessed with α-, β- and γ-secretase activity kits from R&D according the protocol guidelines (R&D Systems; Wiesbaden-Nordenstedt, Germany). Principle of the test is the addition of secretase-specific peptides conjugated to the reporter molecules EDANS and DABCYL. Cleavage of the secretase-specific peptide by the secretase physically separates the EDANS and DABCYL allowing for the release of a fluorescent signal. The level of secretase enzymatic activity in the cortex samples is proportional to the fluorometric reaction. The amount of total protein was determined as described in the BCA kit protocol (Pierce Biotechnology; Rockford, IL, USA). Fluorescence and absorbance measurements were performed using an infinite™ F200 multifunctional reader (Tecan; Crailsheim, Germany).

2.9 Statistical analysis

Statistical calculations were conducted with SPSS Version 13.0 (SPSS; Munich, Germany). To compare different group means one-way ANOVA was performed with the LSD test or the Games-Howell test (heterogeneity of var-

Table 1. Nucleotide sequences of primers used for the real-time qRT-PCR experiments

Gene	Primer sequence *forward* (5′-3′)	Primer sequence *reverse* (5′-3′)
β-Actin	GACAGGATGCAGAAGGAGATTACT	TGATCCACATCTGCTGGAAGGT
ADAM10	CCATGCTCATGGAAGACAGTT	CCTTCTTCACCATAAATATGTCCA
BACE-1	GGAGCATGATCATTGGTGGT	ACTCCTTGCAGTCCATCTTGA
APP	CCGTTGCCTAGTTGGTGAGT	GCTCTTCTCGCTGCATGTCT
Cyclin D1	ATCAAGTGTGACCCGGACTG	ACCAGCCTCTTCCTCCACTT
Cyclin E1	TGCAGATCGCAGAGCTTCTA	TCGCACCACTGATAACCTGA
Cyclin A2	GCAATGTTTTTGGGAGAACTG	ATGACTCAGGCCAGCTCTGT
Cyclin B1	TCTTGACAACGGTGAATGGA	TGACAGTCATGTGCTTTGTGAG
$p19^{ARF}$	GCTCTGGCTTTCGTGAACAT	TCGAATCTGCACCGTAGTTG

iances), as post-hoc tests. In addition, data were analyzed by two-way ANOVA, a model that tested for apoE genotype, α-toc supply and their interactions. Data of Western blot analysis were compared with a *t*-test for independent samples. Results are expressed as means with SEM and significance was accepted at $p < 0.05$.

3 Results

3.1 α-tocopherol concentration in cortex homogenates

The α-toc concentration in the cortex of apoE3 and apoE4 transgenic mice fed with the –VE and +VE diets is given in Fig. 1. Comparable increases in tissue α-toc levels were evident in both genotypes (apoE3 and apoE4) in response to dietary α-toc supplementation. A significant increase in cortex α-tocopherol levels from 4.85 nmol/g tissue (apoE3/–VE) and 4.88 nmol/g tissue (apoE4/–VE) to 8.71 nmol/g tissue (apoE3/+VE) and 8.50 nmol/g tissue (apoE4/+VE), respectively, were detected ($p < 0.01$).

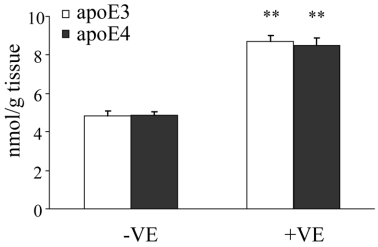

Figure 1. α-Tocopherol level in cortex homogenates from apoE3 and apoE4 transgenic mice fed either a diet with low (–VE) or high (+VE) α-tocopherol content. The α-tocopherol concentration in the cortex was determined by the RP HPLC. Values are means with SEM of six animals per group. Statistical analysis was performed with ANOVA with the LSD post hoc test. $^{**}p < 0.01$ comparing –VE and +VE diets in apoE3 and apoE4 mice.

3.2 Membrane fluidity and free cholesterol in SPM

Synaptosomal membrane fluidity assessed by DPH and TMA-DPH anisotropy was not different between apoE3 and apoE4 mice fed –VE and +VE diets. Values for DPH and TMA-DPH anisotropy averaged 0.19 and 0.22, respectively. Results for determination of free cholesterol in SPM were also not different between the four groups and varied between 490 and 518 μg/mg protein.

3.3 Differential expression of ADAM10, BACE-1, APP

Relative hippocampal mRNA expression of the α-secretase "a disintegrin and metalloproteinase 10" (ADAM10) was about 25% significantly higher in apoE3-transgenic mice compared to the apoE4 genotype ($p < 0.05$). On the other hand, no apoE genotype effects were evident on the expression of β-secretase "beta-site APP cleaving enzyme-1" (BACE-1). Differences in dietary vitamin E supply did not result in significant changes in mRNA levels of ADAM-10 and BACE-1 (Fig. 2). The mRNA expression of the amyloid precursor protein APP was not different between apoE3 and apoE4 transgenic mice and did not change in response to dietary α-toc supplementation (data not shown).

3.4 Processing of APP by α-, β- and γ-secretase

Activity levels of α-, β- and γ-secretase were measured and related to the protein content in cortex homogenates of apoE3 and apoE4 transgenic mice (Figs. 3A, B and C). Activity of α-secretase was modestly, but significantly higher in apoE3 mice compared to apoE4 mice ($p < 0.05$), but different dietary α-toc supply (–VE or +VE) exerted no effects on enzyme activity. No significant impact of genotype or α-toc supply on the activity levels of β- and γ-secretase was observed.

3.5 Differential expression of cell cycle proteins (cyclin D1, E1, A2, B1, $p19^{ARF}$, p53)

Relative hippocampal mRNA level of cell cycle proteins in apoE3 and apoE4 transgenic mice are summarized in Fig. 4. The cyclin D1 (cycD1) mRNA concentration in apoE4 mice was about 50% lower than in apoE3 mice ($p <$

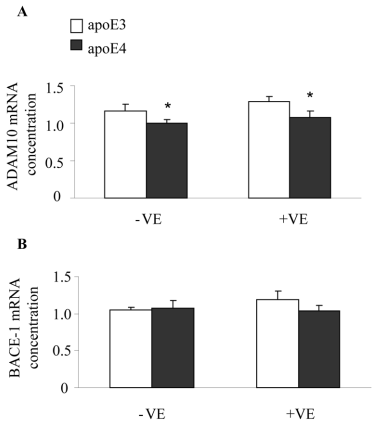

Figure 2. Expression of (A) "a disintegrin and metalloproteinase 10" (ADAM10) and (B) "β-site APP cleaving enzyme-1" (BACE-1) in apoE3 and apoE4 mice fed a diet with low (–VE) or high (+VE) α-tocopherol content. Relative mRNA levels were assessed with real-time qRT-PCR, corrected for β-actin mRNA levels. Values are means from at least five animals per group with SEM. *$p < 0.05$ stands for a genotype effect determined by two-way ANOVA.

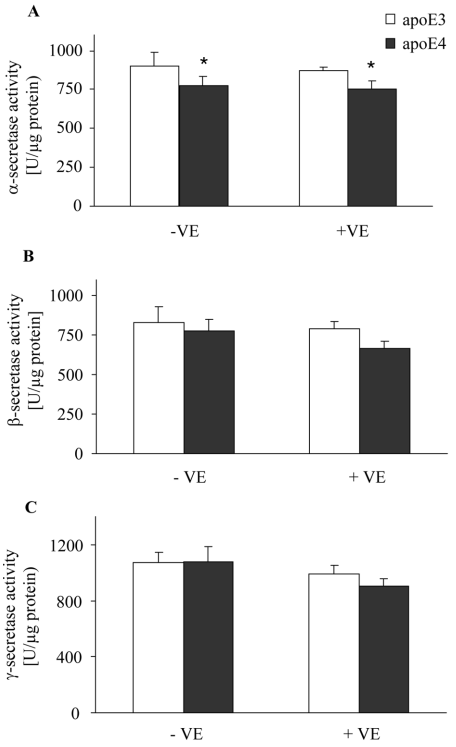

Figure 3. Activity of α-secretase (A), β-secretase (B) and γ-secretase (C) in the cortex of apoE3 and apoE4 transgenic mice fed a diet with low (–VE) or high (+VE) α-tocopherol content. Secretase activity levels were measured with specialised kits (R&D Systems) and results were corrected for protein content. Values are means from at least five animals per group with SEM. *$p < 0.05$ stands for a genotype effect determined by two-way ANOVA.

0.001). However, dietary α-toc treatment exerted no evident effect on cycD1 expression. Relative expression of cyclin E1 was similar in both apoE3 and apoE4 mice either fed the –VE or the +VE diet.

Significant interactive effects of apoE genotype and α-toc treatment were observed on relative mRNA levels of cyclin A2 (cycA2) and cyclin B1 (cycB1) ($p < 0.001$). In apoE4 mice cycA2 and cycB1 expression levels decreased ($p < 0.05$) in response to α-toc supplementation. In contrast, cycA2 and cycB1 mRNA levels in apoE3 mice increased ($p < 0.01$) in response to dietary α-toc supplementation.

The expression of $p19^{ARF}$, an inhibitor of the cell cycle, was strongly affected by α-toc supplementation in apoE4 mice with more than 50% increase in the mRNA level in apoE4/+VE mice compared to apoE4/–VE mice ($p < 0.001$). In apoE3 mice, no significant difference in the $p19^{ARF}$ expression was evident between +VE and –VE mice. However, relative mRNA expression of $p19^{ARF}$ was apparently lower in apoE4/–VE mice relative to apoE3 mice fed the –VE diet ($p < 0.01$) (Fig. 4).

Increased expression of the $p19^{ARF}$ protein may contribute to higher levels of the tumour suppressor protein p53 that can induce cell cycle arrest. Due to the rather post-translational than transcriptional regulation of p53, the protein concentration was determined in apoE4 cortex homogenates. No changes were observed between apoE4/–VE and apoE4/+VE mice (data not shown).

4 Discussion

An apoE4 genotype is associated with an increased risk for AD [2], with a relative risk (RR) of 1.76 and 4.03 reported in heterozygous (E3/E4) and homozygous (E4/E4) individuals, respectively [25]. However, underlying molecular mechanisms that contribute to a higher vulnerability are still uncertain. The present study contributes to the current understanding of the aetiology of apoE genotype-AD associations with a focus on APP metabolism and cell cycle regulation.

The apoE4 genotype is associated with higher plasma cholesterol levels [26] and impaired neuronal cholesterol efflux [27]. Amyloidogenic cleavage of APP is supposed to occur in cholesterol-rich micro domains of membranes called lipid rafts [28] and has been shown to depend on the membrane cholesterol level [29]. In the present study, total levels of unesterified cholesterol and membrane fluidity of synaptosomal plasma membranes were unaffected by the apoE genotype and dietary α-toc supply.

However, data from the current study suggest that apoE genotype may affect the APP cleavage on the transcriptional level. The mRNA concentration of the α-secretase ADAM10 were significantly lower in apoE4 relative to apoE3 transgenic mice. In accordance, cortical α-secretase activity was lower in apoE4 mice. No effect of apoE genotype or dietary VE has been observed on mRNA concentra-

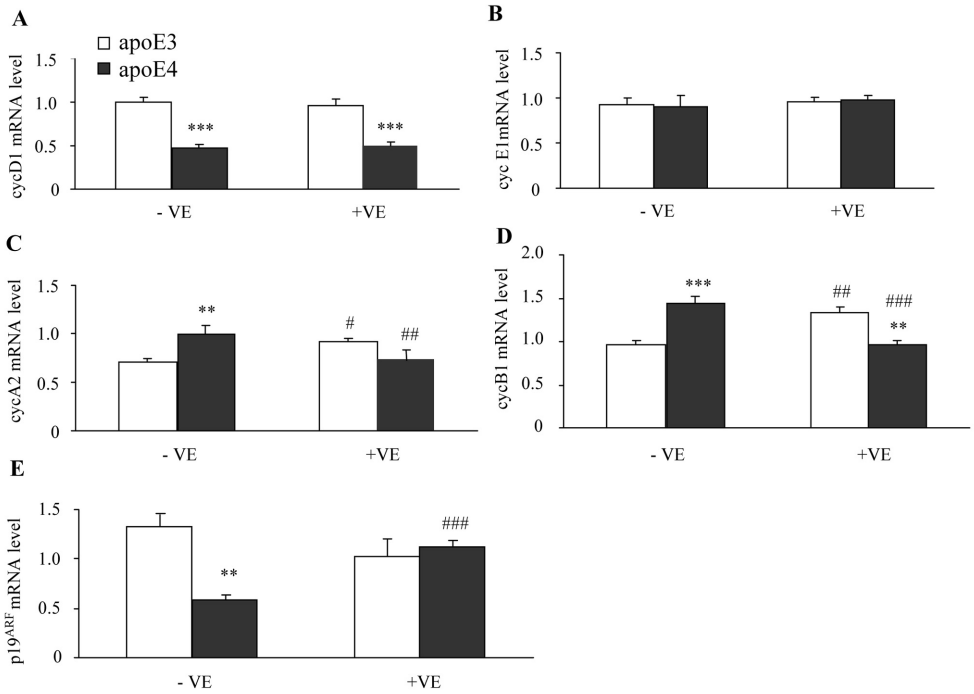

Figure 4. Relative hippocampal mRNA concentration of cell cycle proteins in apoE3 and apoE4 transgenic mice fed a diet with low (−VE) or high (+VE) α-tocopherol content. Relative mRNA concentration of cyclin D1 (A), cyclin E1 (B), cyclin A2 (C), cyclin B1 (D) and $p19^{ARF}$ (E) were assessed with real-time qRT-PCR and related to the β-actin mRNA concentration. Values are means from at least five animals per group with SEM. For statistical analysis one-way ANOVA was performed with the LSD or Games-Howell post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ comparing between apoE3 and apoE4 mice and #$p < 0.05$, ##$p < 0.01$, ###P < 0.001 between −VE and +VE diets.

tion of β-secretase BACE-1 and activities of β- and γ-secretase. Possibly, the lower α-secretase levels found in apoE4 relative to apoE3 mice contribute to the amyloidogenic pathway that has been observed to be elevated in apoE4 transgenic mice expressing APP^{V717F} [15]. However, since the apoE concentration has been shown to be lower in the hippocampus and cortex of apoE4 compared to apE3 transgenic mice [30], it cannot be ruled out that the α-secretase activity is affected by differential expression levels of the apoE protein.

During the last decade, a variety of studies has demonstrated the involvement of abnormal expression of cell cycle proteins and dysfunction of cell cycle control in AD [31–33]. However, those studies point to the cell cycle reentry of post-mitotic neurons, but no studies have been conducted on regulation of glial cell cycle with relevance to AD pathogenesis. Moreover, to the best of our knowledge, no research has been carried out on differential regulation of the brain cell cycle by apoE isoforms with respect to dietary α-toc supply. Therefore, we examined the expression of different cell cycle proteins (cyclins) in the hippocampus, a brain region that is mainly affected in AD. Due to the strong transcriptional regulation of cyclins the relative mRNA concentration was determined.

Based on lower expression of cyclins D, A and B relative to apoE3 mice we propose that the hippocampal cell cycle is possibly arrested or the number of proliferating cells is reduced in apoE4 mice. In the adult brain, only glial cells, such as astrocytes and microglia, are able to proliferate whereas terminally differentiated neurons remain to be post-mitotic. Hence, a reduced number of proliferating cells in apoE4 transgenic may be caused by a shift of the ratio between glial cells and neurons.

The mRNA concentration of cyclin A and B, cyclins of the S- and G_2-phase (DNA synthesis and preparation of mitosis), were lower in response to decreased α-toc concentrations in the brain of apoE3 mice. In contrast, lower α-toc levels were associated with higher cyclin A and B levels in the brain of apoE4 mice. These results indicate that the cell cycle may be regulated differentially in apoE3 and apoE4 mice.

One essential regulator of the cell cycle is the transformation related protein p53. Accumulation and activation of p53 lead to increased expression of numerous downstream proteins that suppress the cell cycle such as p21 [34]. The p53 protein concentration was similar between −VE and +VE apoE4 transgenic mice, but it needs to be taken into account, that (due to lack of tissue) the protein concentration was measured in another tissue (cortex) than mRNA measurements were performed (hippocampus). Nevertheless, the p21 mRNA concentration in the hippocampus was not different between apoE4/−VE and +VE mice (data not

shown). However, $p19^{ARF}$ mRNA expression, a prominent candidate upstream factor of p53, was significantly increased in apoE4 mice in response to high α-toc brain levels. The alternate reading frame (ARF), that is located within the $p16^{INK4a}$ gene (murine $p19^{ARF}$, human $p14^{ARF}$) [35], has been proposed to arrest the cell cycle without p53 action [36]. Thus, higher $p19^{ARF}$ expression may contribute to cell cycle arrest and decreased cyclin expression in apoE4 mice.

Our results suggest that the cell cycle is differentially regulated in apoE3 and apoE4 transgenic mice and that the number of proliferating cells is altered in an apoE genotype-dependent manner. However, it needs to be taken into account that in the present study the effect of apoE genotype and dietary α-toc on cyclins was only examined at the mRNA level. Thus, additional studies are required to investigate whether changes found on gene expression are also reflected at the protein and activity level.

Current data indicate that apoE genotype has an impact on the transcriptional regulation of the non-amyloidogenic cleavage of APP in the hippocampus of transgenic mice, which may contribute to apoE4's role as a risk factor for developing AD. Additionally, our study has been the first to indicate differential regulation of the cell cycle in the hippocampus of apoE3 and apoE4 transgenic mice. Further studies are required to investigate how apoE genotype affects the cell cycle in different cell types of the brain including astrocytes, microglia or even post-mitotic neurons that have re-entered the cell cycle. Differential alterations of the cell cycle may be a major contributor to apoE genotype-mediated differences in AD risk and pathology.

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