JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2007, 58, 4, 683–698
www.jpp.krakow.pl

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EFFECT OF APOE GENOTYPE AND VITAMIN E ON BIOMARKERS OF OXIDATIVE STRESS IN CULTURED NEURONAL CELLS AND THE BRAIN OF TARGETED REPLACEMENT MICE

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The aetiology of apoE4 genotype-Alzheimer's disease (AD) association are complex. The current study emphasizes the impact of apoE genotype and potential beneficial effects of vitamin E (VE) in relation to oxidative stress. Agonist induced neuronal cell death was examined 1) in the presence of conditioned media containing equal amounts of apoE3 or apoE4 obtained from stably transfected macrophages, and 2) after pretreatment with α- and γ-tocopherol, and -tocotrienol. ApoE3 and apoE4 transgenic mice were fed a diet poor or rich in VE to study the interplay of both apoE genotype and VE status, on membrane lipid peroxidation, antioxidative enzyme activity and glutathione levels in the brain. Cytotoxicity of hydrogen peroxide and glutamate was higher in neuronal cells cultured with apoE4 than apoE3 conditioned media. VE pre-treatment of neurons counteracted the cytotoxicity of a peroxide challenge but not of nitric oxide. No significant effects of apoE genotype or VE supplementation were observed on lipid peroxidation or antioxidative status in the brain of apoE3 and apoE4 mice. VE protects against oxidative insults *in vitro*, however, no differences in brain oxidative status were observed in mice. Unlike in cultured cells, apoE4 may not contribute to higher neuronal oxidative stress in the brain of young targeted replacement mice.

Key words: *apoE genotype, neurones, brain, oxidative status, lipid peroxidation, vitamin E, tocopherols and tocotrienols*

INTRODUCTION

Apolipoprotein E (apoE) is a key mediator of cholesterol distribution and cellular uptake with particular relevance to the central nervous system. Beside apolipoprotein J, apoE is the major apolipoprotein in the brain where it is mainly produced by astrocytes. To date 54 individual single nucleotide polymorphisms (SNPs) of the apoE gene located on chromosome 19 have been described (www.ncbi.nlm.nih.gov). The most widely studied of these are the common apoE epsilon SNPs, which give rise to three homozygous (E2/2, E3/3, E4/4) and three heterozygous (E2/3, E2/4, E3/4) phenotypes. The ε3 allele is most frequent (78%) in the general Caucasian population with the frequencies for ε2 and ε4 being 8% and 14%, respectively (1). The common isoform apoE3 contains cysteine and arginine at positions 112 and 158 whereas apoE2 has cysteine and apoE4 arginine at both positions in the amino acid sequence of the mature protein. The ε4 allele appears to be a major risk factor for developing Alzheimer's disease (AD) and is suggested as the only known genetic risk factor for late-onset, sporadic cases of AD (2) with an apparent gene dosage effect. The estimated onset of distribution is shifted to considerably younger ages in AD cases with ε4/4 (50% by age of onset of 66 y/o), than the ε4/3 (50% by 73 y/o) or the ε3/3 (50% by 86 y/o) (3).The prevalence of the ε4 allele has been shown to increase to around 40% in AD population (4).

Alzheimer's disease is a neurodegenerative disorder whose pathology is associated with increased oxidative stress (5). Beside detrimental effects of the apoE4 isoform on a number of factors that are linked to AD pathogenesis such as neurite outgrowth, cytoskeletal stabilization, Aβ clearance (for review see ref. 6), the apoE4 genotype contributes to higher vulnerability towards oxidative insults among AD patients (7). This effect may be attributable to the lower antioxidative capacity of apoE4 relative to apoE3 and E2 found in *in vitro* assays (8). Furthermore, higher hydroxyl radical levels (9), greater nitric oxide (NO) production (10) and higher levels of lipid peroxidation in frontal cortex (1) have been observed in AD patients with an apoE4 phenotype relative to non-E4 carriers.

Dietary vitamin E (VE) supplementation has been shown to delay the progression of AD and improve cognitive functions (11-13). For this reason it was suggested that apoE4 carriers may benefit from a dietary antioxidant supplementation particularly with VE (14). In the current study, we examine the impact of apoE genotype on neurotoxicity and investigate the potential neuroprotective effects of tocopherols and tocotrienols on neuronal cells *in vitro*. Furthermore, the interplay of apoE genotype and dietary VE supplementation is studied in an oxidative stress relevant mouse model.

MATERIALS AND METHODS

Cell culture

Murine neuroblastoma cells (Neuro-2a) and human neuroblastoma cells (SH-SY5Y) were purchased from DSMZ (Braunschweig, Germany). Cell culture media, foetal bovine serum, L-

glutamine, MEM non essential amino acids (NEAA), MEM vitamins, sodium pyruvate, PBS, HEPES, trypsin/EDTA and penicillin/streptomycin were obtained from PAA Laboratories (Coelbe, Germany). Murine neuroblastoma cells (Neuro-2a) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 4 mmol/l L-glutamine, and 100 U/ml penicillin and 100 µg/ml streptomycin. SH-SY5Y cells were cultured in RPMI 1640 medium with 5% FBS, 2 mmol/l L-glutamine, 1x MEM NEAA, 1 mmol/l sodium pyruvate, 1x MEM vitamins and 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified incubator at 37 °C and 5% CO_2.

Determination of cell viability

Cell viability was measured by the Neutral Red assay (15) with slight modifications. After 3 h of incubation with the dye, cell viability (Neutral Red uptake) was assessed by reading absorbance at 540 nm (Labsystems iEMS Reader; Labsystems, Finland). Results were expressed (as absorbance observed) as percentage of control cultures (untreated cells). Hydrogen peroxide (H_2O_2) and Neutral Red dye were purchased from Carl Roth (Karlsruhe, Germany), buthionine sulfoximine (BSO), glutamate and diethylenetriamine nitric oxide adduct (DETA/NO) were from Sigma (Taufkirchen, Germany) and tert-butyl hydroperoxide (tBHP) from Acros Organics (New Jersey, USA). Tocopherols and tocotrienols were obtained from BASF (Ludwigshafen, Germany).

Experimental series 1: effect of the apoE isoform on agonist induced neuronal cell death

Neuro-2a cells were used to test apoE isoform specific effects from murine conditioned media on agonist induced neuronal death. A murine cell line was employed to avoid interference with native apoE isoform background from human neuronal cells. ApoE conditioned media was obtained from murine RAW 264.7 cells stably transfected with either apoE3 or apoE4 (16). Neuro-2a cells were seeded at 5 x 10^5 cells/well in 12-well-plates and grown to 80% confluence for 24 h before apoE containing cell culture supernatant from RAW 264.7-E3 or -E4 monocytes was added. ApoE concentration varied from 0.26 - 0.60 µg/ml and was adjusted by adding normal medium so that both isoform conditioned media contained the same apoE concentration. Preliminary studies showed that varying apoE concentrations did not affect neuronal cell viability. Neuro-2a cells were cultured in apoE3 or apoE4 conditioned media for 24 h prior to treatment with agonists (at >95% confluence). For induction of cell death oxidative agonists (H_2O_2 and glutamate) were chosen, and to gain further insights into mode of action, additional exposure to BSO was used to deplete intracellular glutathione levels. Cytotoxicity of H_2O_2 (incubation for 3 h), glutamate (incubation for 24 h) and 2 mmol/l BSO (pre-incubation for 24 h) with subsequent application of H_2O_2 (incubation for 3 h), was assessed at two concentrations in the presence of apoE.

Experimental series 2: effect of tocopherols and tocotrienols on agonist induced neuronal cell death

In the second experimental series beneficial effects of vitamin E pre-treatment on neuronal vulnerability towards agonists that have particular relevance to AD were examined. Due to different sensitivities of murine neuroblastoma cells to tocopherols and tocotrienols a human neuroblastoma cell line (SH-SY5Y) was used. SY5Y cells were grown to confluence in medium with 5% FBS and seeded in 12-well-plates (2% FBS). Twenty-four hours after seeding (80% confluence) cells were incubated with either α- or γ-tocopherol or α- or γ-tocotrienol at a final concentration of 25 µmol/l. At this concentration the test substances showed no effect on viability of SH-SY5Y cells (data not

shown). Subsequent to tocopherol or tocotrienol pre-treatment the medium was removed and fresh agonist containing medium was applied. Cell death was induced by exposure to oxidative agonists a) that induce lipid peroxidation, tBHP (3 h of exposure), b) that release NO to induce nitrosative stress, DETA/NO (24 h of exposure), c) with prior depletion of intracellular glutathione, BSO (24 h of pre-treatment), combined with H_2O_2 (3 h of exposure). The concentrations of the agonists were chosen to induce moderate cell death of 40-60%.

Experimental series 3: apoE3 and apoE4 transgenic mice

Mice and diets

Twenty-four female apoE3 or apoE4 gene targeted replacement mice (Taconic Ltd., Ry Denmark) aged 6-8 weeks were randomly assigned to 2 diet groups of 6 animals each. The mice were housed in macrolon cages according to the German regulations of animal welfare. The animals had free access to tap water and the experimental diets. The semi-synthetic (ssniff; Soest, Germany) diets were based on casein and corn starch with 10% fat from tocopherol stripped corn oil. The first diet was poor in vitamin E (-VE) (analyzed α-tocopherol content: 3 mg/kg). For the VE containing diet (+VE) 200 mg all-rac-α-tocopheryl acetate was added per kg diet (analyzed content: 235 mg/kg). After 12 weeks the mice were killed by cervical dislocation and the cortical neurons were extracted for determination of lipid peroxidation. The remaining brain tissue (excluding the hippocampus that was used for further analyses, data not shown) was dissected and stored at -80 °C.

Determination of a-tocopherol concentration in mouse brain homogenates

Approximately 100 mg of brain tissue (excluding cortex and hippocampus) was homogenized in 1500 µL of cold phosphate buffer (50 mmol/l Na_2HPO_4, 0.5 mmol/l EDTA, 0.5% ascorbic acid) and mixed with 200 µL of ethanol/1% ascorbate. Homogenates were vortexed for 15 s and 2000 µL of hexane was added. Homogenates were vortexed again and centrifuged at 1700x g and 10 °C for 10 min, with 1200 µL of the hexane phase collected and dried under N_2. The samples were resuspended in methanol (0.005% BHT). For the HPLC analysis, the mobile phase was methanol:water (98:2) isocratically delivered at a flow rate of 1.2 ml/min The tocopherol content was analysed using a Jasco HPLC system on a Waters Spherisorb ODS-2 3 µm column (100x 4.6 mm) with the fluorescence detector set to an excitation wavelength of 290 nm and emission wavelength of 325 nm and the concentration was calculated by the use of an external standard curve.

Antioxidant enzyme activities in brain homogenates (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR)) and glutathione (GSH) level

Brain tissue (excluding cortex and hippocampus) was diluted 1:5 in cold PBS and homogenized. The homogenates were subsequently centrifuged at 3500x g and 4 °C for 10 min, and the supernatants were collected and used for the measurement after appropriate dilution in PBS. The SOD enzyme activity assay was based on the method of Marklund *et al*. (17) using inhibition of autoxidation of pyrogallol by SOD as an index of enzyme activity. CAT enzyme activity was assessed according to a previously described method (18) with the use of Purpald® (Sigma). An external CAT standard curve (CAT from *Aspergillus niger*, Sigma) was applied to calculate activity in the homogenates. Methods for GR and Se-dependent GPx activity measurement (19, 20) were based on oxidation of reduced glutathione (GSH) and reduction of oxidised glutathione disulfide (GSSG) associated with $NADPH/H^+$ utilization which was determined by decrease of absorption. GR from baker's yeast and bovine GPx from erythrocytes,

(Sigma) were used to generate standard curves. GSH levels were measured in homogenates according to Griffith *et al*. (21) whereby GSH is oxidised by 5,5`-dithiobis-(2-dinitrobenzoic acid) and absorbance of 2-nitro-5-thiobenzoic acid is assessed. All measurements were carried out on the DU 800 spectrophotometer (Beckman Coulter; Krefeld, Germany); with the exception of CAT activity that was measured on the multifunctional Infinite F200 plate reader (Tecan; Crailsheim, Germany). Results for antioxidant enzyme activities and GSH concentration were adjusted for sample protein content assessed by the BCA kit (Pierce; Illinois, USA).

Preparation of dissociated mouse cortical cell aggregates

We examined the extent of *ex vivo* lipid peroxidation in the cortex, a vulnerable brain area with particular relevance to AD. Lipid peroxidation is supposed to be a contributing factor to neurodegeneration. On that account cortical cells were dissociated by a previously published method (22) with minor modifications. After decapitation one half of the cortex hemisphere was minced immediately in ice cold phosphate buffer. Tissue was dissociated by repeatedly pipetting up and down prior to filtering the cell suspension through a nylon mesh (100 µm pore diameter, BD biosciences; Heidelberg, Germany). Dissociated cell aggregates were washed twice and centrifuged at 500x g and 4 °C for 3 min. Finally, cells were resuspended in DMEM with 10% FBS and 20 mmol/l HEPES and cultured for no longer than 2 h until the determination of lipid peroxidation.

Determination of lipid peroxidation by oxidation of C11-Bodipy$^{581/591}$

Membranous lipid peroxidation was assessed *ex vivo* by fluorescence of C11-Bodipy$^{581/591}$ (Molecular Probes, Invitrogen; Karlsruhe, Germany) which is a lipophilic substance that incorporates into membranes (23). For this purpose, dissociated cortical cell aggregates were incubated with 10 µmol/l C11-Bodipy$^{581/591}$ of the dye for 30 min. The process of oxidation of C11-Bodipy$^{581/591}$ is comparable to polyunsaturated fatty acids and therefore serves as an index of the extent of lipid peroxidation of membrane polyunsaturated fatty acids. Since fluorescence of the oxidised (green) and reduced (red) form of C11-Bodipy$^{581/591}$ was assessed at different wavelengths a ratio of oxidised to total fluorescence was calculated. C11-Bodipy$^{581/591}$ oxidation was measured under basal conditions and after oxidative stimulation by addition of 80 µmol/l cumene hydroperoxide (CumOOH) and 80 nmol/l hemin for 1 h. A multifunctional reader (GENios Pro, Tecan) was used to measure green (485/535 nm) and red (535/590 nm) fluorescence intensity.

Statistical analysis

Statistical calculations were conducted with SPSS Version 13.0 (Munich, Germany). T-Tests for independent samples (n = 6 - 8) were performed to compare the outcomes of experimental series 1. One-way analysis of variance (ANOVA) with a one-sided Dunnett-test (>control) as the post-hoc analysis was performed to compare outcomes of experimental series 2 (n = 6 - 8). In the absence of normally distributed data the non parametric Mann-Whitney-U test was conducted. The data derived from the apoE transgenic mouse model were analyzed by two-way ANOVA, a model that tested for independent impact of apoE genotype and VE supplementation, plus apoE-vitamin E interactions. In addition, one-way ANOVA was applied to test for treatment of differences among the four groups (n = 6) followed by the post hoc-test Scheffé (homogenous variances) and Games-Howell (heterogeneous variances), respectively. Results are expressed as means with SEM and significance was accepted at $P<0.05$.

RESULTS

Experimental series 1: effect of the apoE isoform on the viability of agonist treated neuronal cells

The cytotoxicity of H_2O_2 was significantly ($P<0.05$) affected by the apoE isoform. Neuro-2a cells cultured in apoE4 conditioned medium had lower viability levels (14.8%) at 250 µmol/l H_2O_2 than cells cultured in apoE3 medium (22.5%) (*Fig. 1A*). After pre-treatment with BSO, a known inhibitor of glutathione synthesis, the cytotoxicity of H_2O_2 was lower due to decreased intracellular antioxidant defence capacity. However, no differences in vulnerability were evident between cells cultured in apoE3 and apoE4 conditioned medium (*Fig. 1B*). Besides, Neuro-2a cells cultured in apoE4 medium were more susceptible towards glutamate induced cytotoxicity (*Fig. 1C*) than neurons cultured in apoE3 conditioned medium. Significant differences were observed at 10 mmol/l glutamate ($P<0.05$).

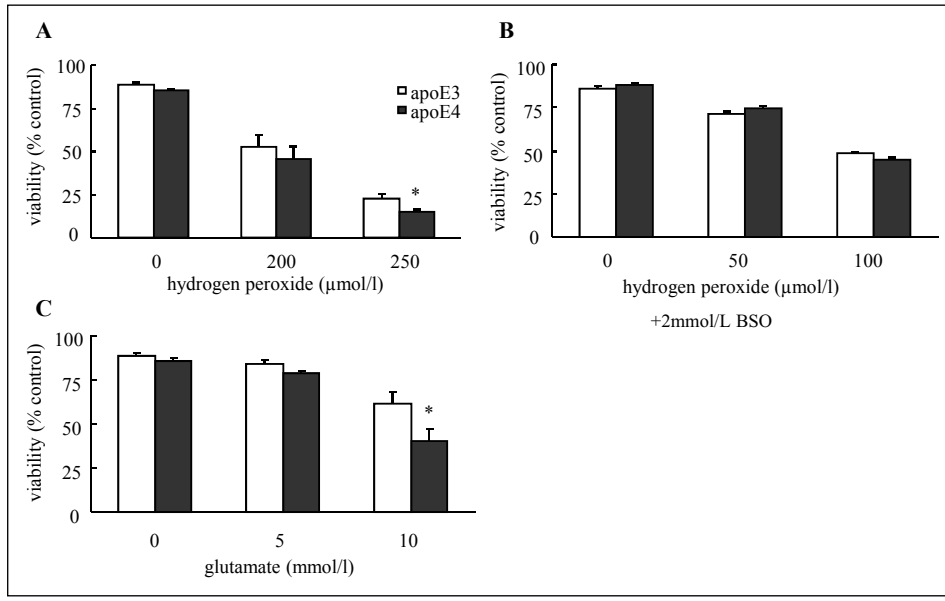

Fig. 1. Effect of the apoE isoform on agonist induced cell death of Neuro-2a cells. Neuro-2a cells were cultured in apoE3 or apoE4 conditioned media for 24 h. Cell death was induced either by incubation with hydrogen peroxide (1A), hydrogen peroxide following pre-incubation with 2 mmol/l BSO (1B), or by glutamate (1C). Viability of cells was determined with the Neutral Red assay and data were expressed as % viability of control (cells cultured in non-conditioned medium without agonists). Values are means of at least 3 independent experiments with SEM. ($n \geq 6$). The *t-test* for independent samples was performed to compare outcomes between cells cultured in apoE3 and apoE4 conditioned media. $P<0.05$ (*)

Experimental series 2: effects of tocopherols and tocotrienols on neuronal agonist induced cell death

Pre-treatment of SH-SY5Y neuronal cells with tocopherols and tocotrienols significantly ($P<0.001$) counteracted the cytotoxicity of tBHP. The highest protection was achieved with α-tocotrienol which completely prevented tBHP induced cell death (*Table 1*). VE also protected against BSO/H_2O_2 induced cell death, with a significant difference compared to BSO/H_2O_2 alone reached by pre-incubation with γ-tocopherol and α-tocotrienol ($P<0.05$). Lower but nevertheles significant protection rates were evident in the case of α-tocopherol pre-incubated cells. Neither tocopherol nor tocotrienol pre-treatment attenuated the cytotoxicity induced by DETA/NO.

Experimental series 3: apoE3 and apoE4 transgenic mice

Concentration of a-tocopherol in brain homogenates

The levels of α-tocopherol were determined in brain homogenates of apoE3 and apoE4 transgenic mice (*Fig. 2*). When mice were fed with a diet poor in vitamin E (-VE) α-tocopherol concentrations of 2.9 nmol/g tissue (apoE3) and 3.1 nmol/ g tissue (apoE4) were found in the brain. Approximately 2-fold higher concentrations were observed in response to dietary α-tocopherol supplementation (+VE) ($P<0.05$). Although there was a trend towards post-supplementation higher α-tocopherol concentrations in apoE3 (6.4 nmol /g tissue) than in apoE4 mice (5.7 nmol/ g tissue), no statistical difference was found between the two genotypes.

Table 1. Neuroprotective effects of α-/γ-tocopherol and α-/γ-tocotrienol pre-treatment on susceptibility of SY5Y cells towards agonist induced cell death. Cells were pre-incubated with VE at 25 µmol/l for 24 h. After removal of tocopherol or tocotrienol containing media different agonists were applicated for 3 h (tBHP, H_2O_2) or 24 h (BSO, DETA/NO). Following, viability was assessed with the Neutral Red assay. Data is expressed as % absorbance of control (cells cultured in VE free medium, with no agonist added). Values are means with SEM of at least 3 independent experiments in duplicate (n=6-8). Differences between different VE pre-treatments in a row were significant at $P<0.05$ (*), $P<0.001$ (***)

Treatment		Viability (% of untreated cells) no vitamin E	α-tocopherol	γ-tocopherol	α-tocotrienol	γ-tocotrienol
no agonist	mean	100	97	110	109	101
	SEM		*2.2*	*2.1*	*1.5*	*2.6*
TBHP	mean	54	87***	86***	100***	79***
	SEM	*1.7*	*1.8*	*1.4*	*1.7*	*1.1*
BSO/H_2O_2	mean	41	62*	76***	74***	51
	SEM	*2.3*	*2.5*	*1.6*	*1.8*	*1.9*
DETA/NO	mean	49	50	57	63	55
	SEM	*1.4*	*1.6*	*1.8*	*2.1*	*1.9*

Effect of vitamin E and apoE genotype on SOD, CAT, GPx, GR and GSH in mice brains

The effects of VE and apoE genotype on antioxidant enzyme activity and glutathione levels in the brain of mice are summarized in *Table 2*. Superoxide dismutase, glutathione peroxidase and glutathione reductase activity as well as glutathione levels were similar between the four groups. Although a >2-fold higher catalase activity was evident in apoE3 *vs*. apoE4 mice the differences did not reach statistical significance, attributable to the relatively high inter-individual variations.

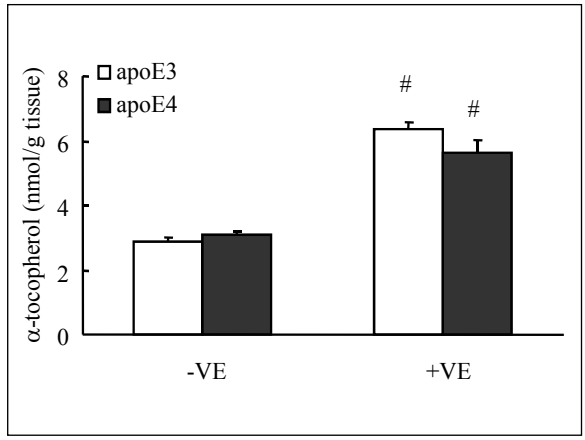

Fig. 2. Concentration of α-tocopherol in brain homogenates of apoE3 and apoE4 mice fed diets with low vitamin E (-VE) content or enriched with a-tocopherol (+VE). After 12 weeks mice were killed by cervical dislocation, the cortex and hippocampus were removed, and the remaining tissue was homogenized and α-tocopherol concentration determined by reversed phase HPLC. Values are means with SEM of 6 animals per group. Differences between -VE and +VE groups were significant at $P<0.05$ ($^{\#}$).

Table 2. Antioxidant enzyme activities and glutathione levels in brain homogenates of apoE3 and apoE4 transgenic mice fed with a diet poor in vitamin E (-VE) or enriched with α-tocopherol (+VE).

Group		Superoxide dismutase [U/mg protein]	Catalase [U/mg protein]	Glutathione reductase [mU/mg protein]	Glutathione peroxidase [mU/mg protein]	Glutathione [nmol/mg protein]
apoE3/ -VE	mean	17.1	57.8	50.8	133.8	29.1
	SEM	*0.7*	*13.0*	*3.7*	*2.7*	*0.6*
apoE3/ +VE	mean	20.9	68.3	51.7	147.2	29.6
	SEM	*1.0*	*11.0*	*7.2*	*5.2*	*1.1*
apoE4/ -VE	mean	18.1	24.6	47.8	131.4	27.0
	SEM	*0.6*	*4.0*	*6.1*	*2.3*	*0.8*
apoE4/ +VE	mean	18.5	29.8	51.5	132.8	28.6
	SEM	*0.5*	*6.3*	*7.5*	*3.7*	*0.5*

No statistical differences were observed with two-way ANOVA testing for impact of apoE genotype, VE supplementation and apoE-vitamin E interactions.

Effect of vitamin E and apoE genotype on lipid peroxidation of dissociated cortical cells

Under baseline conditions the lipid oxidation rate (oxidized/total fluorescence of C11-Bodipy$^{581/591}$, measured as biomarker of lipid peroxidation in the brain) in dissociated cortical cells was similar in apoE3 and apoE4 mice (*Fig. 3*). The oxidation rate increased in all groups after oxidative stimulation with CumOOH/hemin, with statistical significance reached for apoE3/+VE mice ($P<0.05$). No statistically significant effect of dietary VE supplementation on lipid peroxidation was evident in apoE4 mice, however, apoE3/+VE mice exhibited slightly lower (17%) oxidation rates after stimulation than apoE3/-VE mice. Furthermore, apoE3 mice exhibited 26% lower levels of lipid peroxidation after oxidative stimulation compared to apoE4 mice when fed the +VE diet. However, these differences did not reach statistical significance.

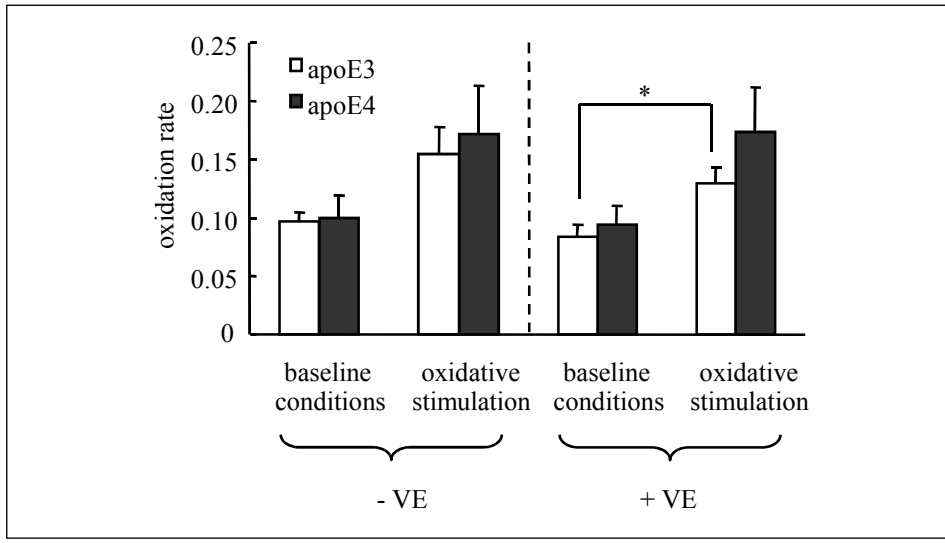

Fig. 3. Comparison of C11-BODIPY $^{581/591}$ dye oxidation in dissociated cortical neurons of apoE3 and apoE4 transgenic mice fed with either a diet poor in vitamin E (-VE) or enriched with α-tocopherol (+VE). Dissociated cell aggregates were labelled with C11-BODIPY $^{581/591}$ and oxidation induced by addition of cumene hydroperoxide (CumOOH) (80 µmol/l) and hemin (80 nmol/l) for 1 h at 37 °C. Baseline cells were incubated with PBS subsequent to incubation with C11-BODIPY $^{581/591}$. Fluorescence intensity was measured at green (485/520 nm) and red (544/590 nm). Oxidation of C11-BODIPY $^{581/591}$ is expressed as the ratio between green fluorescence (oxidised) and total fluorescence (oxidised plus reduced). Data is expressed as mean with SEM of 6 animals per group. The T-Test for independent samples was performed to compare outcomes between baseline conditions and oxidative stimulation, $P<0.05$ (*).

DISCUSSION

ApoE4 is associated with both early and late onset AD (3, 24). Although underlying mechanisms remain unclear, currently available data is suggestive of a lower antioxidative activity of apoE4 compared to apoE3 and apoE2 proteins. This may result in lower protection of susceptible neurons towards oxidative stress (8) and may be in part responsible for the disease differentials. Indeed, there is increasing evidence suggesting that oxidative damage is elevated in AD (25-27). The approach of this study was to examine whether the apoE isoform has an impact on neuronal vulnerability towards AD relevant oxidative agonists. On the other hand, we chose an apoE transgenic mouse model to study the genotype effects on oxidative status in healthy mice with adequate VE supply (+VE) compared to mice that are rather vulnerable to oxidative stress (with insufficient VE supply, -VE).

ApoE conditioned medium was used in order to investigate the effects of the human apoE3 and apoE4 isoform on agonist induced neuronal cell death. Conditioned medium was obtained from stably transfected macrophages that secrete either apoE3 or apoE4 at physiological concentrations. Furthermore, the concentration of ApoE secreted was identical between apoE3 and apoE4 macrophages (16). Macrophages are a comparable cell type to microglia (28), which secrete apoE in the brain (29). For the first time, glutamate induced cell death, along with hydrogen peroxide and BSO cytotoxicity, was systematically studied in the presence of apoE3 or apoE4 conditioned media.

Importantly, our results clearly demonstrate that apoE4 conditioned medium exhibits less protection against hydrogen peroxide or glutamate induced cell death compared to apoE3. Cells detoxify hydrogen peroxide through conversion by catalase or coupling its reduction with oxidation of glutathione by glutathione peroxidase. Furthermore, hydrogen peroxide is suggested to increase extracellular concentrations of glutamate (30). Thus, the toxicity of hydrogen peroxide is at least partly mediated by secondary excitotoxic damage. Excitotoxic concentrations of the neurotransmitter glutamate lead to stimulation of N-methyl-D-aspartate (NMDA) receptor activity resulting in an increase of intracellular cation (such as Ca^{2+}) levels, and finally to mitochondrial dysfunction (30). ApoE has been shown to decrease the activation of the NMDA receptor thereby reducing glutamate toxicity (31). Qiuand and co-workers (2003) found that recombinant apoE4 but not apoE3 increased the calcium response to NMDA receptor activation and associated neurotoxicity (32), which is in line with our results for apoE4 conditioned media.

It has been shown that both glutamate and hydrogen peroxide cause a decrease in cellular glutathione levels and an accumulation of intracellular peroxides (33). When the intracellular glutathione pool is depleted by inhibition of *de novo* synthesis due to BSO, no apoE isoform dependent differences in cell viability after agonist (H_2O_2) treatment were observed. These data may indicate that glutathione is involved in the apoE isoform dependent effect on neuronal

susceptibility towards agonist induced cell death and that apoE4 conditioned media may consume more glutathione than apoE3.

Taken together, our results demonstrate that relative to the apoE3 isoform apoE4 exhibits less protection of neuronal cells against agonists that are of relevance in the pathogenesis of AD.

VE is considered the most important lipid soluble antioxidant in the body and has been shown to protect neuronal cells against agonist induced cell death *in vitro* (34, 35). So far, neuroprotective effects have been mostly studied by co-incubation of VE and agonists in the cell culture media. In contrast, our experimental approach was to examine whether protection can be also achieved by pre-treatment of neuronal cells with VE prior to the induction of oxidative and nitrosative stress. This is of particular interest as neuroprotective properties of VE may not only be attributable to primary antioxidative action but may also be mediated through other cellular functions. The concentration of vitamin E chosen (25 μmol/l) in the cell culture media is comparable to the level of a-tocopherol present in human plasma that can be physiologically achieved via diet (36). For comparison of the neuroprotective action all vitamers were tested at the same concentration although physiological levels of tocotrienols and γ-tocopherol are much smaller (37).

In the current study, the most potent protection by tocopherol and tocotrienol pre-treatment of SH-SY5Y cells was observed when cell death was induced by tBHP, which has been shown to induce marked increase in lipid peroxidation (38). Since VE is a component of lipid membranes and a very potent scavenger of reactive oxygen species and chain breaker, it is reasonable that tocopherols and tocotrienols could counteract tBHP cytotoxicity by prevention of membrane oxidation. In the current study, VE pre-treatment attenuated cytotoxicity of BSO/H_2O_2 with γ-tocopherol and α-tocotrienol being most effective; α-tocopherol prevented BSO/H_2O_2 induced cell death to a lesser extent. This may be at least partly due to the higher cellular accumulation of γ-tocopherol and α-tocotrienol, as previous studies have shown that these isoforms are taken up to a much higher extent than α-tocopherol in cultured cells (39, 40). Reduction of BSO/H_2O_2 cytotoxicity through VE pre-treatment indicates that neuroprotective effects of tocopherols and tocotrienols are possibly mediated by cellular effects which may include modulation of glutathione metabolism.

DETA/NO is an agonist that continuously releases small amounts of NO and maintains low NO concentrations in the incubation medium over 24 h (41). Although γ-tocopherol has been proposed as an efficient trap for nitrogen oxide species (42), DETA/NO induced cell death was not prevented by pre-treatment of neuronal cells with γ-tocopherol or any other vitamer in the current study. It could be speculated that the pre-treatment with VE led to a cellular accumulation of tocopherols and tocotrienols differing from the localization of NO (that was released subsequently by incubation of cells with DETA/NO). Due to dissimilar localization VE may have possibly failed to counteract NO cytotoxicity in cultured neuronal cells.

Overall, our data show that VE pre-treatment of neuronal cells is capable of reducing oxidative insult induced cell death *in vitro*, and particularly against an agonist that results in lipid peroxidation.

When the response to all three agonists applied is considered together, α-tocotrienol emerged as the most potent neuroprotective form of VE, which is in line with previous findings, when VE was incubated simultaneously with agonists (43). However, it needs to be taken into account that *in vivo*, hepatic α-tocopherol preferentially secretes α-tocopherol into VLDL for transport to the peripheral tissue, including the brain. Furthermore hepatic tocopherol-ω-hydroxylase, which catalyzes the initial step of VE degradation has preferential affinities for non-α-vitamers (37, 44). For these reasons α-tocopherol concentration in blood and most other tissues is several times higher than that of other tocopherols and tocotrienols, which will increase its relative antioxidant importance in the whole body setting.

To the best of our knowledge, the interactions of dietary VE and apoE genotype on brain function have not been systematically investigated to date. Furthermore, in the current study the effects of the apoE4 genotype on oxidative status were examined in the absence of disease patterns. We studied the effect of low in comparison to high dietary α-tocopherol supply on lipid peroxidation and antioxidative enzyme activity levels in the brain of apoE3 and E4 mice. Levels of α-tocopherol in the brain of apoE3 and apoE4 transgenic mice fed the diet poor in VE were approximately half of those mice fed the VE supplemented diet. Nevertheless, results of lipid oxidation of dissociated neurons were similar in both VE treatment groups. Hence, in our study dietary VE deficiency was possibly insufficient to cause significant oxidative stress in the brain which is perhaps due to the relatively short, 12 week duration of the feeding trial.

In contrast to current findings which failed to demonstrate an evident association between apoE genotype and the extent of lipid peroxidation, apoE4 phenotype has been reported to be linked with increased lipid peroxidation in the brain in humans (45, 46). However, these observations were made in patients that suffer from AD, with no data available on the lipid peroxidation levels in brains of healthy apoE3 *vs*. apoE4 carriers, which could have been compared to the data of the present study. In any case, small (non-significant) trends towards higher vulnerability to lipid peroxidation in apoE4 relative to apoE3 mice were found after oxidative stimulation with CummOOH/hemin. This observation may be comparable to apoE genotype effects in humans in disease states (45).

Interestingly, in our study no significant associations were observed between apoE genotype or VE supplementation on antioxidant enzyme activity and glutathione in the mouse brain. On the other hand, differences in catalase activity are rather apparent between apoE3 and apoE4 mice although statistical significance has not been reached due to high inter-individual variations. Ramasammy and co-workers reported an association between apoE4 phenotype and decreased levels of antioxidant enzyme activity and glutathione levels in AD patients (1); however these results were not consistent with other groups (9, 47).

Again, no studies have been conducted on antioxidative enzyme activities in brains of healthy and young apoE3 homozygotes versus apoE4 carriers. It is likely that, since oxidative stress accumulates with age, feeding older mice a VE deficient diet would be more likely to observe differences in markers of antioxidative status between apoE3 and apoE4 genotypes.

Moreover, it needs to be taken into account that the duration of the experimental trial was relatively short (12 weeks). For example, changes in differential gene expression in rat hippocampus were observed after long-term VE deficiency (9 months) (48), whereas differential gene expression in rat liver was not significantly altered within 7 weeks of dietary VE deficiency (49, 50). Therefore, further studies should be carried out to test the hypothesis whether apoE genotype, impacts on responsiveness of oxidative stress markers, including antioxidant enzymes, to low VE intakes over several months.

In conclusion, the apoE4 allele contributes to higher neuronal susceptibility towards oxidative damage compared to the apoE3 isoform, as indicated by our *in vitro* data. These data significantly contribute to the current understanding of the aetiology of apoE genotype-AD associations. By contrast, results of our mouse model do not show apoE genotype dependent effects on oxidative damage or antioxidative enzymes under the experimental conditions investigated. Further studies are merited which investigate the impact of apoE genotype on oxidative status in the brain in older animals, and examine the impact of diet to manipulate the apoE genotype-oxidative status association, using longer supplementation periods.

Acknowledgments: G. R. is supported by a grant from the International Foundation for Nutrition Research and Education (ISFE). This work was further funded by a grant from the German Ministry of Education and Science (BMBF 0313856A).

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Received: April 17, 2007
Accepted: June 26, 2007

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