

Aus dem Institut für Humanernährung und Lebensmittelkunde
der Christian-Albrechts-Universität zu Kiel

**EFFECTS OF DIETARY VITAMIN E FROM RAPESEED OIL ON GENE
EXPRESSION IN BRAIN AND LIVER OF RATS**

DISSERTATION

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SUMMARY

Epidemiological studies associated the intake of vitamin E-rich foods, but not of vitamin E supplements, with significant risk reductions for neurodegenerative disorders, including Alzheimer's disease (AD), and other lifestyle related diseases. The underlying mechanisms were suggested to include the reduction of oxidative stress as well as the modulation of the expression of disease-relevant genes by the antioxidant. Rapeseed oil is a particularly rich source of natural vitamin E (VE) and considered to be of great nutritional value because of its high content of monounsaturated fatty acids and the polyunsaturated ω -3 fatty acid α -linolenic acid.

The present thesis aimed at studying the effects of graded dietary concentrations of VE from the natural source rapeseed oil on the occurrence of oxidative stress and on gene regulation in brain and liver of Fisher 344 rats during growth and maturation. To this end, three groups of 8 male rats each were fed diets with deficient (<1 mg α -tocopherol equivalents (α TE)/kg diet), marginal (9 mg α TE/kg diet) or sufficient (18 mg α TE/kg diet) concentrations of VE for 6 months; a fourth group was fed the VE sufficient diet fortified with natural α T (total VE, 146 mg α TE/kg diet).

Feeding of the experimental diets dose-dependently altered α T concentrations in liver, cortex, and plasma. The responsiveness of the tissues for α T-depletion or -enrichment differed significantly. Dietary VE deficiency for 6 months almost completely depleted α T in the liver, but not in the brain. In agreement, no changes in the oxidative stress markers F_2 -isoprostanes and heme oxygenase-1 (HO-1) mRNA, and in activities of antioxidative enzymes and concentrations of glutathione were observed in the brains of VE-depleted rats. Furthermore, no differences in the expression of AD-relevant genes were observed between groups. In the liver of VE-depleted rats, compared to rats fed VE-sufficient diets, HO-1 mRNA and protein concentrations were significantly increased and selected VE-regulated genes were differentially expressed.

Recently, micro RNA (miRNA) were discovered as novel posttranscriptional gene-regulators. Dietary VE-depletion for 6 months reduced the relative concentrations of miRNA 122a, important in lipid metabolism, and miRNA 125b, important in inflammation and carcinogenesis, in the liver compared to sufficiently fed rats. To the best of our knowledge, this is the first report of a role of dietary VE in regulating hepatic miRNA in vivo.

In summary, feeding of graded dietary VE for 6 months affected mRNA levels in brain and liver of rats tissue-specifically. Furthermore, VE deficiency resulted in significantly altered hepatic miRNA concentrations. Thus, the gene regulatory activity of vitamin E seems to be mediated by both changes in the mRNA and also miRNA levels.

Epidemiologische Studien weisen darauf hin, dass der Verzehr Vitamin E-reicher Lebensmittel, jedoch nicht die Aufnahme von Vitamin E Supplementen, möglicherweise mit einem signifikant erniedrigten Risiko für die Entstehung neurodegenerativer Erkrankungen, wie z.B. Morbus Alzheimer, und Zivilisationskrankheiten, wie Atherosklerose, koronare Herzerkrankung und Krebs, assoziiert ist. Die zugrunde liegenden Wirkmechanismen potentiell neuroprotektiver Effekte scheinen sowohl die Verminderung von oxidativem Stress als auch die Modulation der Expression erkrankungsrelevanter Gene durch das Antioxidans Vitamin E zu implizieren. Rapsöl ist eine gute Quelle für Vitamin E und gilt, auch durch seinen hohen Gehalt an einfach ungesättigten Fettsäuren und der mehrfach ungesättigten ω -3 Fettsäure α -Linolensäure, als qualitativ hochwertiges Öl für die Ernährung des Menschen.

Ziel der vorliegenden Arbeit war es, den Einfluss steigender Konzentrationen an Vitamin E aus der natürlichen Quelle Rapsöl auf Parameter des oxidativen Stresses und auf die Genregulation in Gehirn und Leber in wachsenden Fisher 344 Ratten zu untersuchen. Zu diesem Zweck wurden drei Gruppen von jeweils 8 männlichen Albinoratten für 6 Monate mit halbsynthetischen Diäten gefüttert, die entweder eine defiziente (<1 mg α -Tocopherol-Äquivalente (α TÄ)/kg Diät), marginale (9 mg α TÄ/kg Diät) oder adäquate (18 mg α TÄ/kg Diät) Versorgung mit Vitamin E gewährleisteten; eine vierte Gruppe wurde mit einer Diät gefüttert, die mit natürlichem α T angereichert war (Vitamin E Gehalt insgesamt: 146 mg α TÄ/kg Diät).

Die α -Tocopherolgehalte in Leber, Cortex und Plasma stiegen in Abhängigkeit von den in den Diäten enthaltenen Vitamin E-Konzentrationen an. Die Responsivität der Gewebe hinsichtlich α T Depletion bzw. Anreicherung unterschied sich signifikant voneinander. Die Aufnahme der Vitamin E defizienten Diät über einen Zeitraum von 6 Monaten führte nahezu vollständig zur Depletion von α T in der Leber, jedoch nicht im Gehirn. Damit übereinstimmend wurden weder Veränderungen der F_2 -Isoprostan-Konzentrationen, der Expression von Hämoxxygenase 1 (HO-1), der Aktivitäten antioxidativer Enzyme oder der Glutathionkonzentration in den Gehirnen der Vitamin E-defizienten Ratten beobachtet. Des Weiteren kam es zu keinen Veränderungen in den mRNA Spiegeln Alzheimer-relevanter Gene zwischen den einzelnen Gruppen. In den Lebern der Ratten, welche Vitamin E defizient ernährt wurden, konnten, im Gegensatz zu den Tieren, welche adäquat mit Vitamin E ernährt wurden, signifikant erhöhte HO-1 mRNA- und Proteinkonzentrationen gemessen werden.

In jüngster Zeit werden Mikro-RNA (miRNA) als post-transkriptionelle Genregulatoren diskutiert. Defiziente Versorgung mit Vitamin E führte, im Vergleich zu adäquater Versorgung, zu einer Abnahme der relativen Konzentrationen von miRNA-122a, welche eine Rolle im Fettstoffwechsel spielt, und von miRNA-125b, die an Entzündungsreaktionen und Krebsentstehung beteiligt ist. Diese Ergebnisse sind möglicherweise erste Hinweise für einen Einfluss von Vitamin E auf die Regulation hepatischer miRNA in vivo.

Die vorliegenden Versuchsergebnisse zeigen, dass die Aufnahme steigender Konzentrationen an Vitamin E über einen Zeitraum von 6 Monaten die mRNA Konzentrationen in Gehirn und Leber der Ratte gewebespezifisch modulieren. Des Weiteren wird postuliert, dass die genregulatorische Aktivität von Vitamin E sowohl durch Veränderungen der mRNA- als auch der miRNA-Konzentrationen vermittelt werden kann.

ABBREVIATIONS

5 α -R1	5- α -steroid reductase type 1
Abeta	amyloid β peptide
AChE	acetylcholinesterase
AD	Alzheimer's disease
ADAM-10	a disintegrin and metalloprotease domain 10
AGE	advanced glycation endproduct
APP	amyloid beta (A4) precursor protein
Apba1	APP binding family member 1
α TE	α -tocopherol equivalents
α TTP	α -tocopherol transfer protein
BACE-1	beta-site APP cleaving enzyme 1
α TE	alpha tocopherol equivalents
Bax	Bcl2-associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
BHT	butylated hydroxytoluene
CD36	Scavenger receptor CD36
dVE	vitamin E-deficient
ECE	endothelin-converting enzyme
fVE	vitamin E-fortified
γ GCSm	regulatory subunit gamma-glutamylcysteinyl synthetase
GSH	glutathione
HDL	high-density lipoproteine
HO-1	heme oxygenase 1
IC ₅₀	concentration at which activity is 50% of control
IDE	insulin-degrading enzyme
LDL	low-density lipoprotein
miRNA	micro RNA
mRNA	messenger RNA
mVE	marginal vitamin E
nt	nucleotides
PCR	polymerase chain reaction
PKC	protein kinase C
RNA	ribonucleic acid
RNAse	ribonuclease
SeGPx	selenium dependent glutathione peroxidase
SOD	superoxide dismutase
sVE	vitamin E sufficient
RISC	RNA-induced silencing complex
T	tocopherol
VE	vitamin E
VLDL	very low-density lipoprotein

CHAPTER I

GENERAL INTRODUCTION

Molecular structure of vitamin E

Vitamin E (VE) is the generic term for a group of eight lipid-soluble substances, named tocopherols (T) and tocotrienols, respectively, that are composed of a chromanol ring, bearing a free hydroxyl group, attached to a 16-carbon saturated phytyl (tocopherols) or unsaturated isoprenoid side chain (tocotrienols). The tocopherols and tocotrienols are further subdivided into α -, β -, γ -, δ - tocopherols and -tocotrienols depending on the number and positions of methyl groups attached to the chromanol ring (Figure 1). The three chiral centres at positions 2, 4' and 8' give rise to eight different stereoisomers (RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS) for each tocopherol. Tocotrienols only have one chiral centre at position 2, and can therefore exist in R- or S-configuration. However, the double bonds at the 3' and 7' positions of the tocotrienol side chain give rise to four *cis/trans* geometrical isomers. Hence, theoretically eight isomers are possible for each tocotrienol (Kamal-Eldin & Appelqvist, 1996).

Naturally occurring tocopherols exist exclusively as RRR-stereoisomers, whereas synthetic tocopherols are composed of a racemic mixture (all rac), containing equal amounts of all eight stereoisomers (Kamal-Eldin & Appelqvist, 1996).

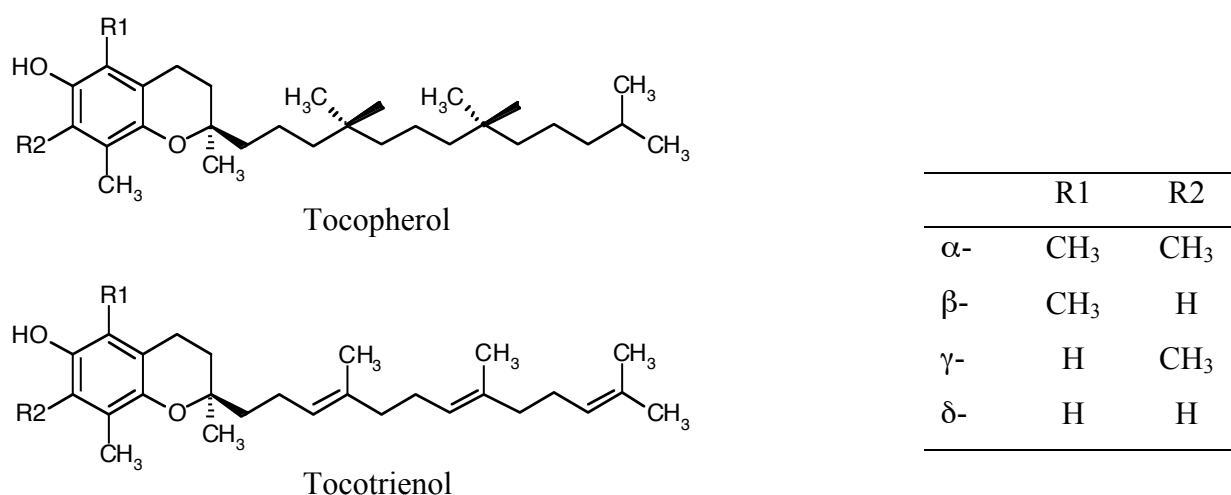


Figure 1: Chemical structures of tocopherols and tocotrienols.

Dietary sources and intake of vitamin E

Natural tocopherols and tocotrienols are exclusively synthesized by photosynthetic organisms i.e. plants and some cyanobacteria. Plants accumulate α T mainly in their green tissues, while tocotrienols are predominantly found in the bran and germ fractions of certain seeds and cereals (Kamal-Eldin & Appelqvist, 1996). Vegetable oils are the richest sources of vitamin E, with wheat germ, safflower, and sunflower oils being particularly rich in α T, and soybean, corn, and sesame oils in γ T. Other good sources of VE include lipid-rich plant parts such as nuts, seeds and grains. Tocotrienols are present at high concentrations in palm, rice-bran, and coconut oils and soybeans (Packer et al., 2001). Compared to other Western populations, where α T is the predominant form in the diet, γ T is the major dietary form of VE in the USA because of the widespread use of soybean and corn oils (Wagner et al., 2004).

Rapeseed oil, a natural food source for vitamin E

Rapeseed oil, because of its fatty acid composition with a high percentage of monounsaturated fatty acids, its high content of the polyunsaturated ω -3 fatty acid α -linolenic acid (Table 1), and its high concentrations of natural vitamin E (Table 2), is considered to be of great nutritional value (Schöne et al., 1998).

Table 1: Fatty acid compositions of various vegetable oils (Kamal-Eldin & Andersson, 1997).

Oil type	Fatty acids (% of total fatty acids)					
	16:0	16:1	18:0	18:1	18:2	18:3
Cottonseed	23.0	0.0	2.3	15.6	55.6	0.3
Linseed	5.6	0.0	3.2	17.7	15.7	57.8
Olive	13.8	1.4	2.8	71.6	9.0	1.0
Palm	44.8	0.0	4.6	38.9	9.5	0.4
Rapeseed	4.6	0.3	1.7	60.1	21.4	11.4
Sesame	9.6	0.2	6.7	41.1	41.2	0.7
Soybean	10.1	0.0	4.3	22.3	53.7	8.1
Sunflower	5.2	0.1	3.7	33.7	56.5	0.0

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Table 2: Tocopherol contents of selected oils, margarines, seeds, nuts, and cereals (Wagner et al., 2004). Ordered by α -tocopherol concentration.

Products	Tocopherols, mg/100 g			
	α	β	γ	δ
<i>Oils</i>				
Wheat-germ	133	71	26	27
Sunflower ¹	49	-	5	0.8
Cottonseed	39	-	39	-
Rapeseed¹	32	-	38	2
Palm	26	-	32	7
Sesame	14	-	29	-
Peanut	13	-	21	2
Olive	12	-	0.7	-
Corn ²	11	5	60	1.8
Linseed	8	-	58	2
Soybean ²	8	1.5	80	27
<i>Margarines (based on)</i>				
Sunflower oil ¹	6	-	10	3
Corn oil ²	5	-	46	-
Soybean oil ²	5	-	34	10
<i>Seeds</i>				
Sunflower seeds	50	3	-	-
Sesame seeds	-	-	23	-
<i>Nuts</i>				
Almonds	45	19	-	-
Pecan nuts	-	-	16	-
Peanuts	11	-	8	-
Brazil nuts	11	-	5	3
<i>Cereal grains</i>				
Rice, white	0.3	-	0.3	-
Sweet corn	0.2	-	4.5	-

¹Preferential consumption in Europe

²Preferential consumption in the USA

A rapeseed variety with especially high tocopherol concentrations (~850 mg/kg total tocopherols) was developed by Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Holtsee, Germany) and kindly provided for the production of the rapeseed oil used in the

present experiments. For more information regarding the production of the rapeseed oil used in this thesis see chapter II.

Absorption, distribution, and metabolism of vitamin E

Vitamin E is comprised of a group of lipophilic substances and, as such, absorbed and transported in the body together with other food lipids. In the gut, vitamin E is incorporated into mixed micelles and taken up into the enterocytes. The efficiency of vitamin E absorption is in the range of 15-45% of the ingested dose, while the remainder is excreted with the faeces (Traber & Sies, 1996). The absorption of vitamin E was long thought to occur by passive diffusion (Kayden & Traber, 1993), but more recently, the ATP-binding cassette (ABC) transporter scavenger-receptor class B type I (SR-BI) was found to be involved in α T uptake (Reboul et al., 2006). In the enterocytes, vitamin E is incorporated into chylomicrons, which are secreted into the lymphatic system and then pass through the thoracic duct into the systemic circulation. Upon contact with lipoprotein lipase, an enzyme located on vascular endothelial cells, chylomicrons are catabolized to chylomicron remnants and a part of the transported vitamin E is transferred to extra hepatic tissues and other lipoproteins. With the chylomicron remnants, the remaining vitamin E reaches and is taken up into the liver (Kayden & Traber, 1993).

The absorption and trafficking of tocopherols and tocotrienols does not differ for all eight vitamin E congeners until they reach the liver. In the liver, however, RRR- α T is preferentially incorporated in VLDL (very low density lipoproteins) over the non-RRR- α -tocopherols and tocotrienols, involving the action of α -tocopherol transfer protein (α TTP) (Kayden & Traber, 1993). α TTP recognizes the fully methylated chromanol head and the R-configuration at carbon 2 (2R) (Meier et al., 2003). The binding affinities of α TTP for tocopherols and tocotrienols relative to that of RRR- α T have been determined as: β T, 38%; γ T, 9%; δ T, 2%; SRR- α T, 11%; and α T3, 12% (Hosomi et al., 1997).

In the liver, non- α T congeners and, to a much lesser extent, excess α T are metabolized to side-chain truncated, water-soluble carboxyethyl hydroxychroman (CEHC) metabolites and eventually excreted in urine (Sontag & Parker, 2002). Tocopherols and CEHCs also undergo biliary excretion, although it is not known to what extent (Traber & Kayden, 1989; Kiyose et al., 2001). Initial ω -hydroxylation of the vitamin E side-chain appears to be facilitated by cytochrome P₄₅₀ (CYP) enzymes and was reported to occur with a higher catalytic activity

towards the non- α T congeners, suggestive of a central role for the involved enzymes in the selective retention of α T in the body and the regulation of γ T plasma concentrations (Sontag & Parker, 2002). Indeed, the selective accumulation of α T in the fruit fly *Drosophila melanogaster*, which does not express α TTP, has been associated with CYP activity (Parker & McCormick, 2005). Following ω -hydroxylation, side-chain shortening is facilitated by enzymes of the β -oxidation pathway (Sontag & Parker, 2002).

From the liver, α T and to a lesser extent γ T is secreted into the bloodstream, incorporated into very-low-density lipoproteins (VLDL). During VLDL catabolism, other lipoproteins become enriched with tocopherols and eventually deliver the vitamin to target tissues (Traber & Sies, 1996). The largest fraction of VE is transported in low-density (LDL) and high-density lipoproteins (HDL), a smaller amount in VLDL (Behrens et al., 1982). Uptake into tissues is facilitated by either the LDL-receptor or, in the case of HDL-associated vitamin E, the scavenger receptor class B type I (SR-BI) (Qian et al., 2005). The rate at which absorbed vitamin E is taken up by the various tissues depends on the turnover of the respective tissue. Plasma and liver are tissues with a fast turnover, whereas in brain or neural tissues the turnover is slow (Burton & Traber, 1990).

Biological functions of vitamin E

Vitamin E was initially discovered, because its absence in the diet of female rats resulted in infertility caused by foetal resorption (Evans & Bishop, 1922). In the following decades, vitamin E deficiency syndromes were discovered and described for many species, including pigs, chicken, rats, and mice, but its essential biological function remained elusive (Mason, 1977). After the discovery of the antioxidant properties of tocopherols (Olcott & Emerson, 1937), this antioxidant activity was assumed to be the biological function of the vitamin in vivo (Dam, 1952). This assumption was driving vitamin E research for many decades, until presumed non-antioxidant functions of tocopherols, such as inhibition of protein kinase C (PKC) (Mahoney & Azzi, 1988) and regulation of cellular signal transduction pathways and gene expression (Rimbach et al., 2002) were discovered. However, the notion that α T might exert biological functions independent of its antioxidant activity is currently a matter of debate (Azzi, 2007; Traber & Atkinson, 2007). One group of researchers put forward the idea that α T or its derivative α -tocopheryl phosphate may act as cellular signalling molecules or redox sensors via binding to as yet unknown transcription factors or receptors (Azzi, 2007), while an opposing school of thought supports the notion that all biological activities exerted

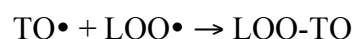
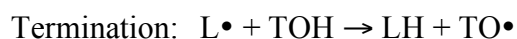
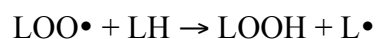
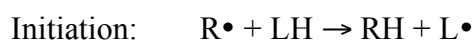
by α T, including the regulation of gene expression and cellular signalling cascades, can be explained on the basis of its antioxidant protection of polyunsaturated fatty acids and the resulting effects on membrane qualities (integrity, fluidity, phase separation, etc.) (Traber & Atkinson, 2007). The most important discoveries in vitamin E research are summarized in Table 3.

Table 3: Milestones in vitamin E research.

Year	Milestone
1922	Evans and Bishop discovered a then unknown essential factor for the successful reproduction of rats, which they called substance “X” (Evans & Bishop, 1922)
1925	This substance “X” was assigned its vitamin status and named “E”, because of the earlier discovery of the vitamins A-D (Mason, 1977)
1936	An alcohol preventing female sterility and foetal resorption in rats was isolated from wheat germ oil by Evans and co-workers and named α -tocopherol, from the Greek word “tokos” (child birth), “phero” (to bear), and the suffix “ol” indicating an alcohol (Evans et al., 1936).
1968	The RDA for vitamin E was established at 300 mg (300 IU) for a 65 kg adult male (Food and Nutrition Board, 1968; Food and Nutrition Board)
1982	Burton, Joyce, and Ingold claim the first proof that VE is the major lipid-soluble chain-breaking antioxidant in human blood plasma (Burton et al., 1982)
1988	α -Tocopherol was shown to inhibit protein kinase C activity (Mahoney & Azzi, 1988)
1991	Purification and characterization of α TTP (Sato et al., 1991)
1993	Primary structure of α TTP (Sato et al., 1993)
1998	The expression of the α TTP gene in the liver is induced by α T and δ T (Fechner et al., 1998)
1999	Supernatant protein factor (SPF), a protein stimulating cholesterol biosynthesis, was shown to be identical with α -tocopherol-associated protein (TAP) (Stocker et al., 1999)
2000	RDA values were replaced by Dietary Reference Intakes (DRI); the DRI were set at 15 IU for both men and women (Food and Nutrition Board, 2000)
2001	α T identified as a transcriptional regulator of gene expression by binding of TAP (Yamauchi et al., 2001)
2001	The first use of α TTP ^{-/-} mice as a genetic model of vitamin E-deficiency (Terasawa et al., 2000)
2002	Crystal structure of the human TAP was reported (Stocker et al., 2002)

Antioxidant function

Free radicals are highly reactive atoms, molecules or ions capable of autonomous existence with at least one unpaired electron. Free radicals and other reactive species can damage biological molecules, such as membrane lipids, nucleic acids and proteins, thereby starting radical chain reactions (Ames et al., 1993). Lipid peroxidation proceeds via such a self-propagating chain reaction, which can be subdivided into initiation, propagation and termination, unless stopped by formation of stable products through the reaction of two radicals with each other or of a radical with an antioxidant (Kamal-Eldin & Appelqvist, 1996).



$R\bullet$ = free radical species, L = lipid, TOH = tocopherol, $TO\bullet$ = tocopheroxyl radical

Barry Halliwell defined an antioxidant as: “any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell, 1990).

Oxidative and reductive processes are elemental in metabolism, during which highly reactive free radicals are generated as by-products of the energy production in the respiratory chain. The organism also employs free radicals to kill exogenous cells as part of its immune defence (Halliwell, 1990). VE protects lipids against oxidative damage, especially polyunsaturated fatty acids (PUFA) in the lipid phase of biomembranes and in plasma lipoproteins. VE incorporates into (cell) membranes, where it reacts with free radicals by donating hydrogen atoms, thereby breaking the membrane-damaging free radical reaction. The thus formed VE radical is much less reactive and does not efficiently react with nearby macromolecules. The in vitro antioxidant capacities (that is, the chemico-physical reactivities) of the tocopherols and tocotrienols depend on the number of methyl groups substituted at the chromanol head. The abilities of the different tocopherols to donate hydrogen atoms increase with the number

of methyl substituents in the order: $\alpha\text{T} > \beta\text{T} > \gamma\text{T} > \delta\text{T}$ (Kamal-Eldin & Appelqvist, 1996). In vivo, however, the situation is more complex. In the body, the antioxidant capacities of the VE congeners do not only depend on their chemical reactivities, but also on factors such as absorption, transport, metabolism, excretion, and compartmentalisation. Therefore, the fully methylated congeners with 2R-stereochemistry are recognized as the most important in vivo antioxidants (Kamal-Eldin & Appelqvist, 1996). In agreement, VE, and in particular αT , was identified as the major lipid-soluble antioxidant in human plasma (Burton et al., 1982).

Regulation of cellular signalling and gene expression

In 1988, Mahoney and Azzi (1988), with their finding of a reduced protein kinase C (PKC) activity in the presence of very high concentrations of αT (IC_{50} , 456 $\mu\text{mol/L}$ αT), were the first to suggest a role of αT in cellular signalling pathways. Three years later, the same group discovered that αT inhibits PKC activity in vascular smooth muscle cells resulting in growth arrest. This effect was specific for αT , whereas βT , trolox (a synthetic tocopherol analogue) and αT esters had no such effect. Furthermore, the effect occurred at concentrations of αT that are physiological in healthy adults (25 μM) (Boscoboinik et al., 1991). Subsequently, the mechanism of action was found to involve the dephosphorylation of PKC by activated protein phosphatase 2A (Ricciarelli & Azzi, 1998). Since the original finding in 1988, other functions of αT related to cellular signalling and gene expression have been revealed. In vitro and in vivo experiments demonstrated that αT inhibits monocyte-endothelial cell adhesion (Devaraj et al., 1996), platelet adhesion and aggregation (Freedman et al., 1996; Williams et al., 1997) and cyclooxygenase 2 activity (Wu et al., 2001).

Some of the above mentioned studies conclude that the observed effects of αT were independent of its antioxidant activity, despite lacking a proper control treatment that would support such a conclusion. The use of βT as control, for example, in cell culture experiments (Fazzio et al., 1997; Breyer & Azzi, 2001) neglects the known differences regarding the metabolism of αT and βT (see above), which may result in rapid catabolism of βT and thus significant differences in the cellular (and thus active) concentrations of αT and βT (Sontag & Parker, 2002).

Supplementation with vitamin E (primarily αT) was shown to regulate the expression of several genes at the transcriptional level, including genes that are i) involved in the uptake and degradation of tocopherols, ii) implicated with lipid uptake and atherosclerosis, iii) involved

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in the modulation of extracellular proteins, iv) connected to adhesion and inflammation, and v) implicated in cell signalling and cell cycle regulation (classification according to Azzi et al. 2004; Table 4).

Gene regulation may also occur as a result of vitamin E depletion: VE deficiency increased rat liver γ GCS_m and γ GCS_c (catalytic subunit) and decreased p21 (cyclin-dependent kinase inhibitor 1A) mRNA concentrations (Morante et al., 2005). Furthermore, VE deficiency increased mRNA concentrations of HO-1 (heme oxygenase 1), a gene with cytoprotective functions (Ito et al., 1997).

Table 4: Genes determined by PCR or Northern blot to be differentially regulated in various organs and cultured cells from different species after supplementation and incubation, respectively, with α -tocopherol.

Gene name	Gene symbol	Regulation by α T supplementation	Tissue	Species	Function of the gene product	Reference
<i>Uptake and degradation of tocopherols</i>						
α -Tocopherol transfer protein	α TTP	↑ ¹	Liver	Rat	α T transfer	(Fechner et al., 1998)
γ -Glutamyl-cysteine synthetase modifier subunit	γ GCS _m	↑	Liver	Rat	Rate-limiting enzyme for glutathione de novo synthesis	(Barella et al., 2004)
<i>Lipid uptake and atherosclerosis</i>						
Scavenger receptor CD36	Cd36	↓	Liver	Rat	Fatty acid metabolism; receptor for oxidized LDL	(Barella et al., 2004)
Scavenger receptor class B type I	SrbI	↓	Type II pneumocytes	Rat	Receptor for HDL	(Kolleck et al., 1999)
Scavenger receptor class A	SR-AI/II	↓	Peritoneal macrophages	Rabbit	Receptor for modified LDL	(Teupser et al., 1999)
Low-density lipoprotein receptor	LDL-R	↑	HepG2 hepatoma cells	Human	Uptake of LDL	(Pal et al., 2003)
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	↑	HepG2 hepatoma cells	Human	Rate-limiting enzyme for cholesterol biosynthesis	(Pal et al., 2003)
<i>Modulation of extracellular proteins</i>						
α -Tropomyosin	Tpm1	↑	Vascular smooth muscle cells	Rat	Actin binding protein; actin-myosin interaction	(Aratri et al., 1999)
Collagen α 1	Col1a1	↓	Liver	Mouse	Matrix protein	(Chojkier et al., 1998)
Matrix metalloproteinase 1 (interstitial collagenase)	MMP-1	↓	Skin fibroblasts	Human	Breakdown of interstitial collagens, types I, II, III	(Ricciarelli et al., 1999)
Matrix metalloproteinase 19	MMP-19	↓	PBMC ²	Human	Cellular proliferation, migration and adhesion to type I collagen	(Mauch et al., 2002)
Connective tissue growth factor	CTGF	↑	Aortic vascular smooth muscle cells	Human	Endothelial cell function	(Mauch et al., 2002)

Table 4 (continued)

<i>Adhesion and inflammation</i>						
Selectin E	SELE	↓	Aortic endothelial cells	Human	Cell adhesion molecule	(Wu et al., 1999)
Intercellular adhesion molecule 1	ICAM-1	↓	HAEC ³	Human	Recruitment of monocytes	(Wu et al., 1999)
Vascular cell adhesion molecule-1	VCAM-1	↓	HAEC ³	Human	Recruitment of monocytes	(Wu et al., 1999)
Integrin alpha 2b	glycoprotein IIb	↓	Erythroleukemia cells	Human	Platelet adhesion	(Chang et al., 2000)
Interleukin 2	Il-2	↑	Naive T cells	Mouse	Proliferation of T and B lymphocytes, immune response	(Adolfsson et al., 2001)
Interleukin 4	Il-4	↓	Peripheral blood T cells	Human	Inflammation and chemotaxis of inflammatory cells	(Li-Weber et al., 2002)
Transforming growth factor-β	Tgf-β	↓	Liver	Rat	Cell metabolism and growth	(Parola et al., 1992)
Integrin, α M	MAC-1	↓	HUVEC ⁴	Human	Immune response	(Terasawa et al., 2000)
Inducible nitric oxide synthase	iNOS	↓	Gastric mucosa	Rat	Nitric oxide production	(Oh et al., 2005)
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IκBα	↑	Activated splenocytes	Mouse	Inhibitor of NFκB	(Hsieh et al., 2006)
<i>Cell signaling and cell cycle regulation</i>						
Peroxisome proliferators-activated receptor gamma	PPAR-γ	↑	SW480 cells ⁵	Human	Regulator of adipocyte differentiation	(Stone et al., 2004)
Cyclin D1	CCND1	↓	LNCaP cells ⁶	Human	Cell cycle regulation	(Gysin et al., 2002)
Cyclin E	CCNE1	↓	LNCaP cells	Human	Cell cycle regulation	(Gysin et al., 2002)
CD95 APO-1/Fas ligand	CD95L	↓	Peripheral blood cells	Human	Induction of apoptosis	(Li-Weber et al., 2002)
Steroid-5-alpha-reductase 1	5α-R1	↓	Liver	Rat	Catalyzes the conversion of testosterone into dihydrotestosterone	(Barella et al., 2004), (Ricciarelli et al., 2000)

Table 4 (continued)

<i>Cytoarchitecture of the lung</i>						
Tropomyosin 2 beta	Tpm2	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Myosin, heavy polypeptide 1	Myh1	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Myosin, heavy polypeptide 8	Myh8	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Troponin I skeletal fast 2	Tnni2	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Myosin light chain	Mylpf	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Actin alpha1 skeletal muscle	Acta1	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Myosin light polypeptide 3	Myl3	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Keratin complex1 acidic gene 15	Krt1-15	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Troponin C2 fast	Tnnc2	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Keratin complex 2 basic gene 4	Krt2-4	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Repetin	Rptn	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Keratin complex1 acidic gene 13	Krt1-13	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
Loricrin	Lor	↑	Lung	Mouse	Cytoskeleton, muscle contraction	(Oommen et al., 2007)
<i>Miscellaneous</i>						
Coagulation factor IX	FIX	↓	Liver	Rat	Intrinsic coagulation pathway	(Barella et al., 2004)
Cellular retinoic acid binding protein 2	CRABP-II	↑	Dermal fibroblasts	Human	Skin growth and differentiation	(Gimeno et al., 2004)
Pro-caspase 3	Pro-CASP3	↑	Jurkat ⁷ ; HL-60 ⁸ ; WiDr ⁹	Human	Induction of apoptosis	(Miyoshi et al., 2005)
Glutathione peroxidase 4	Gpx4	↓	HUVEC	Human	Inhibition of lipid peroxidation	(Sneddon et al., 2003)

¹ ↑ up-regulation and ↓ down-regulation of the respective gene in response to αT

² PBMC: peripheral blood mononuclear cell

³ HAEC: human aortic endothelial cells

⁴ HUVEC: human umbilical vein endothelial cells

⁵ SW480 cells: human colon cancer cell line

⁶ LnCaP cells: human prostate carcinoma cells

⁷ Jurkat cell: human T-cell leukaemia cells

⁸ HL-60 cells: human promyelocytic leukemia cells

⁹ WiDr cells: human colorectal adenocarcinoma cells

Global gene expression profiling with DNA microarrays

In order to obtain a comprehensive understanding of the biological processes in which vitamin E is involved, global gene expression profiling with DNA microarray technology was utilized to understand the consequences of chronic VE deficiency in laboratory rats and mice in a number of tissues including hippocampus (Rota et al., 2005), cortex (Hyland et al., 2006), heart (Vasu et al., 2007), lung (Oommen et al., 2007), liver (Barella et al., 2004; Rimbach et al., 2004), skeletal muscle (Nier et al., 2006), testes (Barella et al., 2004; Rota et al., 2004), and prostate (Siler et al., 2004), as summarized in Table 5.

Table 5: Effects of α -tocopherol depletion on tissue specific transcriptomes and associated biological processes. Analyses from gene chip experiments.

Tissue	Associated biological processes as a result of α-tocopherol depletion
Hippocampus	<ul style="list-style-type: none"> – Hormones/hormone metabolism – Neuronal survival – Dopaminergic system – Amyloid beta/ advanced glycation endproduct clearance
Cortex	<ul style="list-style-type: none"> – Neuronal signal transduction
Heart	<ul style="list-style-type: none"> – Lipid metabolism – Inflammation
Lung	<ul style="list-style-type: none"> – Cytoskeleton
Liver	<ul style="list-style-type: none"> – Androgen metabolism – Blood coagulation – Redox homeostasis – Inflammation
Skeletal muscle	<ul style="list-style-type: none"> – Muscle structure – Inflammation – Growth/development
Testes	<ul style="list-style-type: none"> – Testosterone synthesis – Cholesterol homeostasis – Androgen metabolism
Prostate	<ul style="list-style-type: none"> – Androgen metabolism – Estrogen metabolism

Post-transcriptional gene regulation

Ribonucleic acids (RNA) can be subdivided into coding RNA (messenger RNA, mRNA) and non-coding RNA (transcriptional RNA and small RNA) (Buckingham, 2003) (Figure 2).

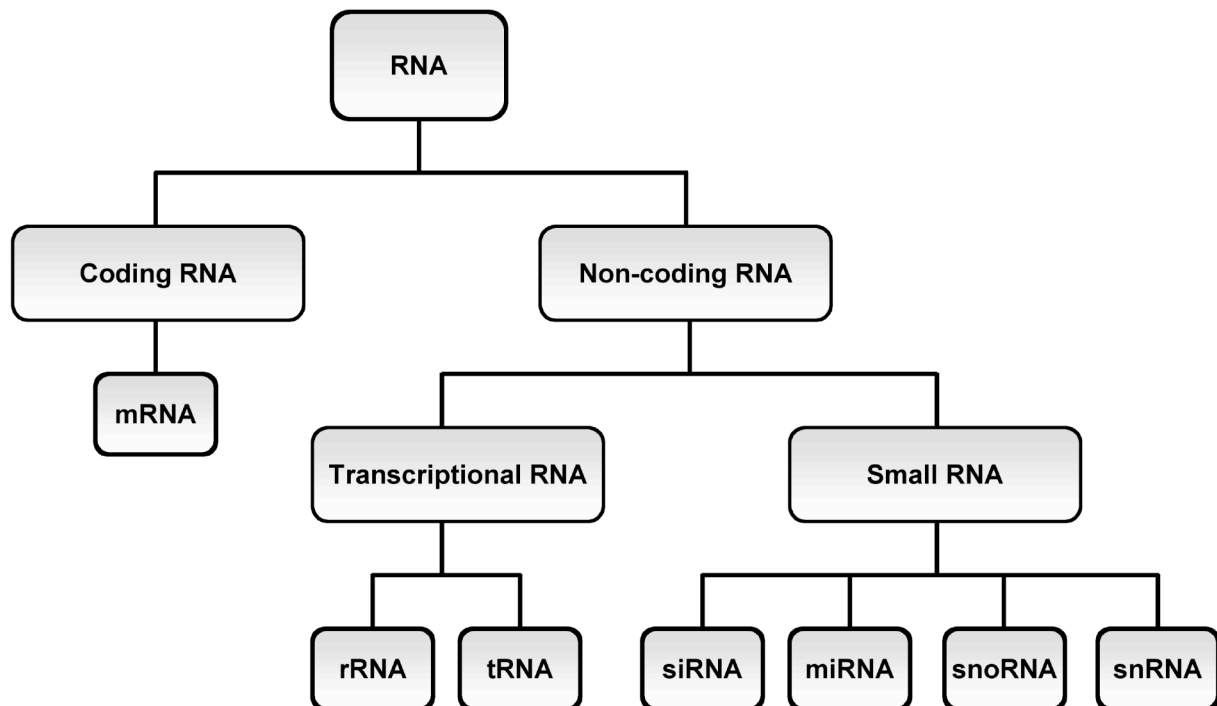


Figure 2: Classification of RNA family members. Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; siRNA, small interfering RNA; miRNA, micro RNA; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA (Buckingham, 2003).

During the processes of gene transcription and translation, in addition to coding RNA, the organism makes use of a number of RNA that do not code for a peptide but serve other molecular functions. A number of these non-coding RNA, such as ribosomal and transfer RNA, which effectuate the ribosomal assembly of peptides, have been known for decades. Small interfering RNA (siRNA) such as microRNA (miRNA), on the other hand, are relatively recent discoveries and facilitate post-transcriptional gene-silencing by a process named RNA interference (RNAi, reviewed in Rana, 2007). miRNA are short, double-stranded RNA species comprised of ~19-25 nucleotides (nt) that are encoded in the genome and transcribed by an RNA polymerase into long (hundreds to thousands of nt) primary miRNA.

Primary miRNA are cut by the ribonuclease (RNase) Droscha and its co-factor Pasha into stem-loop structured precursor miRNA (pre-miRNA) of ~70 nt length, which are shuttled from the nucleus to the cytoplasm by Exportin 5, where they are further processed by the RNase Dicer into small (~22 nt) miRNA. These mature miRNA are unwound and one strand – the so-called guide strand – is loaded into the RNA-induced silencing complex (RISC), a multi-protein complex containing known (including argonaute and Dicer) and as yet unknown proteins, while the other strand – the passenger strand – appears to be degraded. Several of these miRNA-loaded RISC complexes then bind to the target mRNA at the 3' untranslated region, inhibit its translation and direct it toward processing bodies (P-bodies), where it may be stored or degraded. The exact fate of the target mRNA is determined by the degree of base complementarity between the miRNA and its target mRNA. Perfect complementarity activates the siRNA pathway that causes complete degradation of the target mRNA and involves the RNase in RISC. Gene-silencing via the siRNA pathway is mainly observed in plants and to a much lesser extent in animals. Imperfect base complementarity triggers the miRNA pathway, which results in the inhibition of gene translational at the initiation or elongation step and/or in partial RNA degradation through destabilisation due to poly(A)-shortening of the transcript (Cowland et al., 2007; Rana, 2007) (Figure 3).

It has been computed that each miRNA binds on average ~100 different target mRNAs, thus a single miRNA could, at least theoretically, facilitate post-transcriptional silencing of many different genes. It was calculated that ~30% of the genes of the vertebrate genome might be under the regulation of miRNA (Lewis et al., 2005).

While there is ample evidence showing that VE affects gene expression by altering mRNA transcription (Barella et al., 2004; Rimbach et al., 2004), at present, nothing is known about the effects of dietary VE on the expression of miRNAs (see also chapter IV).

GENERAL INTRODUCTION

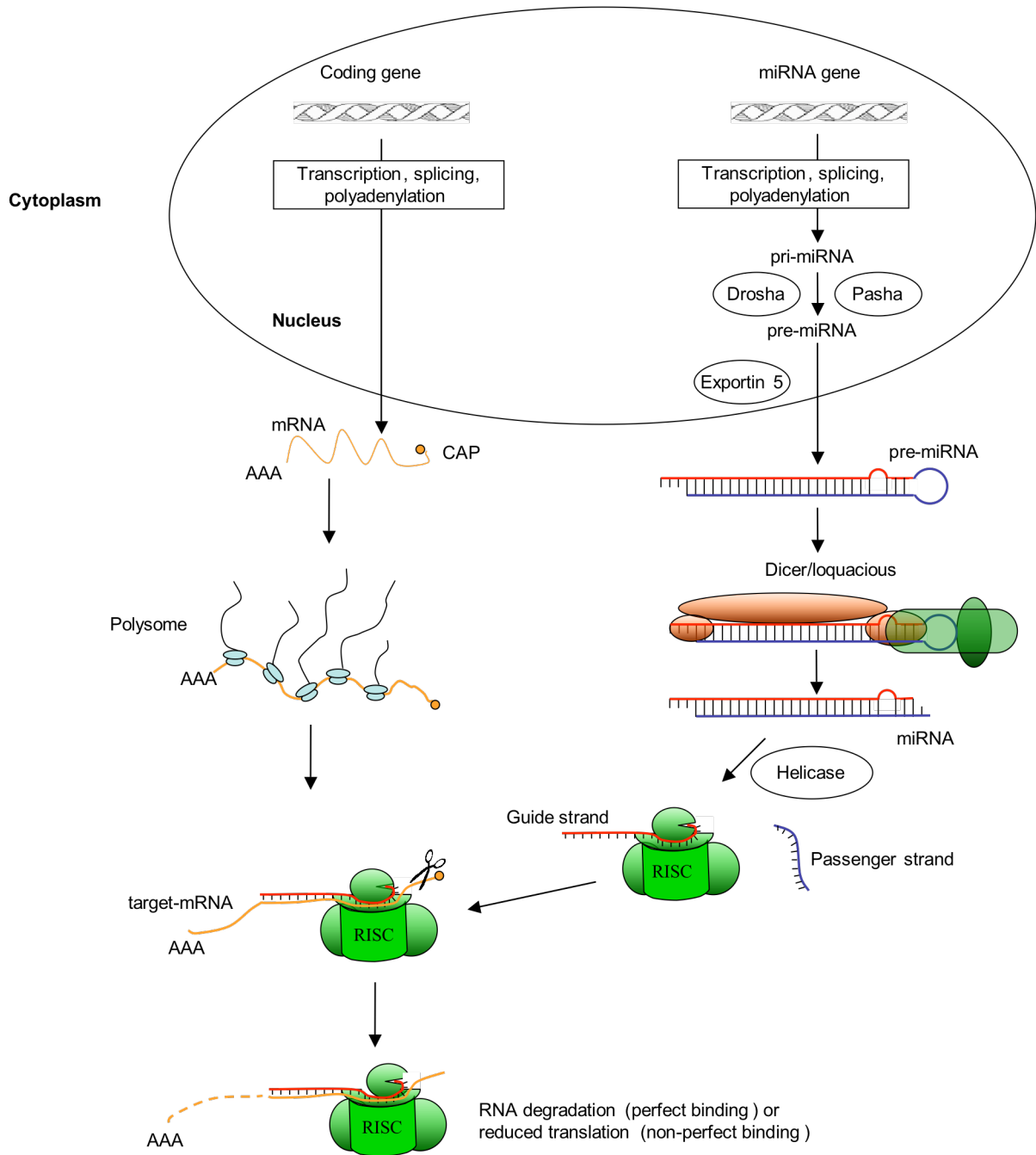


Figure 3: Biogenesis and processing of, and gene-silencing by micro RNA (modified from Kosik, 2006). miRNA are genomically encoded and transcribed by an RNA polymerase into long primary miRNA (pri-miRNA), which are cleaved by the ribonuclease Drosha and its cofactor Pasha into a 70-100 nucleotide precursor (pre-miRNA) with stem-loop structure. The pre-miRNA is translocated from the nucleus by exportin 5, an evolutionary conserved nuclear export factor (Bohnsack et al., 2004), into the cytoplasm, where it is cleaved by an enzyme complex including the proteins Dicer and loquacious. The resulting miRNA duplex is then unwound by a helicase, releasing a guide and a passenger strand. The guide strand is translocated to the RNA-induced silencing complex (RISC) where it binds to a target mRNA, leading to either degradation (perfect binding to the mRNA) or reduced translation (non-perfect binding to the mRNA), while the passenger strand is destroyed (Bartel, 2004; Kosik, 2006).

Vitamin E and neuroprotection

Life expectancy and the number of people aged >60 years, and in parallel with this, the number of people suffering from age-related neurological disorders, are constantly increasing in industrialised countries (Fratiglioni et al., 2000). Alzheimer's disease (AD), also known as Morbus Alzheimer, is the most common neurodegenerative disorder affecting the brain. Clinical symptoms include progressive loss of memory and reduced cognitive ability. The underlying histological changes in the brain include the extra-cellular accumulation of amyloid β peptide, resulting in so-called amyloid plaques, and the formation of intra-cellular neurofibrillary tangles consisting of hyper-phosphorylated tau protein. The characteristic pathophysiological hallmarks of AD are synaptic dysfunction, massive progressive loss of neurons by apoptosis, especially in the hippocampus and cortex, and a selective depletion of neurotransmitter systems (e.g. acetylcholine) (Leuner et al., 2007).

On the cellular level, the processes leading to neuronal death and, thus, neurodegenerative events and AD, are caused by or result in an increased formation of reactive oxygen and nitrogen species. Such an imbalance between free radical generation and elimination is termed "oxidative stress" and was causally connected with the incidence of AD (Lin & Beal, 2006). Epidemiological studies indicate that a high intake of vitamin E from food sources, but not from dietary supplements, may contribute to the prevention of age-related neurodegenerative disorders such as AD (Engelhart et al., 2002; Morris et al., 2002; Morris et al., 2005). Morris and colleagues evaluated the contribution of the different vitamin E congeners toward the prevention of AD. While a higher dietary intake of both α T and γ T was associated with a decreased risk of developing AD, their data suggested that the combination of the different vitamin E congeners may be more effective in preventing AD than the intake of α T alone (Morris et al., 2005). In line with this, another epidemiological study (Fillenbaum et al., 2005) did not find a delayed onset of dementia after vitamin E supplementation. Zandi and co-workers (2004) demonstrated, that vitamin E and vitamin C in combination are more effective in reducing AD incidence.

Some clinical intervention studies employing high dosage α T supplements, on the other hand, found a significant reduction in AD progression (Sano et al., 1997; Onofrj et al., 2002), while in another study by Petersen and colleagues (2005), supplementation of high doses of α T failed to slow down the progression of AD (Table 6).

Table 6: Clinical intervention trials studying the role of vitamin E in the prevention of Alzheimer's disease.

Reference	VE dose and duration	Study design	Conclusion
(Sano et al., 1997)	2000 IU/day 2 years	341 patients double-blind, placebo-controlled, randomized, multicenter study; AD patients, matched healthy controls	VE slowed progression of AD
(Onofrj et al., 2002)	2000 IU/day 6 months	60 patients randomized, controlled clinical trial	VE delayed onset of AD
(Petersen et al., 2005)	2000 IU/day 3 years	212 mild cognitive impairment double-blind, placebo-controlled	no effect of VE on AD progression

Findings from human intervention trials are supported by observations from animal studies where vitamin E altered physiological processes relevant for the progression as well as the prevention of AD. Gene chip technology was used to identify vitamin E-sensitive genes that play an important role in the prevention of AD by global gene profiling in laboratory rats (Rota et al., 2005). The authors reported the modulation by vitamin E of mRNA concentrations of genes that are involved in the clearance and stabilization of the neurotoxic amyloid β peptide. The aggregation of amyloid β is a molecular key event in the pathogenesis of AD and responsible for the formation of so-called senile plaques. Furthermore, vitamin E significantly altered the transcription levels of genes that inhibit programmed cell death (apoptosis) and, thereby, loss of neurons (Rota et al., 2005).

At present, no effective therapies are available for the treatment of AD, therefore research into the prevention of the disease is of high importance. Because of the association of neurodegenerative disorders with increased levels of oxidative stress, the neuroprotective activities of natural antioxidants and especially of vitamin E deserve further attention.

Aim

The overall aim of the present thesis was to investigate the influence of vitamin E from a natural source on gene expression in the brain and liver of laboratory rats. Rapeseed oil was chosen as a rich source of natural vitamin E. The production of the rapeseed oils and experimental diets used in the feeding trial are presented in detail in chapter II. In chapter III, the impact of long-term feeding of graded dietary doses of natural-source vitamin E on tocopherol concentrations, parameters of oxidative stress, and the expression of AD-relevant genes in cortex and hippocampus is described. Chapter IV presents the impact of vitamin E depletion on the expression of selected hepatic miRNAs in rats. In chapter V, the presented experiments and data are discussed in the context of the current literature.

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GENERAL INTRODUCTION

Zandi, P. P., Anthony, J. C., Khachaturian, A. S., Stone, S. V., Gustafson, D., Tschanz, J. T., Norton, M. C., Welsh-Bohmer, K. A. & Breitner, J. C., 2004. Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study. *Archives of Neurology* **61**, 82-8.

CHAPTER II

PREPARATION OF RAPESEED OILS AND EXPERIMENTAL DIETS

Oil production

S-NAP 944 is a rapeseed variety rich in α T, which was developed within the project NAPUS 2000 and was provided by Dr. Leckband (*Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ)*). Pressing of the oil was carried out by *Pilot Pflanzenoeltechnologie Magdeburg e.V. (PPM e.V.)*. Rapeseeds underwent an initial quality check and were mature, without stocking, with medium- to large-sized grains, and a neutral to rapeseed-typical odour. Seeds were flaked on roller mills to increase the oil yield. Warm-pressing usually results in a higher oil yield, but also in the formation of undesirable by-products, which make subsequent refining necessary. Refining, however, causes thermal degradation of vitamin E and, thus, decreases the tocopherol content of the resulting oil (Willner et al., 1997). To ensure a maximum vitamin E-yield during oil production, S-NAP 944 rapeseeds were cold-pressed under nitrogen atmosphere. The crude oil was of strong yellow-green colour and vacuum-filtered to increase stability, after which it was gold-yellow and had a rapeseed oil-typical smell.

Purification of the oil and removal of vitamin E and other natural antioxidants

The cold-pressed rapeseed oil was stripped of tocopherols and other antioxidants by adsorption chromatography according to the method of Lampi and Kamal-Eldin (1992). Aluminium oxide (250 g) was activated at 100°C for 8 h and at 200°C for 12 h. Glass columns (60 x 3 cm) were plugged with wads of cotton, which were soaked with n-hexane. Proper care was taken to avoid the inclusion of air in order to prevent oxidation at the bottom of the column before putting aluminium oxide. The activated aluminium oxide (stationary phase, adsorbent) was suspended with n-hexane (mobile phase) and the suspension was filled into the glass columns. The columns were capped with cotton to exclude air and to prevent stirring of the stationary phase during application of the oil. The eluent n-hexane was drained until the liquid level was ca. 1 cm above the top cotton wad. The prepared column was free of fissures and air bubbles. In order to avoid exposure of the oils to light, the column was wrapped in aluminium foil and dark glass jars were used for the collection of the stripped oils. Rapeseed oil was mixed 1:1 (v/v) with n-hexane (500 ml each) and applied to the column. Approximately 300 ml n-hexane was used to elute the remaining oil, after the oil-hexane-mixture had passed through the column. Finally, the stripped rapeseed oil was purged with a stream of nitrogen gas and stored at -20°C until analysis and removal of hexane. The absence of tocopherols from the purified oils was verified by HPLC. Therefore, 100 mg oil were

PREPARATION OF RAPESEED OILS AND EXPERIMENTAL DIETS

weighed into caps, 400 μ l n-heptane (with 1 mg/ml BHT) were added, the mix was vortexed and injected into the HPLC (column: LiChrosphere Si 100, 5 μ m, 10 x 250 mm; mobile solvent: n-heptane/isopropanol 100:1; flow rate: 1,0 ml/min; $\lambda_{\text{Excitation}}$: 290 nm; $\lambda_{\text{Emission}}$: 328 nm).

Hexane was removed from the stripped oil in a vacuum rotary evaporator with the water bath initially set to 65°C. For the last 10 minutes, the water temperature was increased to 90°C. Subsequently, the oil was purged with a stream of nitrogen gas and stored at -20°C until further use.

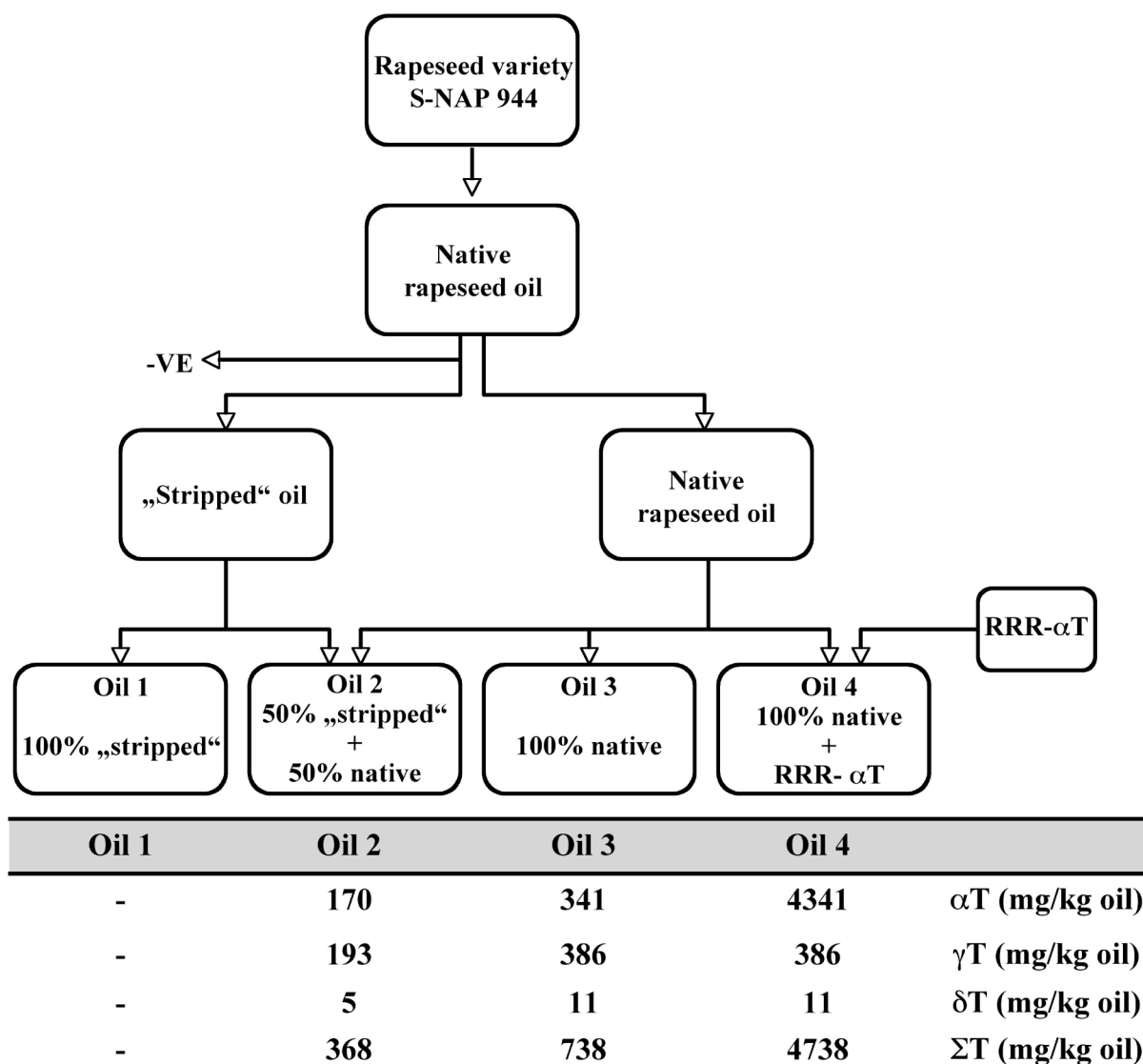


Figure 1: Flow chart of the oil preparation and tocopherol concentrations in the resulting oils.

Preparation of oils with different vitamin E concentrations

In order to obtain oils with increasing vitamin E concentrations, the stripped and native oils were blended at varying ratios (1:0, 1:1, 0:1; v/v; Figure 1). The native oil was fortified with RRR- α -tocopherol to prepare a fourth oil with a particularly high content of alpha-tocopherol.

Fatty acid composition of the native and stripped oils

Vitamin E, carotinoids, and other antioxidants as well as free fatty acids and mono- and diglycerides were adsorbed at the stationary phase during column chromatography and thus removed from the oil. Because fatty acids affect gene expression (Sessler & Ntambi, 1998; Jump et al., 2005), the fatty acid profiles of the prepared oils were determined by gas chromatography (column: DB-WAX; detector: GC-FID). As shown in Figure 2, the fatty acid profiles of the rapeseeds, the native rapeseed oil, and the stripped rapeseed oil did not differ significantly.

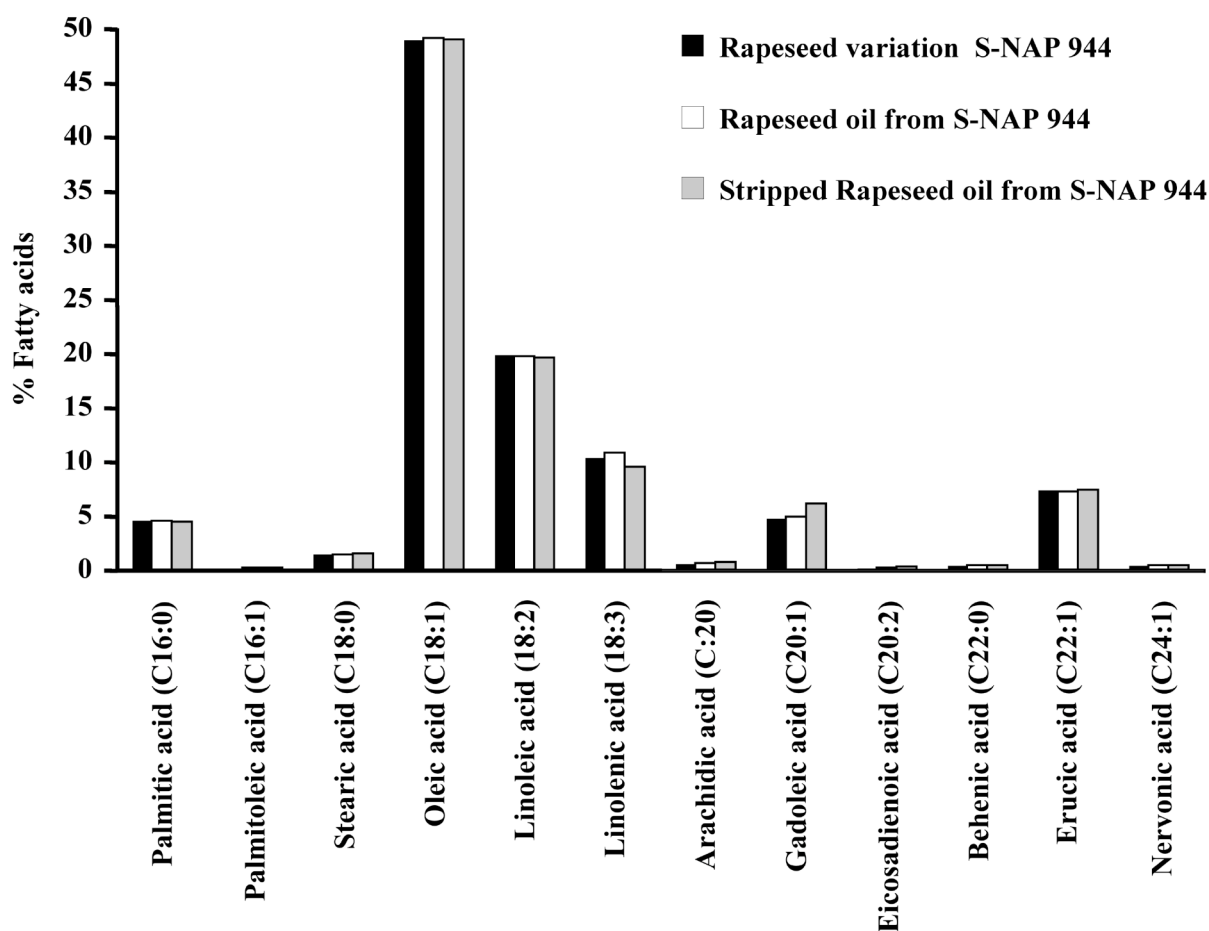


Figure 2: Fatty acid profiles of rapeseed variation S-NAP 944, its native and stripped oil.

Preparation of the animal diet

The composition of the semi-synthetic experimental diets is shown in Table 1. The prepared rapeseed oils were used as the sole source of fat and tocopherols in the diets. All diets were cold-palletized and vacuum-packed with oxygen absorber pads in polyethylene bags in units of 1.5 kg. Diets were stored at -20°C until used.

Table 1: **Composition of the diets used in the feeding experiment.**

Compound	g/kg diet
Casein	240
Corn starch, modified	480
Glucose	110
Cellulose powder Arbocell B 800	50
Vitamin E-free vitamin premix (E15313-2)	10
Mineral premix (E15000)	60
Rapeseed oil*	50

*All vitamin E in the diet originated from the rapeseed oil. Vitamin E concentrations of the diets prepared with tocopherol-stripped oil, tocopherol-stripped oil plus native oil (blended at a ratio of 1:1), native oil or α -T-fortified oil (4 g RRR- α -T/kg native oil) were <1, 9, 18 or 146 mg/kg diet, respectively (see Chapter III).

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CHAPTER III

VITAMIN E, REDOX STATUS, AND GENE REGULATION

**DIETARY VITAMIN E, BRAIN REDOX STATUS AND EXPRESSION OF ALZHEIMER'S
DISEASE RELEVANT GENES IN RATS**

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ABSTRACT

Oxidative stress is one of the major pathological features of Alzheimer's disease (AD). Here we investigated whether dietary vitamin E depletion may induce adverse effects and supplementation with alpha tocopherol (α T) may result in beneficial effects on redox status and the regulation of genes relevant in the pathogenesis of AD in healthy rats. Three groups of 8 male rats each were fed diets with deficient (<1 mg α T equivalents (α TE)/kg diet), marginal (9 mg α TE/kg diet) or sufficient (18 mg α TE/kg diet) concentrations of natural-source vitamin E for 6 months; a fourth group was fed the VE sufficient diet fortified with α T (total vitamin E, 146 mg α TE/kg diet). Feeding of the experimental diets dose-dependently altered α T concentrations in cortex and plasma. No significant changes in F_2 -isoprostane concentrations, activities of antioxidative enzymes (total SOD, SeGPx) and concentrations of glutathione, or the expression of AD-relevant genes were observed. In this non-AD model, depletion of vitamin E did not induce adverse effects and supplementation of α T did not induce positive effects on parameters related to the progression of AD.

INTRODUCTION

Oxidative stress appears to be centrally involved in the pathogenesis of Alzheimer's disease (AD) and many of the pathological processes involved in its development and progression are either caused by or lead to an augmented generation of reactive oxygen and nitrogen species (Christen, 2000). Therefore, the importance of vitamin E for the maintenance of neuronal integrity and brain function is becoming increasingly recognized.

Epidemiological studies indicate that a high intake of vitamin E from food sources, but not from dietary supplements, may contribute to the prevention of age-related neurodegenerative disorders such as AD (Morris *et al.*, 1998; Engelhart *et al.*, 2002; Morris *et al.*, 2005). Morris and colleagues evaluated the contribution of the different vitamin E congeners towards the prevention of AD. While a higher dietary intake of both α -tocopherol (α T) and γ -tocopherol (γ T) was associated with a decreased risk of developing AD, their data suggested that the combination of the different vitamin E isoforms may be more effective in preventing AD than the intake of α T alone (Morris *et al.*, 2005). In line with this, in some intervention studies supplementation of high doses of α T, the major lipid-soluble antioxidant in human plasma and the vitamin E congener with the highest biological potency (Food and Nutrition Board of the Institute of Medicine, 2000), failed to delay the onset of dementia (Fillenbaum *et al.*, 2005) and to slow down the progression of AD (Petersen *et al.*, 2005). Other clinical intervention studies employing high dosage α T supplements, on the other hand, found a significant reduction in AD progression (Sano *et al.*, 1997; Onofrij *et al.*, 2002).

Little is known regarding the long-term effects of dietary vitamin E on the expression of genes involved in the development or prevention of AD and on the antioxidant status in the healthy brain. The aim of the current study was therefore to investigate (i) if suboptimal supply of dietary vitamin E may cause unfavorable physiological changes with regard to parameters of oxidative stress and the expression of genes relevant in the pathogenesis of AD in the healthy brain and (ii) whether supplementation of a high dose of α T on top of an already sufficient dietary supply of vitamin E may induce AD-preventive effects.

MATERIALS AND METHODS

Experimental diets

The tocopherol-rich rapeseed variety S-NAP 944 (a gift from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany) was cold-pressed under a nitrogen atmosphere. The resulting oil was vacuum-filtered and contained 341 mg α T, 386 mg γ T and 11 mg δ T per kg oil. A part of the native rapeseed oil was stripped of tocopherols by column chromatography as described by Lampi et al. (1992). The fatty acid composition of the oil (oleic acid, 49.1% of total fatty acids; linoleic acid, 19.7%; linolenic acid, 10.8%; erucic acid, 7.2%; gadoleic acid, 4.9%; palmitic acid, 4.5%) was not altered by the removal of antioxidants (data not shown). The tocopherol-stripped oil was used (i) unmodified or (ii) blended at a ratio of 1:1 with the native oil, (iii) the native oil was used unaltered or (iv) fortified with 4 g natural RRR- α -tocopherol (Cognis, Monheim am Rhein, Germany) per kg oil. Butylated hydroxytoluene (Carl Roth GmbH, Karlsruhe, Germany; 200 mg/kg) was added to all oils as a preservative to protect the tocopherols from oxidative decay.

These oils were used to prepare diets (ssniff Spezialdiaeten GmbH, Soest, Germany) with vitamin E concentrations that, according to the *Nutrient Requirements of Laboratory Rats* (1995), can be classified as vitamin E-deficient (dVE), -marginal (mVE), -sufficient (sVE), and -fortified (fVE).

The composition of the four semi-synthetic experimental diets was as follows (g/kg diet): casein, 240; corn starch, modified, 480; glucose, 110; cellulose, 50; vitamin E-free vitamin premix (E15313-2), 10; mineral premix (E15000), 60; rapeseed oil, 50. All vitamin E in the experimental diets originated from rapeseed oil and were as follows (as analyzed by HPLC and given in mg/kg diet): dVE: α T, <1; γ T, <1; mVE: α T, 6; γ T, 11; sVE: α T, 12; γ T, 24; and fVE: α T, 140; γ T, 24.

Experimental animals and study design

The animal experiment was conducted in accordance with the German regulations on animal care and with permission of the responsible authority. Thirty-two male Fisher 344 rats (Charles River Laboratories, Sulzfeld, Germany) with a mean initial body weight of 51 ± 5 g were randomly divided into 4 groups of 8 animals each and fed the experimental diets for 6 months. The rats were housed in Macrolon III cages in a conditioned room ($22 \pm 2^\circ\text{C}$, 55%

relative humidity, 12 h light-dark cycle). The animals had free access to tap water and the experimental diets throughout the experiment. Food consumption and animal weight were recorded daily and weekly, respectively. At the end of the experiment, the rats were fasted for 12 h prior to CO₂-anaesthesia and decapitation. Blood samples were collected in tubes containing heparin as anticoagulant and plasma was obtained by centrifugation (4°C, 3000 x g, 10 min) and stored at -80°C until analyzed. The brain was excised and dissected; one part of the cortex and the hippocampus were stored in RNAlater (Qiagen, Hilden, Germany). The remaining brain was snap-frozen in liquid nitrogen and stored at -80°C.

Quantification of tocopherols

Tissues (100 mg) were weighed into test tubes with screw caps on ice. Ethanol (2 ml) containing 1% (by weight) ascorbic acid, 700 µl H₂O, and 300 µl saturated aqueous KOH were added. The samples were saponified at 70°C in a shaking water bath for 30 min, and then cooled on ice. Fifty µl BHT in ethanol (1 mg/ml) and 2 ml n-hexane were added, and the samples were centrifuged (4°C, 5 min, 167 x g) to aid phase separation. One ml of the supernatant were transferred to a clean tube and dried under a stream of nitrogen gas. The residue was dissolved in 250 µl mobile phase (methanol:water (98:2, v/v)) and injected into the HPLC system. Plasma samples (100 µl) were processed as described above, but without saponification. Tocopherols were analyzed using a Jasco (Gross-Umstadt, Germany) HPLC system (pump PU2080Plus, autosampler AS2057Plus, detector FP2020Plus). The separation of tocopherols was performed on a Waters Spherisorb ODS-2 column (100 mm x 4.6 mm, 3 µm). The fluorescence detector was set to an excitation wavelength of 296 nm and emission wavelength of 325 nm. The concentrations of α- and γ-tocopherols were quantified by use of authentic tocopherols (Calbiochem, Schwalbach, Germany) as external standards (limit of quantification, ≈ 50 pg). Analyses were performed in duplicate. Porcine plasma with known concentrations of αT and γT was analyzed in parallel as quality control. Plasma vitamin E concentrations were normalized for plasma cholesterol concentration, which was determined spectrophotometrically using a commercial kit (Fluitest[®] CHOL 4241, Biocon Diagnostik, Voehl/Marienhausen, Germany) according to the manufacturer's protocol.

Determination of rat cortex concentrations of F₂-isoprostanes

Cortex samples were homogenized using Dounce homogenizer to yield 10% homogenates in 180 mM KCL, 10 mM EDTA, 0.1 mM BHT, pH 7.4. To hydrolyse esterified F₂-isoprostanes, the cortex homogenates (100 µl) were treated with 4 M KOH at 45°C for 30 min and neutralized by addition of 4 M HCl (pH adjusted to 2.0 with 0.1 M HCl). 0.5 nanogram of 9α,11α-PGF_{2α}-d₄ (Cayman Chem. Co., Ann Arbor, MI) was used as an internal standard. Fatty acids, inclusively isoprostanes, were extracted two times by ethylacetate. The samples were centrifuged for phase separation and the upper phases were applied onto NH₂ cartridges. Solid phase extraction and derivatisation steps were performed as previously described (Wiswedel *et al.*, 2004). F₂-Isoprostanes were separated and measured by gas chromatography/ negative-ion chemical ionization mass spectrometry (DSQ/Trace GC Ultra, Thermo Fischer Scientific, Dreieich, Germany) with ammonia as reagent gas using selected ion monitoring of the carboxylate anion [M-181] at *m/z* 569 and *m/z* 573 for F₂-isoprostanes and the deuterated internal standard. The F₂-isoprostane peak co-eluted with authentic 8-iso-PGF_{2α}, but it is known that other F₂-isoprostane isomers occur at the same retention time (Morrow *et al.*, 2002). All analyses were performed in triplicate for each tissue sample. The protein content was determined using the method of Bradford (Bradford, 1976).

Antioxidant enzyme activities and glutathione concentration in the cortex

Cortex tissue (100 mg) was diluted 1:10 (by weight) in ice-cold phosphate-buffered saline (PBS) and homogenized. The homogenate was centrifuged (3000 x g, 4°C, 10 min), the supernatant collected and used for the measurements after appropriate dilution in PBS. Superoxide dismutase (total SOD) enzyme activity was measured according to the method of Marklund and Marklund (1974). Se-dependent glutathione peroxidase (SeGPx) activity was measured according to the method of Lawrence and Burk (1976) using bovine GPx (Sigma Aldrich, Darmstadt, Germany) to generate the external standard curve. GSH concentrations were measured in homogenates according to Tietze (1969) with modifications (Dringen & Hamprecht, 1996). All measurements were carried out on a DU 800 spectrophotometer (Beckman Coulter, Krefeld, Germany). Results for antioxidant enzyme activities and GSH concentrations were adjusted for protein content as assessed using a commercial kit (BCA Protein Assay; Pierce, Rockford, IL, USA).

RNA isolation and real-time qRT-PCR

Total RNA was extracted according to the RNeasy[®] Lipid Tissue Protocol (Qiagen). DNA digestion was performed with the RNase-Free DNase Set (Qiagen). The concentration of isolated RNA was spectrophotometrically determined (Beckmann Instruments; Munich, Germany) by measuring the absorbance at 260 nm, the purity was determined by the ratio of 260/280 nm and a ratio of 1.6-1.9 was considered as acceptable. RNA aliquots were stored at -80°C until PCR analysis. Primer sequences for real-time RT-PCR experiments were designed with Primer3 software (version 0.4.0; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pairs (Table 1) were obtained from MWG Biotech AG (Ebersberg, Germany). The Bcl-2 primer was obtained from Qiagen. One-step quantitative reverse transcriptase PCR was carried out with the QuantiTect[™] SYBR[®] Green RT-PCR kit (Qiagen). Real-time PCR amplification was performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Relative mRNA concentrations of genes were quantified by use of a standard curve. Target gene mRNA concentration was normalized to the mRNA concentration of the housekeeping gene β -actin.

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

Gene	Primer sequence <i>forward</i> (5'-3')	Primer sequence <i>reverse</i> (5'-3')
β -Actin	GGG GTG TTG AAG GTC TCA AA	TGT CAC CAA CTG GGA CGA TA
APP	CAG CGT GTC ATC CCA AAG T	GCG TGC TCG TTC TCA TCT C
Apb1	AGC AGT AAG CCG GAT CAA GA	GAT CCT CTG GGT GGA GAT GA
ADAM-10	GGG CAA TGT GCA GGT TCT AT	TGA ACT GCT TGT TCC ACT GC
BACE-1	AAT CAG TCC TTC CGC ATC AC	GGC TCG ATC AAA GAC CAC AT
ECE	CTA TGT CAG TGC CGA CTC CA	CCC AGC TGG ACC ATG TAG TT
IDE	TCA AAG GGC TGG GTA AAC AC	CCT TGC ACT CTT GGA AAA CC
NEP	ACC TAG CAG GTG GAC AGC AT	ACA CCT GGG CAA AGT TCA AG
Bax	AGC TGC AGA GGA TGA TTG CT	GAT CAG CTC GGG CAC TTT AG
AChE	CAG CAA TAC GTG AGC CTG AA	TTC CAG TGC ACC ATG TAG GA
α TTP	GCT TTT CAA ATT ACC CCA TC	GAT CCC ACG AAC TTT CAA TG
HO-1	GGG TGA CAG AAG AGG CTA AG	GCT GAT CTG GGA TTT TCC TC

Western blot experiments in cortex homogenates

Cortex tissue (100 mg) was homogenized in RIPA buffer (NaCl, 150 mM; sodium deoxycholate, 0.5%; SDS, 0.1%; NP-40, 1%; EDTA, 20 mM; Tris-HCl, 50 mM, pH 7.4; DTT, 1mM; protease inhibitor cocktail). Lysates were purified by centrifugation (4°C, 14000 x g, 30 min). Total protein concentrations in each lysate were quantified using a BCA Protein Assay Kit (Pierce). Total proteins of the lysate (40 µg per lane) were separated by SDS gel electrophoresis followed by transferring the proteins to a PVDF membrane, which were then blocked for 1.5 h in blocking buffer (5% non-fat milk in Tris-buffered saline (TBS)). Primary antibodies were diluted in 5% non-fat milk (Bax, Bcl-2, 1:200; β-actin, 1:1000) and the blots were incubated over night at 4°C. The blots were washed and incubated with secondary antibody (Santa Cruz Biotechnology; anti-mouse horseradish peroxidase) in blocking buffer (Bax, Bcl2, 1:5000; β-actin, 1:1000) with gentle agitation for 1 h at room temperature. The blots were washed and exposed to Immun-Star Western Chemiluminescent Kit (Bio-Rad Laboratories, Hercules, CA, USA) and scanned with a Chemidoc (Bio-Rad). Digital images were captured and quantified using the Quantity-One system (Bio-Rad). Relative concentration of proteins were quantified as the ratio between the amount of target protein and the amount of the housekeeping protein β-actin.

Statistical analysis

Statistical calculations were conducted with SPSS Version 13.0 (SPSS, Munich, Germany). For the comparison of group means, one-way ANOVA was performed with the Scheffé test (normal distribution, homogeneity of variances) or the Games-Howell test (normal distribution, heterogeneity of variances) as post-hoc tests. When data were not normally distributed, Mann-Whitney-U-test was performed. Significance was accepted at $P < 0.05$.

RESULTS**Feed intake and bodyweight gain**

Total feed intake (data not shown) and final bodyweights (dVE, 362.8 g ± 11.5 g; mVE, 366.6 g ± 18.3 g; sVE, 372.1 g ± 21.2 g; fVE, 349.9 g ± 21.0 g; means ± SD) did not differ between the dietary groups.

Plasma and tissue concentrations of α - and γ -tocopherols

Plasma concentrations of total cholesterol were significantly lower in the dVE group than in the mVE ($P<0.05$) and fVE ($P<0.001$) groups (dVE, 2.23 ± 0.22 mmol/L; mVE, 2.6 ± 0.24 mmol/L; sVE, 2.51 ± 0.17 mmol/L; fVE, 2.80 ± 0.24 mmol/L as means \pm SD; one-way ANOVA with Scheffé post-hoc test) and were used to normalize plasma tocopherol concentrations.

Consumption of the experimental diets with graded concentrations of VE for 6 months significantly and dose-dependently affected α T concentrations in plasma, lung, muscle, cortex, and hippocampus of male Fisher 344 rats (Table 2).

Table 2: Effect of tocopherol intake on tocopherol concentrations in rat plasma, lung, muscle, cortex, and hippocampus.

Tissue	Diet				<i>P</i> less than
	dVE	mVE	sVE	fVE	
Plasma	<i>n</i> =8	<i>n</i> =8	<i>n</i> =8	<i>n</i> =8	
(nmol/ml)					
α T	0.34 ± 0.01^A	10.8 ± 0.49^B	17.7 ± 0.43^C	38.1 ± 1.26^D	0.001
γ T	0.01 ± 0.001^A	0.57 ± 0.01^B	0.55 ± 0.02^B	0.07 ± 0.01^C	0.001
(nmol/mol cholesterol)					
α T	0.15 ± 0.002^A	4.15 ± 0.09^B	7.05 ± 0.15^C	13.6 ± 0.18^D	0.001
γ T	$<0.01^A$	0.22 ± 0.01^B	0.22 ± 0.01^B	0.02 ± 0.002^C	0.001
Lung (nmol/g)					
α T	0.40 ± 0.04^A	12.1 ± 0.39^B	21.2 ± 1.24^C	43.1 ± 1.21^D	0.001
γ T	0.04 ± 0.02^A	0.54 ± 0.02^B	0.65 ± 0.03^C	0.09 ± 0.02^A	0.05
Muscle (nmol/g)					
α T	0.39 ± 0.04^A	7.37 ± 0.28^B	11.4 ± 0.64^C	20.0 ± 0.92^D	0.001
γ T	0.03 ± 0.004^A	0.21 ± 0.01^B	0.36 ± 0.03^C	0.06 ± 0.005^D	0.05
Cortex (nmol/g)					
α T	3.78 ± 0.44^A	16.7 ± 0.97^B	21.2 ± 1.27^B	30.0 ± 1.59^C	0.001
	<i>n</i> =8	<i>n</i> =8	<i>n</i> =6	<i>n</i> =8	
γ T	0.01 ± 0.001^A	0.13 ± 0.01^B	0.16 ± 0.01^B	0.05 ± 0.005^C	0.001
Hippocampus (nmol/g)	<i>n</i> =7	<i>n</i> =6	<i>n</i> =5	<i>n</i> =6	
α T	2.00 ± 0.39^A	$7.86 \pm 1.60^{A,B}$	$9.64 \pm 1.30^{B,C}$	15.6 ± 1.52^C	0.05
γ T	n.d. ^b	n.d.	n.d.	n.d.	

a: *n*=number of observations; values represent means \pm SEM. Values within each row not sharing a common superscript roman letter are statistically different at *P*.

F₂-Isoprostanes and antioxidant enzymes in the cortex

Concentrations of F₂-isoprostanes in cortex homogenates were not significantly different between the dietary groups (Figure 1).

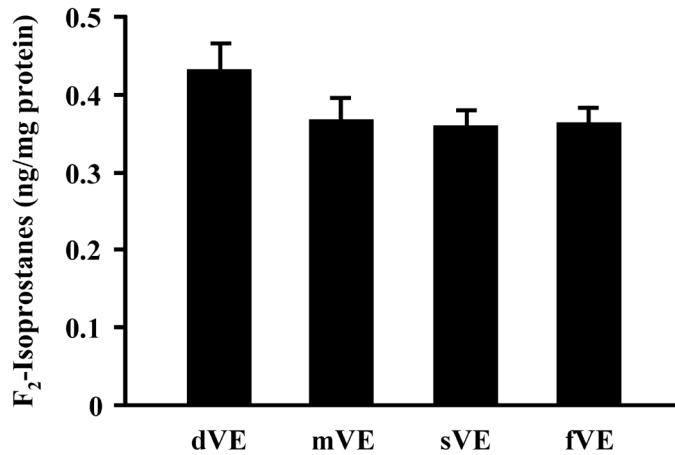


Figure 1: F₂-isoprostane concentrations (ng/mg protein) in the cortex of Fisher 344 rats fed the experimental diets for 6 months. Values are means ± SEM, n=8 per group. No statistical differences were observed.

Superoxide dismutase and glutathione peroxidase activities as well as glutathione concentrations in the cortices of the rats were similar in all groups (Table 3).

Table 3: Antioxidant enzyme activities and glutathione concentrations in the cortex. *

Experimental groups	Antioxidative enzymes		
	SOD (U/mg protein)	SeGPx (mU/mg protein)	GSH (nmol/mg protein)
dVE	13.8 ± 0.77	88.0 ± 4.67	24.9 ± 1.40
mVE	13.0 ± 0.79	95.6 ± 5.20	25.1 ± 0.76
sVE	13.5 ± 0.51	90.4 ± 4.35	23.9 ± 0.76
fVE	14.2 ± 0.55	98.0 ± 6.61	26.0 ± 0.49

* Values are means ± SEM, n=8 per group. No statistical differences were observed.

Gene expression in cortex and hippocampus

In cortex and hippocampus, the differences in dietary supply with vitamin E did not result in significant changes in relative mRNA concentrations of APP (amyloid beta (A4) precursor protein), Apba1 (APP binding family member 1), ADAM-10 (a disintegrin and metalloprotease domain 10), BACE-1 (beta-site APP cleaving enzyme 1), ECE (endothelin-converting enzyme), IDE (insulin degrading enzyme), NEP (neprilysin), Bax (Bcl2-associated X protein), Bcl-2 (B-cell leukemia/lymphoma 2), AChE (acetylcholinesterase), α TTP (α tocopherol transfer protein), and HO-1 (heme oxygenase 1) (Table 4).

Table 4: Relative mRNA expression (related to the housekeeping gene β -actin) of Alzheimer’s disease relevant genes in cortex and hippocampus.

Gene	Experimental group			
	dVE	mVE	sVE	fVE
<i>Cortex</i>				
APP	1.21 ± 0.06	1.13 ± 0.08	1.13 ± 0.10	1.21 ± 0.04
Apba1	1.06 ± 0.11	1.13 ± 0.11	1.35 ± 0.14	1.16 ± 0.09
ADAM-10	0.94 ± 0.08	0.91 ± 0.08	1.06 ± 0.06	0.97 ± 0.08
BACE-1	0.96 ± 0.07	0.88 ± 0.09	1.06 ± 0.08	0.98 ± 0.08
Bax	0.94 ± 0.04	0.89 ± 0.07	1.08 ± 0.07	0.96 ± 0.03
Bcl-2	1.09 ± 0.05	1.04 ± 0.05	1.16 ± 0.07	1.14 ± 0.05
ECE	1.03 ± 0.06	0.87 ± 0.05	1.10 ± 0.09	0.96 ± 0.04
IDE	0.92 ± 0.04	0.86 ± 0.07	1.07 ± 0.06	0.97 ± 0.08
Neprilysin	1.58 ± 0.29	1.18 ± 0.30	1.25 ± 0.17	1.12 ± 0.06
α TTP	1.06 ± 0.06	1.08 ± 0.09	1.17 ± 0.11	1.10 ± 0.06
HO-1	1.35 ± 0.09	1.39 ± 0.10	1.45 ± 0.13	1.31 ± 0.11
AChE	1.35 ± 0.23	1.04 ± 0.09	1.11 ± 0.06	1.27 ± 0.10
<i>Hippocampus</i>				
APP	0.89 ± 0.08	0.88 ± 0.04	1.01 ± 0.08	0.95 ± 0.07
Apba1	0.94 ± 0.06	0.92 ± 0.06	0.95 ± 0.08	0.95 ± 0.06
ADAM-10	0.90 ± 0.06	0.83 ± 0.03	1.00 ± 0.04	0.83 ± 0.05
BACE-1	0.79 ± 0.06	0.79 ± 0.03	0.76 ± 0.04	0.73 ± 0.02
Bax	1.02 ± 0.08	0.84 ± 0.03	0.90 ± 0.02	0.90 ± 0.04
Bcl-2	1.15 ± 0.08	1.01 ± 0.04	1.15 ± 0.05	1.08 ± 0.05
ECE	0.95 ± 0.06	0.90 ± 0.04	0.95 ± 0.04	0.89 ± 0.05
IDE	0.93 ± 0.12	0.88 ± 0.07	0.99 ± 0.08	0.86 ± 0.09

Table 4 (continued)

Neprilysin	0.88 ± 0.07	0.98 ± 0.06	0.94 ± 0.05	0.89 ± 0.05
αTTP	0.99 ± 0.07	0.91 ± 0.04	1.01 ± 0.09	1.02 ± 0.05
HO-1	0.85 ± 0.10	0.68 ± 0.06	0.81 ± 0.05	0.63 ± 0.04
AChE	0.96 ± 0.06	1.02 ± 0.10	1.05 ± 0.06	0.94 ± 0.07

Protein concentrations of apoptosis markers in cortex homogenates

Protein concentrations of Bcl-2 and Bax in the cortex did not differ between the groups (Figure 2). Protein concentrations of activated caspases 3 and 8 were undetectable (data not shown).

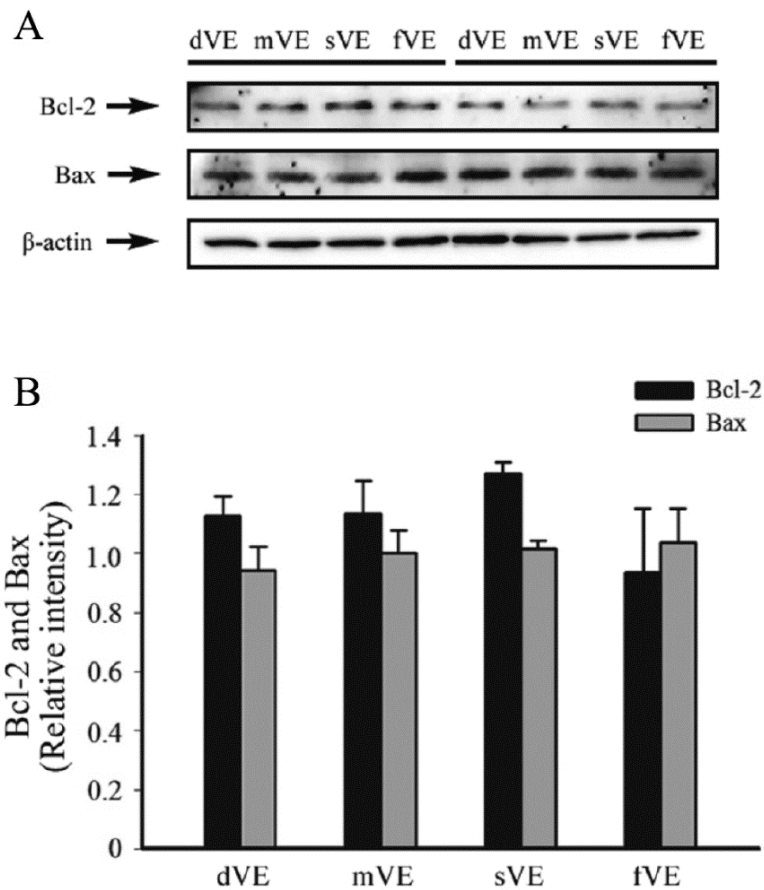


Figure 2: Bcl-2, Bax and β-actin protein concentration in cortex homogenates (A). β-Actin served as endogenous (loading) control. Relative concentration of Bcl-2 and Bax (B). Values are means ± SEM, n=6.

DISCUSSION

Epidemiologic as well as clinical intervention studies indicate that a high intake of vitamin E may be important for the prevention of age-related neurodegenerative disorders such as AD (Grundman *et al.*, 2002). Of the different tocopherols present in foods, RRR- α -tocopherol (RRR- α T) is thought to be the most important to human health and has the highest vitamin E activity in humans and animals (Food and Nutrition Board of the Institute of Medicine, 2000). Plant oils are the major dietary source of tocopherols and rapeseed oil, due to its high concentrations of tocopherols and ω -3 fatty acids (Grusak & DellaPenna, 1999), is considered of high value for human nutrition. Therefore, in the present study, we investigated the effects of graded dietary concentrations of vitamin E from rapeseed oil on the antioxidant status and the regulation of genes relevant in the pathogenesis of AD in the brains of Fisher 344 rats during growth and maturation. During the 6 months-feeding period of the present experiment, no differences in feed intake and life weight gain were observed between the dietary groups, which is in accordance with our previous findings (Barella *et al.*, 2004).

VE in plasma and tissues

In correspondence with the intake of α T, we observed dose-dependent changes in the concentrations of α T in brain (cortex and hippocampus), plasma and other tissues in our rats (Figure 1). In accordance with data from previously published rat experiments (Vatassery *et al.*, 1988; Martin *et al.*, 1999), the changes in α T concentrations in the brain, however, were less pronounced than the changes observed in all other tissues. Similarly, in Parkinson's disease patients who, over a period of 5 months, consumed high amounts of α T (from 400 IU/d during the first month to 4000 IU/d during the last month of supplementation), Pappert and colleagues observed large increases in α T concentrations in plasma (from 19 to 111 μ mol/l) while its concentrations in ventricular cerebrospinal fluid (CSF) remained unchanged (in a range of 0.114-0.164 μ mol/l) (Pappert *et al.*, 1996).

In our rats, a deficient supply of α T (<1 mg/kg diet) resulted in an almost complete depletion of α T in the plasma (0.15 \pm 0.002 mmol/mol cholesterol), while again changes in the brain were less pronounced (Table 2). In the cortex and hippocampus, 3.7 \pm 0.4 and 2.0 \pm 0.4 nmol α T/g tissue, respectively, were retained even after 6 months of deficient vitamin E intake. These data are in agreement with the results of Clement *et al.* (1995), who fed rats from

vitamin E deficient mothers a vitamin E-deficient diet for 8 weeks, and observed a marked reduction in the concentrations of α T in serum, liver, and muscle, while significant concentrations of α T were preserved in all analyzed nervous tissues. Vatassery et al. (1988) observed that even though other tissues could be almost completely depleted of α T by feeding of a VE-deficient diet for 4 months, in the brain low levels of α T were retained. Combined, these results and ours suggest that the brain is especially efficient at maintaining α T within a certain physiological range, preventing both excessive α T accumulation as well as its depletion.

In this study, the dVE, mVE, and sVE diets provided increasing concentrations of α T and γ T. The fVE diet, on the other hand, was prepared by addition of α T to the natural rapeseed oil and thus provided the same amount of γ T as the sVE diet but ~ 10 x more α T. With decreasing γ T concentrations in the dVE, mVE, and sVE diets, we observed dose-dependent decreases in the concentrations of γ T in lung, muscle and cortex tissue but not in plasma. Interestingly, the fVE-fed rats consuming additional α T, also had significantly reduced plasma and tissue γ T concentrations compared to sVE-fed rats despite a similar γ T intake (Table 2). In humans, the phenomenon of reduced γ -T concentrations caused by a high intake of α T, was first described by Handelman and co-workers. In 86 subjects (>50 years of age, mean age 71.8 ± 6.4), Handelman and colleagues observed an inverse relationship between α T and γ T concentration in the plasma. Supplementation of 8 volunteers (30-60 years of age) with 1200 IU all-rac- α T per day for 8 weeks resulted in a significant increase of plasma α T and a significant decrease in γ T (Handelman *et al.*, 1985). These findings were later confirmed in humans by other researchers (Melchert & Pabel, 1998; Huang & Appel, 2003) and are thought to be the result of an induction of cytochrome P₄₅₀ enzymes, which are involved in VE metabolism and preferentially degrade the desmethyl vitamers (Sontag & Parker, 2002), by α T.

F₂-Isoprostanes

With increasing age, lipid peroxidation of membrane fatty acids in the brain appears to augment (Floyd & Hensley, 2002) and oxidative stress in the brain is thought to be an important factor in the pathology of AD (Grundman *et al.*, 2002). Accordingly, concentrations of F₂-isoprostanes, which are non-enzymatic free radical-induced peroxidation products of arachidonic acid (Morrow *et al.*, 1990), have been found to be significantly higher in the CSF or brains of AD patients than those of matched healthy controls (Montine *et al.*, 2004). We

investigated the effects of increasing doses of dietary VE on F₂-isoprostane concentrations in the cortex of male Fisher 344 rats and found a non-significant trend towards higher F₂-isoprostane concentrations in the group fed the dVE diet, but overall no significant differences were observed between the dietary groups (Figure 1). Similar to our findings, Cuddihy and colleagues did not observe significant differences between the F₂-isoprostane concentrations in the cortices of mice fed vitamin E-deficient or -sufficient diets for 40 weeks. Surprisingly, cortex concentrations of F₄-neuroprostanes, which are oxidation products of docosahexaenoic acid (22:6), an abundant fatty acid in the brain, were positively correlated with α T concentrations (Cuddihy *et al.*, 2008).

Our results indicate that the low concentrations of α T retained in the brain may have been sufficient to protect the cortex from lipid peroxidation.

Another aim of this study was to investigate whether or not the intake of different amounts of VE may influence the activity of antioxidative enzymes and modulate cellular glutathione levels. Activities of total SOD and SeGPx as well as concentrations of total GSH in the cortices of our rats did not differ between the experimental groups. It appears likely that the lack of oxidative stress in our animals may be responsible for the absence of differences in the activities and concentrations, respectively, of antioxidant enzymes. Supplementation with α T (fVE group) did not result in enhanced enzyme activities or concentration, either. However, there is no consistent opinion regarding this in the literature: while some groups report no changes of antioxidative enzyme activities or concentrations (Kolosova *et al.*, 2006), others report increases in the activities or concentrations of antioxidant enzymes caused by vitamin E depletion (De & Darad, 1988) as well as supplementation (Devi & Kiran, 2004).

Gene expression

The accumulation and aggregation of misfolded proteins (particularly amyloid- β), the loss of neurons due to programmed cell death (e.g. apoptosis), and impaired neuronal signal transduction in specific parts of the brain are major pathological features of AD (Christen, 2000). Therefore, we measured mRNA concentrations of genes involved in the formation (APP, Apba1, ADAM-10, BACE-1) or degradation (NEP, IDE, ECE) of amyloid- β , programmed cell death (Bcl-2, Bax), and neurotransmitter degradation (AChE) in the brains of our rats.

In cortex and hippocampus of our rats, dietary VE did not affect mRNA concentrations of the measured genes (Table 4). Some of the analyzed genes (e.g. AChE, BACE-1; (Melo *et al.*, 2003; Tong *et al.*, 2005)) are thought to be regulated by oxidative stress, others experience age-related loss of activity and/or protein concentration (NEP, IDE; (Fukami *et al.*, 2002; Caccamo *et al.*, 2005)). As an additional biomarker for oxidative stress, we determined heme oxygenase type 1 (HO-1) mRNA concentrations in the brains of our rats. HO-1 is up-regulated in situations of oxidative and nitrosative stress, presenting a cytoprotective effect against various forms of stress by releasing the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin (Calabrese *et al.*, 2003). A significantly higher expression of HO-1 was observed in neurons and astrocytes of AD hippocampus and cerebral cortex samples relative to age-matched controls (Schipper *et al.*, 1995). Consistent with our F₂-isoprostane data, mRNA concentrations of HO-1 were not affected by VE depletion or supplementation, indicating the absence of oxidative stress in the rat brains in our study.

α TTP mRNA has been found in the human and rat brains, although at lower concentrations than in the liver (Hosomi *et al.*, 1998; Copp *et al.*, 1999). The exact function of α TTP in the brain is not known, but it was suggested to function in maintaining α T concentrations in the brain (Leonard *et al.*, 2002). In our study, α TTP mRNA concentrations were not changed by VE depletion or by α T supplementation. Other groups, however, observed that VE deficiency led to transcriptional alterations in the cortex (Hyland *et al.*, 2006) and hippocampus (Rota *et al.*, 2005) of rats as well as in α TTP^{-/-} mice (Gohil *et al.*, 2003). Rota and colleagues have shown that certain genes related to the pathophysiology of neurodegenerative diseases were regulated in rat hippocampi by dietary VE (Rota *et al.*, 2005). One main difference between these two studies is that we fed our rats for 6 months, while Rota and colleagues fed theirs for 9 months. In both studies, however, animals fed a VE deficient diet had significantly reduced VE concentrations in plasma and hippocampus, although the VE depletion of the brains remained incomplete, suggesting that the brain has a strong capability to retain VE.

Additionally, the diets in our trial contained the synthetic antioxidant BHT to protect the diets from lipid peroxidation during storage and BHT may have exerted additional antioxidant effects in our rats by direct scavenging of reactive species and/or regenerating α T from its radical form (Frank *et al.*, 2003). However, with 10 mg/kg, the concentration of BHT in our diets was low compared to 2000-4000 mg/kg diet employed in rat studies where antioxidant effects of BHT were observed (Kamal-Eldin *et al.*, 2000; Frank *et al.*, 2003). BHT is extensively metabolised in the liver and both BHT and its metabolites are excreted with the

bile, faeces and urine, which in turn results in a low rate of tissue accumulation of BHT (Daniel & Gage, 1965; Conacher *et al.*, 1986; Thompson *et al.*, 1991). Hence, potential antioxidant effects of BHT are likely to be more potent in the liver than in extra-hepatic tissues. However, hepatic HO-1 mRNA concentrations were significantly higher in the dVE than in the sVE animals in conjunction with reduced microRNA expression (Gaedicke *et al.*, 2008). Therefore, the absence of oxidative stress, as assessed by concentrations of F₂-isoprostanes and relative mRNA levels of HO-1, in the brains of our VE depleted as well as α T supplemented rats compared to control (sVE) is unlikely to be a result of the BHT in the diet.

Apoptosis seems to be an important process in AD, as suggested by increased levels of apoptosis markers in the brains of AD patients (Honig & Rosenberg, 2000). Bcl-2 and Bax, anti- and pro-apoptotic members, respectively, of the Bcl-2 family of signaling molecules, play a central role in apoptotic cell death. Both proteins function independently to regulate cell death, but can also form heterodimers. Therefore, the ratio of Bcl-2 to Bax is indicative of a pro- or anti-apoptotic cell status (Lu *et al.*, 2000). In our study, neither Bcl-2 nor Bax mRNA (Table 4) and protein (Figure 2) concentrations were different depending on the diet.

CONCLUSIONS

The present data suggest that stepwise reduction of dietary α T and γ T for 6 months does not impair antioxidant status, induce apoptosis or alter the expression of selected genes in the brains of rats. Similarly, α T supplementation of already VE sufficient animals does not improve antioxidant status, affect apoptosis or alter the expression of AD-relevant genes. In conclusion, VE does not appear to change parameters involved in the etiology and progression of AD in healthy animals. Further investigations into the impact of VE on these parameters in an AD disease model are warranted.

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CHAPTER IV

VITAMIN E DEPENDENT MICRO RNA REGULATION IN RAT LIVER

VITAMIN E DEPENDENT MICRO RNA REGULATION IN RAT LIVER

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ABSTRACT

Dietary vitamin E (VE) is known to regulate gene expression by altering mRNA concentrations. Recently, miRNAs have been discovered as a means of posttranscriptional gene regulation. Since the effect of VE on miRNA regulation is unknown, we fed rats for 6 months diets deficient or sufficient in VE and determined hepatic concentrations of miRNAs involved in processes previously associated with VE (lipid metabolism, miRNA-122a; cancer and inflammation, miRNA-125b). VE deficiency resulted in reduced concentrations of miRNA-122a and miRNA-125b. The findings of the present study demonstrate that differences in dietary VE may affect hepatic miRNA concentrations in vivo.

INTRODUCTION

In rats, dietary vitamin E (VE), particularly its major congener α -tocopherol (α T), has been shown to regulate gene expression by increasing or lowering mRNA concentrations in various tissues (Ricciarelli et al., 2000; Barella et al., 2004; Rimbach et al., 2004; Rota et al., 2004; Hundhausen et al., 2006; Vasu et al., 2007). In mammals, mRNA levels often correlate poorly with the respective protein concentrations (Nelson & Keller, 2007). The recent discovery of microRNAs (miRNAs) revealed a possible explanation for this phenomenon. miRNAs are a class of small, non-coding, double-stranded RNAs that, in their mature form, are single-stranded and ~22 nucleotides long. Mature miRNAs post-transcriptionally suppress gene expression by binding at the 3' untranslated region of mRNAs and inhibiting their translation into proteins (Boyd, 2008). It has been calculated that each miRNA binds on average 100 different target mRNAs, allowing for post-transcriptional silencing of many different genes, or potentially entire pathways, by a single miRNA (Lewis et al., 2005). miRNA are encoded for in the genome and are, thus, liable to regulation. Presently, the potential regulatory effects of dietary VE on the expression of miRNAs are unknown. In order to investigate the role of dietary VE on miRNA expression, we fed rats for 6 months diets deficient or sufficient in VE and analyzed miRNA concentrations in the liver. For this study, we selected miRNAs that were previously shown to be involved in processes that have been associated with VE, namely lipid metabolism (miR-122a) (Esau et al., 2006; Kutay et al., 2006), cancer progression and inflammation (miR-125b) (Porkka et al., 2007; Sonkoly et al., 2007; Ozen et al., 2008).

MATERIALS AND METHODS

Experimental animals and diets

Two groups of 8 recently weaned male Fisher 344 rats (mean body weight, 51±5 g; Charles River Laboratories, Sulzfeld, Germany) were randomly assigned to a vitamin E-containing (VE⁺) or a vitamin E-deficient diet (VE⁻). The composition of the semi-synthetic diets (ssniff Spezialdiaeten GmbH, Soest, Germany) was (g/kg diet): casein, 240; corn starch, modified, 480; glucose, 110; cellulose, 50; vitamin E-free vitamin premix (E15313-2), 10; mineral premix (E15000), 60; rapeseed oil, 50. All vitamin E in the diets originated from native or antioxidant-stripped rapeseed oils, respectively, and were as follows (mg/kg diet; analyzed by

HPLC): VE⁻: α T, <1; γ T, <1; VE⁺: α T, 12; γ T, 24. Because consumption of oxidized lipids may alter gene expression (Koch et al., 2007), great care was taken to protect the used oils and diets from oxidation. Peroxide values of the oils were <0.5 mEq O₂/kg, indicating that no lipid oxidation had occurred. Butylated hydroxytoluene (Carl Roth GmbH, Karlsruhe, Germany; 200 mg/kg) was added to all oils as a preservative. Diets were vacuum-packed with oxygen absorber pads in polyethylene and stored at -20°C. The animals had free access to tap water and the experimental diets throughout the experiment and were housed in pairs in a conditioned room (temperature, 22±2°C; relative humidity, 55%; 12 h light/dark cycle). The animal experiment was conducted in accordance with the German regulations on animal care and with permission of the responsible authority. Food consumption was recorded daily and animal weight weekly. At the end of the experiment, the rats were fasted for 12 h prior to CO₂-anaesthesia and decapitation. The liver was excised and dissected; one part was stored in RNAlater (Qiagen, Hilden, Germany) and the remainder snap-frozen in liquid nitrogen and stored at -80 °C until used.

Quantification of tocopherols

Tocopherols were quantified by HPLC with fluorescence detection as described by Augustin et al. (Augustin et al., 2008).

Total RNA isolation

Total RNA including miRNA were extracted by using the miRNeasy[®] Isolation Kit (Qiagen). DNA digestion was performed with the RNase-Free DNase Set (Qiagen). The concentration of isolated RNA was determined spectrophotometrically by measuring the absorbance at 260 nm; purity was determined using the ratio of 260/280 nm and a ratio of 1.6-1.9 was considered acceptable. Total RNA aliquots were stored at -80°C until PCR or TaqMan analysis.

Real-time qRT-PCR

Primer sequences for real-time quantitative RT-PCR experiments were designed with Primer3 software (version 0.4.0; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pairs (Table) were obtained from MWG Biotech AG (Ebersberg, Germany). One-step qRT-PCR was carried out with the QuantiTect[®] SYBR[®] Green RT-PCR kit (Qiagen). RT-PCR amplification was performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney,

Australia). Relative mRNA concentrations are given as the ratios between the amount of the target gene and the housekeeping gene β -actin.

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

Gene	Primer sequence <i>forward</i> (5'-3')	Primer sequence <i>reverse</i> (5'-3')
β -Actin	GGG GTG TTG AAG GTC TCA AA	TGT CAC CAA CTG GGA CGA TA
CD36	AAG CAA GGT TGC CAT AAT TG	CCA AAC ACA GCA TAG ATG GA
5 α -R1	TTA TGC TGA AGA CTG GGT GA	AAA TAG TTG GCT GCA GAT ACG
γ -GCS _m	TGT GTG ATG CCA CCA GAT TT	GCT TTT CAC GAT GAC CGA GT
α TTP	GCT TTT CAA ATT ACC CCA TC	GAT CCC ACG AAC TTT CAA TG
HO-1	GGG TGA CAG AAG AGG CTA AG	GCT GAT CTG GGA TTT TCC TC

TaqMan analyses

TaqMan[®] MicroRNA Assays were obtained from Applied Biosystems (ABI, Foster City, CA, USA). miRNA quantification was performed as two-step RT-PCR. Reverse transcription was performed in a thermocycler (Biometra, Goettingen, Germany) with specific miRNA primers from the TaqMan[®] MicroRNA Assays and reagents from the TaqMan[®] MicroRNA Reverse Transcription Kit. During the PCR step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan[®] Universal PCR Master Mix (ABI). PCR reaction was performed in an Applied Biosystems 7300 Real-Time PCR System. Target sequences were as follows: hsa-miR-122a, UGGAGUGUGACAAUGGUGUUUGU; hsa-miR-125b, UCCCUGAGACCCUAACUUGUGA; hsa-miR-146a, UGAGAACUGAAUCCAUGGGUU. Relative miRNA concentrations are given as the ratios between the amount of the target gene and the endogenous control snoRNA (rat).

Western blot experiments in liver homogenates

Liver tissue (100 mg) was homogenized in RIPA buffer, lysates were purified by centrifugation (4°C, 14000 x g, 30 min) and total protein concentrations in each lysate were quantified using a BCA Protein Assay Kit (Pierce). Total proteins of the lysate (40 μ g per lane) were separated by SDS gel electrophoresis followed by transferring the proteins to a PVDF membrane. Samples were then incubated with the primary antibodies (diluted in 5% non-fat milk (1:1000)) and the secondary antibodies (Santa Cruz Biotechnology; anti-mouse

horseradish peroxidase) in blocking buffer (anti-HO-1, 1:4000; anti- β -actin, 1:1000). The blots were exposed to Immun-Star Western Chemiluminescent Kit (Bio-Rad Laboratories, Hercules, CA, USA) and scanned with a Chemidoc (Bio-Rad). Digital images were captured and quantified using the Quantity-One system (Bio-Rad). Relative concentration of proteins were quantified as the ratio between the amount of target protein and the amount of the housekeeping protein β -actin.

Statistical analyses

Statistical comparisons were made by means of a one-tailed unpaired t-test (normal distribution, homogeneity of variances), Welch-corrected t-test (heterogeneity of variances), or a Mann-Whitney U test (skewed data) as appropriate using the software InStat 3 for Macintosh (version 3.0b; GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Neither symptoms of ataxia, a classical sign of severe VE-deficiency, nor differences in feed intake and life weight gain were observed in the animals fed vitamin E deficient (VE⁻) or VE-sufficient (VE⁺) diets for 24 weeks (data not shown), which is in accordance with previous findings (Barella et al., 2004). As expected, hepatic α T concentrations were reduced in the VE⁻ rats (Figure 1A), in agreement with the literature (Vatassery et al., 1988).

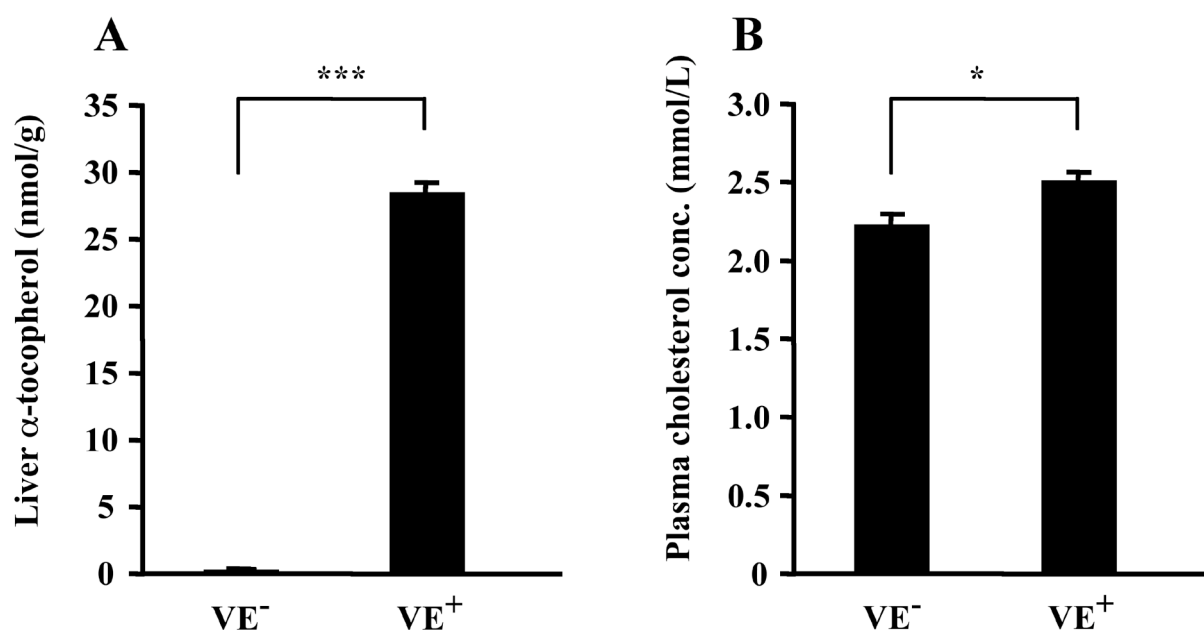


Figure 1: Effect of dietary vitamin E on α -tocopherol concentrations in the liver (A) and plasma cholesterol concentrations (B) of rats fed vitamin E deficient (VE⁻) or sufficient (VE⁺) diets for 6 months. Values are expressed as means \pm SEM, n=8. ***P<0.0001.

Differences in hepatic α T were previously shown to affect relative mRNA concentrations in the liver of rats (Barella et al., 2004). In order to verify the differential expression of genes regulated by α T, we quantified relative mRNA concentrations of known VE sensitive genes in the livers of our rats. As expected, mRNA concentrations of CD36 and 5 α -R1 were significantly increased, and mRNA concentrations of α TTP and γ -GCS_m were significantly decreased in the VE⁻ animals (Figure 2).

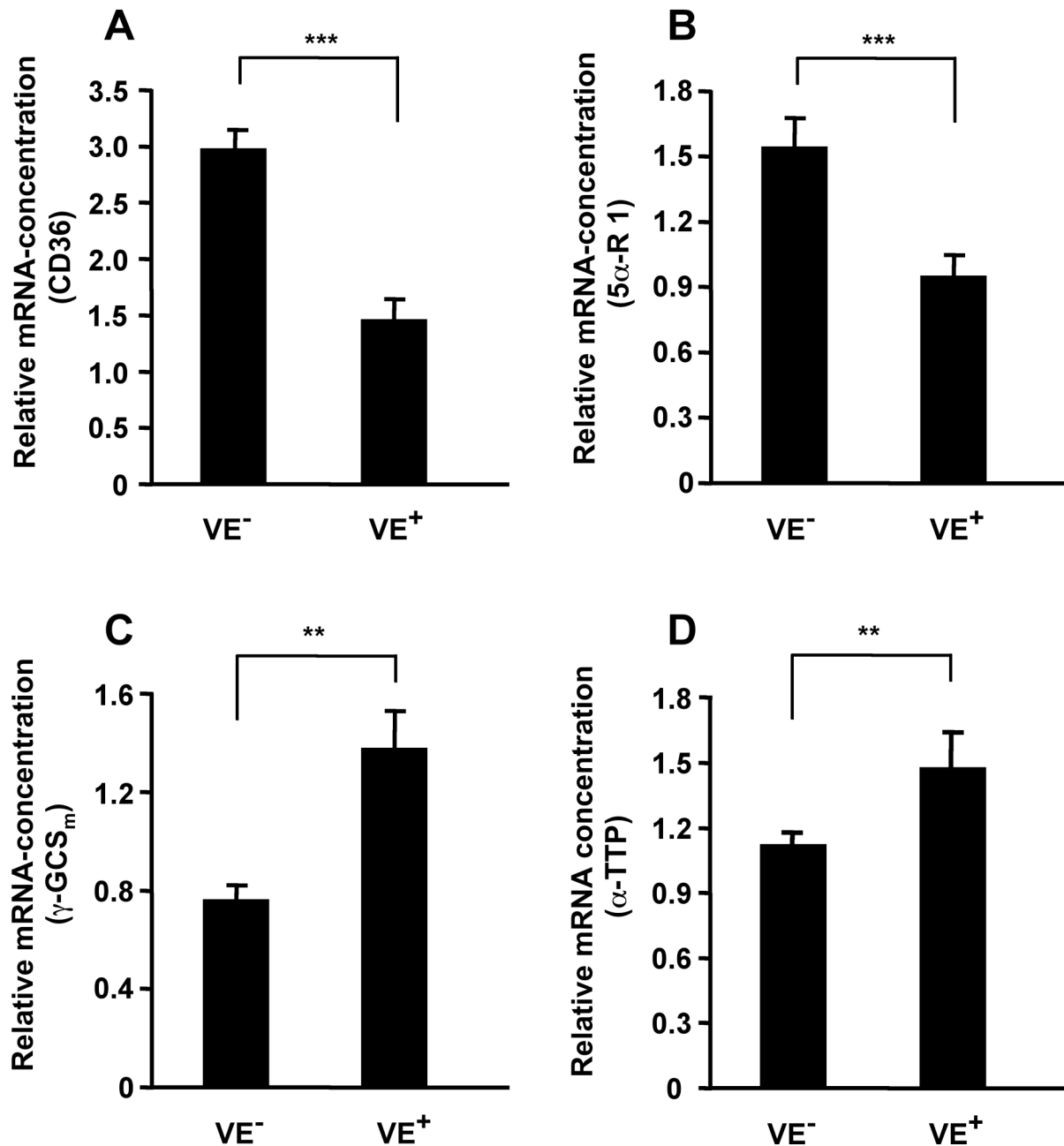


Figure 2: Effect of dietary vitamin E on (A) scavenger receptor CD36, (B) 5- α -steroid reductase type 1 (5 α -R1), (C) regulatory subunit gamma-glutamylcysteinyl synthetase (γ -GCS_m), and (D) α tocopherol transfer protein (α TTP) mRNA concentrations in rat liver. Values are means \pm SEM, n=8. **P<0.001, ***P<0.0001.

Recently, miRNA have been discovered as molecules that effect post-transcriptional gene-silencing by a process termed RNA interference (Rana, 2007). To date, the impact of dietary VE on hepatic miRNA concentrations is unknown. VE has long been investigated for its potential cardiovascular disease (CVD) and cancer preventive properties (Jialal et al., 2001;

Tucker & Townsend, 2005). Increased circulating concentrations of blood lipids, especially cholesterol, and pro-inflammatory cytokines have been associated with increased risk for CVD and cancer (Belluco et al., 2000; Scott, 2004). For the current investigations, we selected two miRNAs (122a, 125b) that were shown to regulate genes associated with lipid metabolism and cancer. Because miRNAs are evolutionary conserved across species, the chosen miRNAs are found in rat as well as in human tissue (Bartel, 2004).

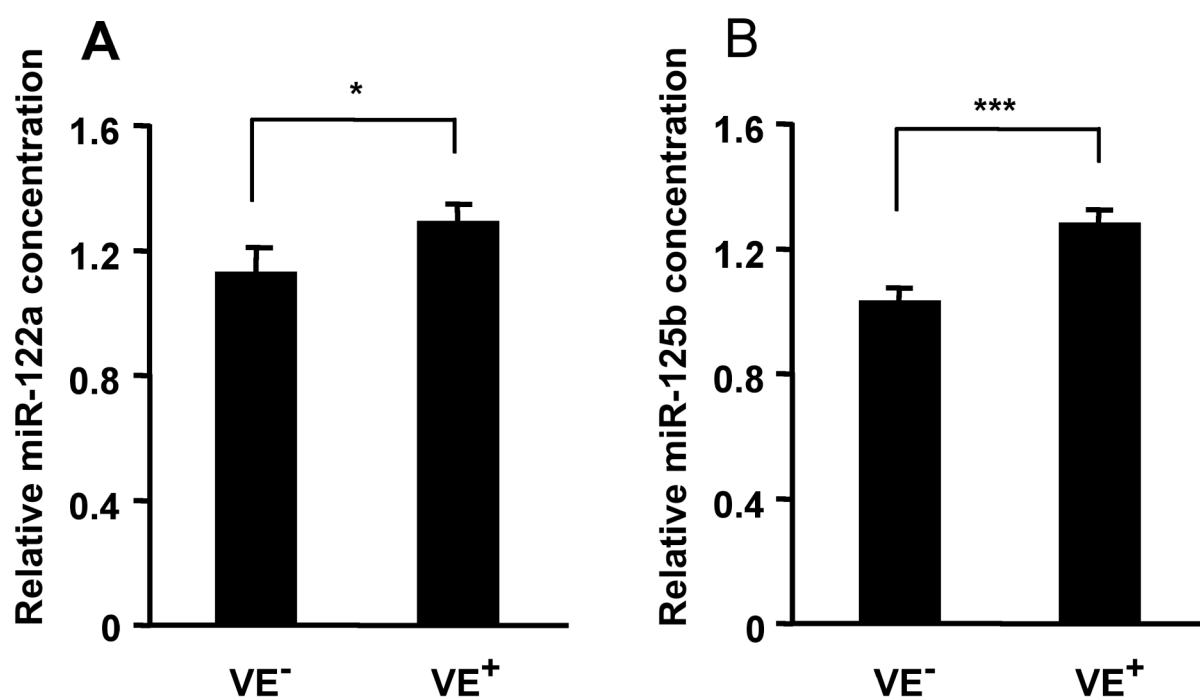


Figure 3: Effect of dietary vitamin E on miR-122a and miR-125b concentrations in rat livers. Values are means \pm SEM, n=8. *P<0.05, ***P<0.0001.

miRNA-122 is liver-specific and the most abundant miRNA in the liver (Girard et al., 2008). In our rats, concentrations of miR-122a in the liver (Figure 3) and cholesterol in plasma (Figure 1B) were significantly lower in the VE⁻ than in the VE⁺ group. Esau and co-workers (2006) used antisense oligonucleotide inhibition to specifically inhibit miR-122 in mice, which resulted in significantly increased hepatic concentrations of 108 mRNAs related to lipid metabolism and significantly reduced plasma cholesterol and triacylglycerol concentrations. A comparable experiment recently supported the finding that cholesterol concentrations are only weakly reduced upon targeted inhibition of miR-122, although a large

number of genes related to lipid metabolism were differentially regulated (Elmen et al., 2008). Apart from its role in lipid metabolism, miR-122 was down-regulated in rodent and human hepatocellular carcinomas (Kutay et al., 2006) and is thought to play a critical role in liver development (Chang et al., 2004). Shan and colleagues demonstrated that inactivation of miR-122 by antagomir 122, a chemically synthesized oligonucleotide with sequence complimentary to miR-122, resulted in decreased Bach-1 and increased HO-1 mRNA concentrations (Shan et al., 2007). Bach-1 is a repressor of the redox-sensitive transcription factor nuclear factor erythroid 2-like 2 (Nrf2), which controls HO-1 expression (Reichard et al., 2007). In agreement, HO-1 mRNA as well as protein concentrations were increased in our VE deficient rats (Figure 4). Because oxidative stress is known to cause Nrf2 activation (Itoh et al., 2004), the induction of HO-1 expression in our rats might also have occurred by a miR-122 independent mechanism.

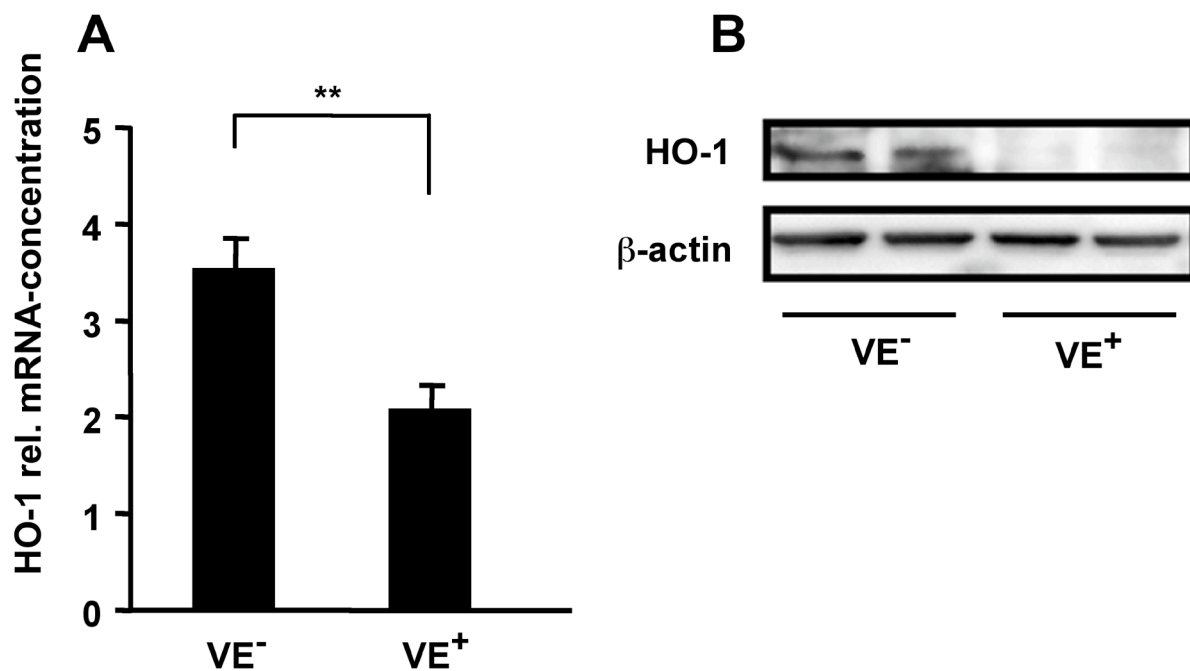


Figure 4: Effect of dietary vitamin E on (A) HO-1 mRNA concentration (values are means \pm SEM, n=8, **P<0.001) and (B) protein levels of HO-1 and β -actin (loading control). Western blots of two representative animals per group are shown.

Vitamin E-depletion in our rats caused significant, but relatively weak changes in miR-122a when compared to targeted inhibition (Esau et al., 2006; Elmen et al., 2008). In fact, dietary

factors are not expected to exert drastic effects on miRNA expression (Barella et al., 2004). Nevertheless, even small changes in hepatic miRNA expression over a prolonged period may be of biological significance.

In our study, miR-125b was significantly down-regulated in the VE⁻ as compared to the VE⁺ animals (Figure 3). A role for miRNA-125b in cancer development has been suggested because miRNA-125b was down-regulated in human prostate cancer tissues (Porkka et al., 2007; Ozen et al., 2008), lung cancer cell lines (Nagayama et al., 2007), breast cancer (Iorio et al., 2005), and in squamous cell carcinoma of the tongue (Wong et al., 2008). Shi and colleagues (2007), on the other hand, found over-expression of miR-125b in prostate cancer cell lines and prostate cancer samples. Retroviral infection of the breast cancer cell line SKBR3 with constructs expressing 125b resulted in ca. 35% suppression of the oncogenic proteins ERBB2 and ERBB3 at the transcript as well as protein levels, and the cells exhibited reduced migration and invasion capacities (Scott et al., 2007). A role of miRNA-125b in inflammation is supported by a study that identified TNF α as a direct target of miRNA-125b. A decrease of miR-125b resulted in increased TNF α production and inflammation in LPS stimulated macrophages (Tili et al., 2007). Thus, the reduced miR-125b levels observed in our VE deficient rats may be associated with an enhanced inflammatory response due to vitamin E deficiency, as previously described (Lim et al., 2008; Yamaoka et al., 2008).

In conclusion, in rats fed a VE-depleted diet for 6 months, VE deficiency resulted in reduced concentrations of miRNA-122a and miRNA-125b, which may play an important role in lipid metabolism, carcinogenesis, and inflammation. Thus, the gene regulating properties of α -tocopherol may be, at least partly, mediated by changes in miRNA levels.

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CHAPTER V

GENERAL DISCUSSION

The present thesis aimed at studying the effects of vitamin E (VE) from the natural source rapeseed oil on the regulation of selected genes in the brain and liver of healthy rats. Rapeseed oil was chosen as source of vitamin E, because a high intake of vitamin E from food but not from dietary supplements was previously shown to contribute to the prevention of age-related neurodegenerative diseases (Engelhart et al., 2002; Morris et al., 2002; Morris et al., 2005). In the brain, the focus was set on genes relevant for the development and prevention of Alzheimer's disease. In the liver, emphasis was placed on the effects of dietary VE on the expression of miRNAs, a group of regulatory RNAs that post-transcriptionally silence genes (see Chapter I).

Vitamin E and neuroprotection

Oxidative stress appears to play an important role in the pathogenesis of AD (see also Chapter I). Decreased vitamin E concentrations were found in the cerebrospinal fluid of AD patients compared to healthy controls (Kontush & Schekatolina, 2004). Sano and co-workers (1997) demonstrated in a placebo-controlled trial, that the daily intake of 2000 IU vitamin E for 2 years could slow down the progression of the disease in patients with mild AD. A combined intake of vitamin E and C supplements resulted in a reduced prevalence and incidence of AD in a population aged 65 years and older (Zandi et al., 2004). In a transgenic AD mouse model, supplementation with vitamin E at an early stage reduced lipid peroxidation and amyloid beta deposits in the brain (Sung et al., 2004). Those trials indicate, that vitamin E intake may prevent or even slow down AD. Other trials, however, were not able to confirm these results. Supplementation of patients suffering from mild cognitive impairment (MCI) with 2000 IU/day vitamin E or placebo for 3 years did not result in significant differences in progression from MCI to AD (Petersen et al., 2005).

While the brain makes up only 2% of the overall body weight, it has the highest oxygen uptake rate of all organs with approximately 20% of the whole bodies oxygen consumption (Smith et al., 1999). Given the fact that, within the mitochondrial electron transport chain, 2-5% of the consumed oxygen is converted to superoxide, the brain is exposed to a constant flux of reactive species. The activities of antioxidant enzymes, such as catalase, superoxide dismutase and glutathione peroxidase, in the brain are only ~10% of the activities in the liver and other tissues (Floyd, 1999). Because of its high content of polyunsaturated fatty acids (PUFA), such as arachidonic acid (C20:4) and docosahexaenoic acid (C22:6), the brain is particularly vulnerable to oxidative insult (Smith et al., 1999). F₂-isoprostanes, for example,

are formed in vivo by non-enzymatic free radical-catalyzed peroxidation of arachidonic acid and contribute to inflammatory processes (Basu, 2008). Therefore, a sufficient supply of antioxidants, such as α T, is likely of particular importance for the brain.

Not only oxidative, but also nitrative stress appears to play an important role in the pathogenesis of AD. Reactive nitrogen species can be formed by the reaction of nitric oxide with reactive oxygen species and elevated concentrations of nitric oxide have been found in brains of AD patients (Floyd & Hensley, 2002). The γ T nitration product 5-nitro- γ T as well as the protein nitration products 3-nitrotyrosine and 3,3'-dityrosine were elevated in cortical regions of brains from AD patients as compared to age-matched controls (Hensley et al., 1998; Williamson et al., 2002). Due to its unsubstituted 5-position, γ T but not α T is capable of scavenging reactive nitrogen species, thus reducing nitrative stress (Williamson et al., 2002). Maintaining optimum γ T concentrations may, thus, be of particular importance in the prevention of age related dementias. Because γ T appears to have biochemical properties that may be important in the prevention of AD, a decrease in γ T concentrations due to high α T intake, as observed in our rats (Chapter III, Table 2), might be unfavourable and may even partly explain the negative outcomes of high dosage α T supplementation trials.

As outlined in the introduction (Chapter I), the lack of effective treatments for AD makes the prevention of the disease all the more important. Because of the observation that VE from food sources but not from supplements was associated with a reduced AD risk, we chose to study the effects of graded dietary doses of VE from a natural food source in a model simulating healthy and not demented subjects, as detailed in Chapter III.

Vitamin E and the expression of Alzheimer's disease-relevant genes

In order to study the effects of dietary vitamin E on the pathology of AD, transgenic mouse models, such as Tg2576 (developing A β plaques), APP23 (neuronal cell loss) or TAPP (exhibiting both A β plaque and neurofibrillary tangle pathology) are frequently employed (Higgins & Jacobsen, 2003). Here, we chose the non-transgenic rat strain "Fisher 344" as a model animal, because the focus of the present work was on the prevention of oxidative stress and neurodegeneration as well as the expression of AD-associated genes rather than the interference with pathological processes in progress.

Oxidative stress and the development of neurodegenerative diseases are tightly connected. Therefore, one aim of this study was to investigate the influence of vitamin E deficiency in

healthy animals on the occurrence of oxidative stress and its potential association with differential gene expression of Alzheimer's disease relevant genes. The VE-dependent expression of these genes (see below) was studied in hippocampus and cortex, the tissues primarily affected by AD.

Amyloid β ($A\beta$) is a 40-42 amino acid peptide that is formed by proteolytic cleavage of its precursor amyloid β precursor protein (APP) and is a major component of senile plaques, a characteristic hallmark of AD (Nunan & Small, 2000). APP processing and $A\beta$ generation can be slowed down by interaction of APP with the APP-stabilizing protein amyloid β (A4) precursor protein-binding, family A, member 1 protein (Apba1), which can preserve APP from cleavage by secretases (Sastre et al., 1998). Initial cleavage of APP may be facilitated by one of two different secretases, namely α - or β -secretase, and is followed by a second scission through a γ -secretase. α -Secretase cleaves APP within the amyloid sequence, which precludes the formation of the amyloidogenic peptide $A\beta$ and leads to the release of the neurotrophic, soluble APPs α into extracellular fluids following γ -secretase cleavage; APPs α represents a normal component of plasma and CSF (Lammich et al., 1999; Rota et al., 2005). Hydrolysis of APP by β -secretases prior to γ -secretase scission leads to the formation of the insoluble, neurotoxic $A\beta$. mRNA concentrations of the major secretases involved in $A\beta$ formation, namely ADAM-10, as a member of the α -secretase family (Nunan & Small, 2000) and BACE-1, the major β -secretase, were quantified. Although it is not yet fully understood how ADAM-10 and BACE-1 are regulated, oxidative stress was suggested to be directly responsible for BACE-1 activation and, thereby, the production of $A\beta$ (Tong et al., 2005).

Under physiological conditions, $A\beta$ is degraded by enzymes including neprilysin (NEP), insulin-degrading enzyme (IDE), and endothelin-converting enzyme (ECE), which all belong to a group of zinc-metalloproteinases. Diminished concentrations and activities of these enzymes may increase the risk for AD, whereas increases may be protective.

Achetylcholinesterase (AChE) is an enzyme cleaving the neurotransmitter acetylcholine, which is produced by cholinergic neurons. In AD, loss of cholinergic neurons in the basal forebrain occurs and leads to a diminished production of acetylcholine and, thus, to impaired signal transduction. In order to prevent rapid neurotransmitter degradation, some AD treatments are directed at enhancing the cholinergic system by inhibiting AChE (Holzgrabe et al., 2007). Bond and Greenfield observed increased mRNA concentrations of AChE as a result of oxidative stress in astroglia (Bond & Greenfield, 2007). In agreement, Melo and

colleagues found increased AChE activity due to A β induced oxidative stress in cultured retinal cells and its reduction by pre-incubation with VE (Melo et al., 2003).

In our model, neither dietary VE-depletion, nor supplementation with α T for 6 months resulted in the differential expression of the above-described genes involved in AD development (Chapter III , Table 4).

Vitamin E and gene regulation

In our study, mRNA concentrations of HO-1, a biomarker of oxidative stress, were significantly increased in the liver of animals fed the dVE diet compared to the other groups, whereas in cortex and hippocampus of the same animals no changes in HO-1 mRNA concentrations were observed. In situations of oxidative and nitrosative stress, the expression of the enzyme HO-1, which acts as a cytoprotectant by releasing the vasoactive molecule carbon monoxide and the potent antioxidant bilirubin, is induced (Calabrese et al., 2003). F₂-isoprostanes are accepted biomarkers for lipid peroxidation (Basu, 2008). Induction of oxidative stress resulted in decreased vitamin E concentration and significantly increased CAT, GPx, and SOD activities in rat cortices (Tejada et al., 2007). The fact that, in the cortex of our rats, neither F₂-isoprostane and glutathione concentrations nor the activities of the endogenous antioxidants SOD and Se-GPx were different between the experimental groups suggests that no oxidative stress had occurred in the brain. In the liver, on the other hand, the increases in HO-1 mRNA and protein concentrations are indicative of oxidative stress. Interestingly, in the liver, where differences in redox status were evident, differential expression of a number of genes involved in atherosclerosis, cancer, and antioxidant defence (see below) was observed as a result of the differences in vitamin E consumption.

Scavenger receptor CD36, which was up-regulated in our vitamin E deficient rats, is involved in lipid uptake and appears to play an important role in the pathology of atherosclerosis (Azzi et al., 2004). CD36 binds oxidized LDL (LDL_{ox}), incorporates LDL_{ox} into macrophages and, thereby, leads to foam-cell formation; consequently, it is found in atherosclerotic lesions (Podrez et al., 2002). It has been previously shown that incubation with α T decreased CD36 expression in human monocyte-derived macrophages (Devaraj et al., 2001) and cultured aortic smooth muscle cells, leading to a reduced uptake of LDL_{ox}. Down-regulation of CD36 by α T may thus be an important mechanism for the reduction of atherosclerosis risk (Ricciarelli et al., 2000).

Steroid-5 α -reductase (5 α -R1), a gene which is expressed in many tissues, especially in the liver (Russell & Wilson, 1994), was significantly up-regulated in response to VE depletion (Chapter IV, Figure 2). Dihydrotestosterone, the most potent androgen, is irreversibly transformed from testosterone by 5 α -R1 (Russell & Wilson, 1994). Androgens play an important role in the development of prostate cancer (Thomas et al., 2008; Tindall & Rittmaster, 2008) and 5 α -R1 mRNA concentrations were reported to be significantly increased in prostate cancer compared to normal tissue (Iehle et al., 1999). Therefore, the inhibition of 5 α -R1 might represent a potential therapeutic benefit against the development of prostate cancer. Conclusive evidence from human intervention trials with vitamin E is still lacking. Nonetheless, vitamin E supplementation resulted in a significant reduction in the incidence of prostate cancer in smokers (Heinonen et al., 1998). A recent meta-analysis supports a protective role of α T in men who are at high risk of developing prostate cancer (Alkhenizan & Hafez, 2007).

The expression of mRNA for α TTP, a protein important for the retention of α T and mainly expressed in the liver, was down-regulated in our rats fed VE deficient diets for 6 months. In agreement, α TTP expression was previously shown to depend on α T intake, although the results reported in the literature are not consistent. While Kim et al. (1998) found significantly increased α TTP mRNA (but not protein) concentrations in the livers of VE depleted rats, Barella et al. (2004) found no differences in the mRNA concentrations of rats fed VE sufficient or deficient diets. Fechner and colleagues (1998) observed that both α T and δ T, when given to VE depleted rats, increased α TTP mRNA concentrations.

Biosynthesis of glutathione, the most important cytosolic antioxidant, requires inter alia the rate-limiting enzyme γ -glutamyl-cysteinyl synthetase (γ GCS). This enzyme consists of two subunits, a heavy catalytic subunit and a light regulatory subunit (= modifier subunit). In our study, γ GCSm (γ GCS modifier subunit) gene expression was down-regulated in VE-deficient animals, verifying results observed by Barella and colleagues (2004), who demonstrated a significant up-regulation of γ GCSm expression by VE supplementation. Because of its involvement in GSH de novo synthesis, decreased γ GCS due to vitamin E deficiency could result in decreased GSH concentrations.

With regard to the mechanisms underlying the gene-regulatory functions of vitamin E, two opposing positions have emerged in recent years. One group of researchers furthers the opinion that VE acts as an antioxidant “and nothing more” (Traber & Atkinson, 2007), while

the other advances the view that VE functions solely as a cellular signaling molecule and gene regulator, because its utilization as antioxidant would be a waste, given the large quantities of endogenous and exogenous antioxidants in the organism (Azzi, 2007).

In this study, vitamin E-deficiency did neither result in significant changes in oxidative stress, as evidenced by similar F₂-isoprostane concentrations and HO-1 mRNA concentrations, nor in differential gene expression in the brain. In the liver, on the other hand, where VE was nearly completely depleted and oxidative stress occurred (as evidenced by increased HO-1 mRNA and protein concentrations; Chapter IV, Figure 4), differential gene expression was observed. This data suggests, that even small amounts of VE are sufficient to protect the brain against oxidative stress and, in consequence, differential gene expression. Our findings furthermore support the hypothesis that the gene regulatory function of VE is linked to its antioxidative properties.

Levels of gene regulation

The data presented within this thesis as well as published literature support the view that vitamin E affects gene expression on a transcriptional level. In mammals, however, mRNA levels often poorly correlate with the respective protein concentrations (Nelson & Keller, 2007). Only a few years back, miRNA were discovered as molecules that effect post-transcriptional gene-silencing by a process termed RNA interference (Rana, 2007). miRNA are now considered as a fundamental component in the regulatory system of gene expression and their biological functions are actively investigated (Girard et al., 2008). Nonetheless, it is important to keep in mind, that post-transcriptional gene-silencing by miRNA is not the only mechanism by which genes are regulated; miRNAs are more likely a means to “fine-tune” transcript and protein levels.

In our study, miRNA-122a and -125b were significantly reduced in the livers of rats fed the vitamin E deficient compared to rats fed vitamin E sufficient diet. miRNA-122a is liver-specific, the most abundant miRNA in the liver and is assumed to play a role in lipid metabolism (Esau *et al.*, 2006). MiRNA-125b is important in inflammatory processes and carcinogenesis. Computational tools (Chang *et al.*, 2004) and antisense targeting (Esau *et al.*, 2006; Elmen *et al.*, 2008) are methods to propose putative miRNA targets. Target genes of miRNA-122a and -125b are listed in Table 1.

Table 1: PCR-verified target genes of miRNA 122a and 125b.

miRNA	Target gene name	Target gene symbol	Function of the gene product	Species	Tissue	Reference
122	<i>Lipid metabolism</i>					
	Cationic amino acid transporter	CAT-1	Facilitates uptake of arginine and lysine in mammalian cells	Human	Huh 7 cells ¹	(Bhattacharyya <i>et al.</i> , 2006)
	Cyclin G1	CCNG1	Cell cycle regulation	Human	Primary liver carcinoma	(Gramantieri <i>et al.</i> , 2007)
	Aldolase A	Aldo A	Catalyzes the irreversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate	Mouse	Liver	(Elmen <i>et al.</i> , 2008)
	Branched-chain α ketoacid dehydrogenase kinase	Bckdk	Catalyzes the oxidative decarboxylation of branched-chain α ketoacids	Mouse	Liver	(Elmen <i>et al.</i> , 2008)
	Scavenger receptor Cd320	Cd320	Putative VLDL receptor	Mouse	Liver	(Elmen <i>et al.</i> , 2008)
	IQ-motif-containing GTPase-activating protein 1	Iqgap1	Regulates cell morphology and motility	Mouse	Liver	(Krutzfeldt <i>et al.</i> , 2005)
	Acetyl-Coenzyme A carboxylase alpha	ACACA/ACCC1	Catalyzes the carbonylation of acetyl-CoA to malonyl-CoA	Mouse	Liver	(Esau <i>et al.</i> , 2006)
	Acetyl-Coenzyme A carboxylase beta	ACACB/ACCC2	Involved in the regulation of fatty acid oxidation	Mouse	Liver	(Esau <i>et al.</i> , 2006)
	Stearoyl-Coenzyme A desaturase 1	SCD1	Involved in the synthesis of unsaturated fatty acids	Mouse	Liver	(Esau <i>et al.</i> , 2006)
	Sterol regulatory element binding transcription factor 1	SREBP1	Transcription factor for sterol synthesis	Mouse	Liver	(Esau <i>et al.</i> , 2006)
	Sterol regulatory element binding transcription factor 2	SREBP2	Transcription factor for sterol synthesis	Mouse	Liver	(Esau <i>et al.</i> , 2006)
	Scavenger receptor CD36	CD36	Receptor for oxidized LDL	Mouse	Liver	(Esau <i>et al.</i> , 2006)

Table 1 (continued)

<i>125 Carcinogenesis</i>						
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	ERBB2	Activation of cell proliferation and differentiation	Human	SKBR30 ²	(Scott <i>et al.</i> , 2007)	
v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	ERBB3	Activation of cell proliferation and differentiation	Human	SKBR30	(Scott <i>et al.</i> , 2007)	
Eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1	Key regulator of translation; inhibits translation initiation of capped mRNAs that can promote tumor progression	Human	Prostate cancer tissue	(Ozen <i>et al.</i> , 2008)	
BCL2-antagonist/killer 1	Bak-1	Induces apoptosis	Human	LNCaP cells ³	(Shi <i>et al.</i> , 2007)	
	lin 28	Oncogene in malignant cells	Mouse	ST2 ⁴	(Mizuno <i>et al.</i> , 2008)	
<i>Inflammation</i>						
Tumor necrosis factor alpha	TNF- α	Proinflammatory cytokine	Human	HEK-293 cells ⁵	(Tili <i>et al.</i> , 2007)	
¹ Huh 7 cells:	hepatocarcinoma cells					
² SKBR30:	human breast cancer cell line					
³ LNCaP cells:	human prostate cancer cell line					
⁴ ST2 cells:	mouse mesenchymal stem cells					
⁵ HEK-293 cells:	human embryonic kidney cells					

Gene expression is a multi-step process that can be subdivided into transcription, post-transcription (splicing, degradation and protection of mRNA), translation, and post-translational modification, which can be regulated at all steps (Moore, 2005). Transcriptional regulation is carried out by transcription factors (TF), which bind in the promoter-regions of the DNA and either enhance or repress the transcription of DNA to RNA. On the post-transcriptional level, mRNA is affected by polyadenylation, 5' capping (mRNA stabilization) and splicing (removal of introns). Alternative splicing is a mechanism by which different proteins can be synthesised from the same messenger RNA (Bolton et al., 1999). Also, mRNA silencing by e.g. miRNA, plays a role in gene regulation (see Chapter I). Post-translational modifications of a protein determine its proper functioning and represent the final checkpoint in gene regulation.

The mechanism(s), by which α T regulates mRNA levels of genes is (are) not completely understood. However, there is some evidence suggesting that α T binds to and activates the transcription factor tocopherol-associated protein (TAP, also known as supernatant protein factor). Upon binding of α T, TAP was found to translocate from the cytosol to the nucleus and to activate reporter gene transcription (Yamauchi et al., 2001). More recent data from competition binding assays, however, suggest that α T is not a specific ligand for TAP, which was shown to have much higher binding affinities for other molecules, including γ T (Schneider, 2005). While specific nuclear receptors, that control gene expression, have been identified for vitamins A and D (retinoic acid and vitamin D receptors; reviewed by Jones et al. (1998)), no specific nuclear receptors have been discovered for vitamin E. However, tocopherols and tocotrienols were shown to activate a reporter gene coupled to the nuclear receptor pregnane X receptor (PXR), with γ -tocotrienol being the most potent activator of all studied congeners (Landes et al., 2003). Similar to TAP, PXR is known to non-specifically bind a wide range of xenobiotics; therefore, PXR activation by vitamin E is likely a mechanism to prevent accumulation of excess vitamin E (Traber & Atkinson, 2007). In the absence of specific vitamin E-dependent nuclear receptors and transcription factors, other gene-regulatory mechanisms related to the antioxidant properties of vitamin E might be considered to be of importance. Polyunsaturated fatty acids (Mater et al., 1999; Xu et al., 2006) as well as oxidized lipids have been shown to alter gene expression (Koch et al., 2007). It might thus be possible, that α T exerts its gene-regulatory activities by protecting these fatty acids from oxidation. Indeed, we found increased CD36 expression in the livers of our VE-

depleted rats concomitantly with increased oxidative stress (Chapter IV). Lipid peroxides and oxidative stress were previously shown to induce CD36 expression, which was prevented by antioxidants, including VE (Fuhrman et al., 2002). After the initial discovery that PKC activity is reduced by incubation with high concentrations of α T (Mahoney & Azzi, 1988), (half inhibitory concentration: 450 μ M; a concentration that can hardly be found in the organism), Azzi and co-workers repeatedly claimed that this phenomenon represented a non-antioxidative function of α T (Fazzio et al., 1997; Azzi et al., 2002; Azzi, 2007). PKC, however, is known to contain redox-sensitive domains that are critical for its activity (Gopalakrishna & Gundimeda, 2002). In agreement, hydrogen peroxide-induced oxidative stress increased (Abdala-Valencia & Cook-Mills, 2006), while antioxidant treatment inhibited PKC activity (Gopalakrishna & Gundimeda, 2002). In an in vitro study with primary dissociated cortical cells from 2 days-old rats, stimulation with α T induced mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways, which are important for neuronal survival (Numakawa et al., 2006), demonstrating that further kinase pathways are influenced by α T. Thus, it seems unlikely that α T-mediated changes in PKC activity are the result of a specific cellular signalling function, but more likely due to an antioxidant phenomenon (Traber & Atkinson, 2007).

CONCLUSION

In conclusion, the data from the present thesis suggest tissue-specific differences in the accumulation and retention of vitamin E in the rat and, consequently, in the susceptibility of different organs to oxidative stress. Furthermore, our data indicate that oxidative stress may be partly responsible for the observed vitamin E-dependent gene regulation. In addition, the regulation of hepatic miRNA concentrations may represent a novel biological function of vitamin E.

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GENERAL DISCUSSION

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APPENDIX

Table 1: Life weight gain of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet for 24 weeks.

Animal	Week	Randomization	Body weight (g)					
	Treatment (mg TE/kg diet)		1	2	3	4	5	6
1	18	59.6	82.1	117.4	149.3	183.6	212.3	238.5
2	18	54.7	74.0	107.9	138.2	175.1	195.2	221.8
3	18	54.2	79.5	113.8	148.6	186.3	211.6	233.8
4	18	51.9	74.2	108.6	142.8	184.8	207.7	234.0
5	18	51.6	70.5	102.2	132.5	174.7	196.7	221.7
6	18	46.8	66.9	103.6	132.5	186.3	203.4	233.4
7	18	45.2	65.9	104.2	137.2	190.2	215.4	241.3
8	18	42.6	63.9	100.5	133.8	182.1	206.0	233.8
9	9	59.1	81.4	112.3	145.9	182.6	201.7	229.3
10	9	54.8	76.8	107.3	140.9	181.3	204.6	224.7
11	9	53.2	75.9	113.4	143.9	182.8	204.4	226.6
12	9	51.9	73.8	113.2	145.3	185.4	210.6	235.5
13	9	51.6	73.7	113.2	139.9	183.8	216.0	239.8
14	9	47.4	69.5	104.8	131.3	171.8	194.9	219.2
15	9	44.9	66.5	102.9	132.7	176.1	202.2	225.6
16	9	41.8	63.2	101.6	135.2	182.6	210.7	234.5
17	<1	59.0	80.9	115.6	150.8	187.4	212.4	233.7
18	<1	57.1	79.2	113.2	149.8	188.4	208.7	229.9
19	<1	52.9	77.0	115.0	152.8	194.7	219.0	242.1
20	<1	52.0	74.9	112.1	149.9	191.4	216.1	244.9
21	<1	50.1	71.2	107.5	137.2	179.3	206.7	229.8
22	<1	48.0	69.0	108.1	141.0	190.1	218.2	240.5
23	<1	44.6	62.5	98.4	130.6	174.8	201.7	227.7
24	<1	43.0	64.0	103.1	137.5	178.9	211.6	240.7
25	146	58.6	79.7	112.2	142.8	186.1	206.0	232.8
26	146	58.6	79.3	117.4	154.3	194.1	216.1	236.8
27	146	52.6	73.0	108.7	139.3	182.4	206.2	232.5
28	146	52.4	74.4	109.1	141.0	177.2	200.9	222.8
29	146	49.6	69.6	104.0	132.7	167.9	191.8	212.6
30	146	49.1	70.5	105.7	133.5	173.9	195.8	215.9
31	146	44.5	64.0	99.1	128.4	167.7	191.7	215.7
32	146	43.8	63.4	100.7	129.8	173.4	201.9	227.4

APPENDIX

Table 1 (continued)

Animal	Week Treatment (mg TE/kg diet)	Randomization	7	8	9	10	11	12
			Body weight (g)					
1	18	59.6	258.0	278.4	292.4	305.5	318.6	330.3
2	18	54.7	241.6	260.2	271.5	283.9	294.5	306.1
3	18	54.2	247.6	266.3	279.8	291.4	304.4	314.0
4	18	51.9	252.2	272.8	282.5	294.7	308.8	318.6
5	18	51.6	242.6	258.9	270.2	283.0	292.4	299.6
6	18	46.8	256.5	278.9	291.4	304.5	313.8	326.2
7	18	45.2	264.9	286.4	300.9	316.4	322.5	342.0
8	18	42.6	255.8	278.8	297.7	308.5	314.9	337.1
9	9	59.1	246.9	262.7	276.1	285.6	297.9	302.4
10	9	54.8	242.8	260.7	274.1	284.9	297.6	305.6
11	9	53.2	241.7	259.2	269.8	282.7	295.2	301.9
12	9	51.9	253.8	272.3	287.1	300.4	313.2	320.7
13	9	51.6	263.3	282.6	296.4	302.1	312.4	324.0
14	9	47.4	241.8	258.8	274.0	281.9	290.3	297.6
15	9	44.9	245.4	261.4	275.4	285.5	287.4	304.3
16	9	41.8	258.6	280.1	295.3	310.9	319.8	334.9
17	<1	59.0	251.2	269.1	285.2	296.5	308.0	314.8
18	<1	57.1	249.0	266.9	279.0	289.0	303.1	312.0
19	<1	52.9	264.1	287.5	299.7	309.6	318.7	332.1
20	<1	52.0	268.8	287.7	300.3	312.9	318.8	335.3
21	<1	50.1	250.9	271.2	284.9	294.2	304.9	313.7
22	<1	48.0	263.8	284.4	298.7	310.2	323.6	332.1
23	<1	44.6	251.8	270.6	287.4	300.1	312.6	329.9
24	<1	43.0	263.6	284.6	299.9	314.5	329.9	342.5
25	146	58.6	249.9	270.0	284.2	294.7	301.5	317.7
26	146	58.6	258.6	278.0	295.3	301.7	315.9	327.1
27	146	52.6	252.8	256.3	285.8	300.0	304.9	311.6
28	146	52.4	241.3	271.4	268.6	277.0	286.4	293.6
29	146	49.6	229.3	246.5	255.1	262.9	274.1	279.2
30	146	49.1	233.5	251.6	262.2	272.1	285.0	291.5
31	146	44.5	245.2	262.6	271.6	283.9	292.2	300.7
32	146	43.8	234.5	255.3	264.9	280.0	286.8	297.1

APPENDIX

Table 1 (continued)

Animal	Week	13	14	15	16	17	18
	Treatment (mg TE/kg diet)	Body weight (g)					
1	18	355.5	342.0	349.6	358.5	365.5	369.0
2	18	312.5	314.1	316.8	322.5	328.0	330.2
3	18	318.6	323.1	329.8	335.9	341.8	347.7
4	18	324.6	329.6	331.7	338.7	346.0	350.7
5	18	304.9	306.4	308.1	311.7	319.5	322.0
6	18	332.1	335.1	333.9	338.0	344.4	345.7
7	18	349.4	351.6	355.6	359.6	373.5	378.1
8	18	347.5	349.8	357.3	361.4	370.2	372.4
9	9	309.5	312.3	314.3	323.0	332.4	333.5
10	9	313.2	315.2	316.7	326.2	332.0	332.7
11	9	308.8	309.7	313.8	316.4	326.0	325.6
12	9	329.5	330.2	338.2	343.2	357.1	354.1
13	9	338.4	344.5	351.3	356.6	362.7	366.7
14	9	309.4	314.4	319.5	324.4	333.2	338.4
15	9	313.6	315.6	322.0	331.0	334.5	339.0
16	9	347.3	347.7	353.0	364.9	368.0	372.1
17	<1	323.7	321.6	328.6	334.2	334.8	340.7
18	<1	320.8	322.4	329.4	340.0	345.5	350.5
19	<1	341.3	346.5	353.4	359.8	365.0	368.6
20	<1	345.0	344.5	348.7	357.0	363.8	368.7
21	<1	322.3	325.4	331.9	338.5	344.4	343.6
22	<1	344.2	345.5	351.3	357.5	364.3	363.8
23	<1	337.6	336.0	343.7	345.7	352.3	356.6
24	<1	355.4	359.3	367.0	373.0	378.2	377.3
25	146	324.9	323.7	327.7	339.5	346.6	354.0
26	146	337.0	336.2	343.1	351.2	357.5	364.0
27	146	322.7	321.3	325.1	330.2	334.3	338.6
28	146	301.8	306.3	312.7	319.1	322.6	326.3
29	146	285.6	286.5	288.4	299.4	302.1	302.8
30	146	296.3	296.7	299.7	307.9	314.7	316.0
31	146	309.9	314.1	320.6	330.0	330.8	336.2
32	146	303.7	306.1	312.6	321.9	329.0	332.1

APPENDIX

Table 1 (continued)

Animal	Week	19	20	21	22	23	24	Final
	Treatment (mg TE/kg diet)	Body weight (g)						
1	18	372.7	374.8	382.5	389.4	390.3	392.6	386.9
2	18	330.6	335.6	343.8	346.3	348.7	350.5	346.7
3	18	352.1	356.2	360.7	361.7	365.8	371.2	364.7
4	18	353.0	360.5	362.4	365.3	369.5	372.3	367.1
5	18	329.7	333.9	339.3	345.2	348.3	352.2	346.2
6	18	355.5	361.4	362.5	364.7	370.7	376.5	367.7
7	18	386.6	391.6	397.0	399.4	402.3	413.9	404.7
8	18	377.3	381.9	384.6	389.9	394.3	401.6	393.0
9	9	336.7	343.3	347.1	353.2	355.0	356.5	350.8
10	9	340.1	345.9	348.2	350.8	352.2	358.8	353.3
11	9	329.0	336.2	337.8	342.0	348.0	351.2	349.5
12	9	362.2	371.5	372.3	378.4	386.2	388.8	384.5
13	9	369.6	377.1	381.8	385.0	389.7	394.6	387.5
14	9	339.2	343.1	349.3	354.0	353.5	361.3	354.5
15	9	342.9	345.8	355.4	356.8	364.2	367.3	360.0
16	9	374.3	378.9	384.1	386.0	393.7	396.4	392.6
17	<1	340.4	345.7	347.3	349.8	348.8	349.3	342.2
18	<1	351.4	351.2	350.1	356.9	361.7	367.8	354.5
19	<1	366.9	365.3	366.0	373.4	375.3	378.9	375.7
20	<1	364.0	359.0	362.2	371.5	374.6	378.6	374.4
21	<1	343.6	349.2	350.5	353.7	359.1	362.8	358.2
22	<1	365.4	368.4	369.1	370.0	374.9	375.9	373.3
23	<1	366.3	369.7	369.2	368.5	369.4	371.9	362.6
24	<1	383.3	384.2	382.4	381.9	361.4	366.5	361.4
25	146	354.2	358.5	366.5	370.6	372.8	377.8	373.9
26	146	362.6	365.4	370.9	378.9	376.5	384.4	378.0
27	146	347.2	350.4	357.0	357.9	364.4	364.2	357.8
28	146	328.6	334.7	339.8	344.7	350.3	354.4	348.3
29	146	304.9	308.7	309.5	311.8	314.4	315.7	313.9
30	146	320.3	324.9	324.7	332.9	332.2	338.2	330.9
31	146	337.7	341.4	346.4	348.5	350.3	354.8	347.7
32	146	336.0	338.9	345.4	351.9	350.3	356.3	348.3

APPENDIX

Table 2: Concentrations of α - and γ -tocopherol in the plasma of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks.

Animal	Treatment mg TE/kg diet	α T Plasma (nmol/mL)	γ T Plasma (nmol/ml)
1	18	16.6	0.472
2	18	19.9	0.654
3	18	17.2	0.509
4	18	17.6	0.616
5	18	19.3	0.557
6	18	16.9	0.552
7	18	17.0	0.589
8	18	17.0	0.481
9	9	11.5	0.532
10	9	10.6	0.509
11	9	12.2	0.882
12	9	11.3	0.670
13	9	11.7	0.517
14	9	11.9	0.585
15	9	8.7	0.444
16	9	8.7	0.423
17	<1	0.34	0.004
18	<1	0.34	0.009
19	<1	0.35	0.008
20	<1	0.32	0.010
21	<1	0.36	0.009
22	<1	0.38	0.010
23	<1	0.37	0.010
24	<1	0.28	0.006
25	146	41.8	0.059
26	146	41.0	0.061
27	146	33.1	0.047
28	146	37.4	0.073
29	146	42.2	0.077
30	146	34.1	0.043
31	146	35.5	0.082
32	146	39.8	0.097

APPENDIX

Table 2 (continued)

Animal	Treatment mg TE/kg diet	Cholesterol (mmol/L)	α T/Cholesterol (mmol/mol)	γ T/Cholesterol (mmol/mol)
1	18	2.52	6.59	0.187
2	18	2.73	7.27	0.239
3	18	2.36	7.27	0.215
4	18	2.70	6.53	0.229
5	18	2.57	7.53	0.217
6	18	2.20	7.65	0.251
7	18	2.49	6.81	0.236
8	18	2.53	6.73	0.190
9	9	2.69	4.27	0.198
10	9	2.44	4.34	0.208
11	9	2.91	4.20	0.303
12	9	2.75	4.11	0.244
13	9	2.58	4.52	0.200
14	9	2.84	4.17	0.206
15	9	2.30	3.79	0.193
16	9	2.27	3.81	0.186
17	<1	2.17	0.16	0.002
18	<1	2.18	0.16	0.004
19	<1	2.37	0.15	0.003
20	<1	2.12	0.15	0.005
21	<1	2.37	0.15	0.004
22	<1	2.33	0.16	0.004
23	<1	2.49	0.15	0.004
24	<1	1.79	0.16	0.004
25	46	3.09	13.5	0.019
26	146	2.86	14.4	0.021
27	146	2.50	13.3	0.019
28	146	2.70	13.8	0.031
29	146	3.00	14.1	0.026
30	146	2.60	13.1	0.017
31	146	2.56	13.9	0.032
32	146	3.07	12.9	0.031

APPENDIX

Table 3: Concentrations of α - and γ -tocopherol in cortex and hippocampus of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks.

Animal	Treatment (mg TE/kg diet)	α T Cortex (nmol/g)	γ T Cortex (nmol/g)	α T Hippocampus (nmol/g)
1	18	25.51	0.189	9.96
2	18	21.22	0.142	5.11
3	18	17.28	0.128	5.38
4	18	15.07	0.111	12.87
5	18	23.13	0.166	9.64
6	18	19.77	0.157	10.19
7	18	23.81	0.186	n.d.
8	18	23.65	0.183	14.31
9	9	22.23	0.151	6.91
10	9	17.32	0.119	3.36
11	9	14.68	0.121	10.35
12	9	12.99	0.106	7.16
13	9	15.56	0.145	n.d.
14	9	16.49	0.135	n.d.
15	9	18.19	0.157	14.32
16	9	16.24	0.132	5.09
17	<1	5.75	n.d.	2.32
18	<1	2.85	0.008	n.d.
19	<1	2.00	0.006	n.d.
20	<1	3.04	0.011	2.74
21	<1	3.12	0.009	n.d.
22	<1	4.17	0.008	1.43
23	<1	4.81	n.d.	2.73
24	<1	4.48	0.008	0.77
25	146	38.53	0.076	19.36
26	146	27.26	0.031	8.65
27	146	23.61	0.043	n.d.
28	146	29.35	0.050	n.d.
29	146	27.54	0.056	16.37
30	146	29.78	0.049	17.51
31	146	30.34	0.058	14.74
32	146	33.84	0.062	16.82

n.d. = not detectable

APPENDIX

Table 4: Concentrations of α - and γ -tocopherol in liver, lung, and muscle of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks.

Animal	Treatment mg TE/kg diet	α T Liver nmol/g	γ T Liver nmol/g	α T Lung nmol/g	γ T Lung nmol/g	α T Muscle nmol/g	γ T Muscle nmol/g
1	18	27.5	0.397	20.0	0.570	11.9	0.584
2	18	32.3	0.441	19.6	0.645	13.1	0.311
3	18	26.1	0.359	21.3	0.606	10.4	0.272
4	18	28.2	0.405	17.7	0.609	11.2	0.384
5	18	26.2	0.442	17.0	0.582	9.2	0.391
6	18	29.2	0.443	27.2	0.759	11.1	0.312
7	18	29.9	0.446	25.3	0.807	15.3	0.339
8	18	28.8	0.357	21.2	0.591	11.5	0.308
9	9	14.2	0.289	11.9	0.443	5.7	0.196
10	9	18.3	0.377	13.0	0.544	7.9	0.197
11	9	12.1	0.357	13.1	0.675	7.3	0.163
12	9	13.6	0.347	12.2	0.544	6.8	0.218
13	9	15.9	0.313	12.5	0.571	8.1	0.229
14	9	14.9	0.345	10.0	0.520	7.8	0.252
15	9	16.8	0.371	11.2	0.466	7.4	0.215
16	9	14.8	0.329	13.2	0.546	8.0	0.221
17	<1	0.39	0.014	0.45	0.015	0.48	0.038
18	<1	0.38	0.016	0.55	0.054	0.42	0.045
19	<1	0.28	0.014	0.35	0.084	0.30	0.038
20	<1	0.31	0.018	0.55	0.141	0.27	0.031
21	<1	0.29	0.007	0.30	0.017	0.52	0.033
22	<1	0.31	0.010	0.40	0.025	0.48	0.017
23	<1	0.30	0.016	0.28	0.011	0.25	0.021
24	<1	0.37	0.014	0.31	0.011	0.36	0.009
25	146	98.8	0.092	47.7	0.044	24.9	0.081
26	146	126.7	0.106	46.9	0.048	22.2	0.078
27	146	72.7	0.095	41.0	0.041	17.8	0.059
28	146	111.2	0.099	42.3	0.047	19.6	0.059
29	146	108.3	0.106	43.4	0.174	20.3	0.052
30	146	103.1	0.109	44.8	0.193	16.7	0.060
31	146	116.7	0.106	37.1	0.085	18.3	0.040
32	146	96.5	0.118	41.5	0.077	19.9	0.054

APPENDIX

Table 5: Relative expression of selective genes in the cortex of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are the ratio of target-gene/ β -actin.

Animal	Treatment (mg TE/kg diet)	Gene					
		APP	Apba1	Adam-10	Bace-1	Bax	Bcl-2
1	18	2.23	1.20	1.24	1.26	1.39	1.08
2	18	0.88	1.07	1.30	1.43	0.93	0.94
3	18	0.97	0.98	1.08	1.08	0.96	0.93
4	18	1.04	0.93	1.01	1.17	1.02	1.05
5	18	1.05	1.89	1.06	0.91	1.36	1.52
6	18	1.16	1.51	0.80	0.68	0.85	1.18
7	18	1.71	1.92	0.97	0.92	1.14	1.31
8	18	1.07	1.33	1.00	1.02	0.99	1.23
9	9	1.08	0.62	0.71	0.67	0.75	1.05
10	9	0.90	0.78	0.95	0.91	0.78	1.00
11	9	1.36	1.35	1.39	1.29	0.99	1.30
12	9	0.91	1.24	1.07	1.16	1.22	1.08
13	9	1.01	1.04	0.82	0.70	0.77	0.90
14	9	1.03	1.06	0.64	0.68	0.66	0.87
15	9	1.29	1.28	0.81	0.70	0.89	1.09
16	9	1.47	1.65	0.87	0.96	1.08	1.05
17	<1	2.17	0.83	1.10	1.09	0.86	1.20
18	<1	0.98	0.87	1.08	1.22	0.92	1.23
19	<1	1.40	0.90	1.32	1.25	1.14	1.04
20	<1	1.09	0.80	0.77	0.87	1.12	1.06
21	<1	1.23	1.24	0.98	0.90	0.90	1.13
22	<1	1.13	1.71	0.90	0.87	0.95	1.25
23	<1	1.21	0.85	0.68	0.63	0.83	0.82
24	<1	1.45	1.28	0.72	0.86	0.81	0.94
25	146	1.10	1.13	0.74	0.71	0.81	1.02
26	146	1.27	1.67	1.08	0.96	1.07	1.15
27	146	1.13	1.17	0.82	0.78	0.97	1.28
28	146	1.19	1.22	0.75	0.83	0.88	0.92
29	146	1.47	1.05	1.03	1.16	0.93	1.36
30	146	1.12	1.01	1.05	1.10	1.01	1.04
31	146	1.18	1.22	1.41	1.40	1.09	1.12
32	146	1.22	0.78	0.88	0.89	0.92	1.20

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Table 5 (continued)

Animal	Treatment (mg TE/kg diet)	Gene					
		ECE	IDE	Neprilysin	α TTP	HO-1	AChE
1	18	1.47	1.28	0.89	1.04	1.42	0.93
2	18	1.03	0.88	1.05	1.13	1.65	0.99
3	18	0.99	1.05	1.01	0.86	0.86	0.98
4	18	0.92	1.08	0.99	0.97	1.46	0.93
5	18	1.42	1.31	2.31	1.87	0.94	1.28
6	18	0.79	0.89	1.59	1.21	1.67	1.11
7	18	1.27	1.11	1.18	1.16	1.90	1.26
8	18	0.90	1.00	1.00	1.11	1.66	1.39
9	9	0.95	0.85	0.77	0.73	1.17	0.61
10	9	0.83	0.65	0.53	0.80	1.25	1.21
11	9	0.98	1.14	1.23	1.28	1.74	0.80
12	9	0.75	0.98	0.66	1.15	1.30	0.89
13	9	0.69	0.67	0.87	0.89	0.98	1.09
14	9	0.70	0.62	3.21	1.03	1.60	1.34
15	9	1.11	0.87	1.04	1.19	1.77	1.28
16	9	0.97	1.14	1.13	1.53	1.32	1.13
17	<1	1.30	0.90	0.60	0.96	1.35	0.92
18	<1	0.91	0.91	1.68	0.97	1.20	1.17
19	<1	1.26	1.12	0.65	1.38	1.73	0.72
20	<1	0.90	0.93	2.69	1.23	1.40	1.97
21	<1	1.01	0.98	1.02	0.91	0.88	1.03
22	<1	0.91	0.87	1.28	1.01	1.56	1.18
23	<1	0.82	0.71	2.40	0.97	1.34	1.14
24	<1	1.13	0.97	2.32	1.07	1.34	2.69
25	146	0.89	0.73	1.39	1.04	1.91	1.05
26	146	0.86	1.20	1.02	1.50	1.54	1.34
27	146	0.93	0.90	1.14	0.98	1.24	1.62
28	146	0.95	0.77	0.99	1.17	1.30	1.33
29	146	1.01	1.03	1.29	1.00	1.01	1.70
30	146	0.87	1.01	1.14	0.95	1.23	0.98
31	146	1.24	1.33	1.17	1.13	1.28	1.02
32	146	0.94	0.81	0.80	1.04	0.97	1.08

APPENDIX

Table 6: Relative expression of selective genes in the hippocampus of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are the ratio of target-gene/ β -actin.

Animal	Treatment (mg TE/kg diet)	Gene					
		APP	Apba1	Adam-10	Bace-1	Bax	Bcl-2
1	18	0.89	0.55	0.93	0.76	0.88	1.01
2	18	1.52	0.73	1.18	0.91	0.84	1.30
3	18	0.91	0.95	0.98	0.90	0.92	1.38
4	18	0.80	1.17	1.00	0.71	0.78	1.15
5	18	0.85	1.13	0.81	0.74	0.95	1.15
6	18	1.00	0.90	0.99	0.73	0.94	1.00
7	18	1.09	1.01	1.17	0.59	0.95	1.13
8	18	0.99	1.11	0.92	0.78	0.96	1.09
9	9	1.02	0.86	0.96	0.68	0.77	1.04
10	9	0.83	1.11	0.88	0.83	0.79	0.95
11	9	0.69	0.71	0.71	0.76	0.73	1.05
12	9	0.98	1.22	0.95	0.94	0.92	1.25
13	9	0.92	0.81	0.81	0.78	0.88	0.88
14	9	0.80	0.99	0.76	0.71	0.82	0.89
15	9	0.86	0.73	0.76	0.82	0.89	0.99
16	9	0.93	0.91	0.84	0.76	0.94	1.03
17	<1	0.75	0.83	0.90	0.81	0.93	1.29
18	<1	0.55	0.84	0.78	0.66	0.72	0.88
19	<1	0.81	0.91	0.73	0.74	0.99	0.93
20	<1	0.83	0.83	0.91	0.65	0.87	1.17
21	<1	1.32	1.30	1.26	1.15	1.48	1.60
22	<1	0.88	0.88	0.84	0.64	1.00	1.12
23	<1	1.13	1.03	0.96	0.90	1.20	1.26
24	<1	0.84	0.93	0.84	0.73	0.94	0.97
25	146	0.84	1.23	0.95	0.76	1.12	1.07
26	146	1.07	1.20	1.00	0.63	0.82	1.11
27	146	1.12	0.96	0.94	0.77	1.03	1.19
28	146	0.74	0.88	0.66	0.74	0.80	0.92
29	146	0.94	0.90	0.85	0.77	0.84	1.37
30	146	1.32	0.89	0.79	0.69	0.76	0.92
31	146	0.82	0.79	0.75	0.69	0.90	1.03
32	146	0.76	0.78	0.65	0.81	0.89	1.02

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Table 6 (continued)

Animal	Treatment (mg TE/kg diet)	Gene					
		ECE	IDE	Neprilysin	α TTP	HO-1	AChE
1	18	0.78	0.86	0.71	0.87	0.72	1.07
2	18	0.94	0.72	0.93	0.69	0.82	0.85
3	18	0.86	0.90	0.84	0.85	1.05	1.10
4	18	0.93	0.77	1.08	0.99	0.74	1.25
5	18	0.98	0.88	0.96	1.13	0.84	0.94
6	18	0.93	1.18	0.85	0.99	0.79	0.96
7	18	1.09	1.36	1.12	1.58	0.57	1.32
8	18	1.09	1.24	1.06	0.97	0.91	0.94
9	9	0.80	0.62	0.77	0.94	0.77	1.18
10	9	0.82	0.66	1.19	1.05	0.68	1.26
11	9	0.81	0.84	0.89	0.88	0.76	1.09
12	9	1.14	0.74	1.24	1.13	0.98	1.49
13	9	0.96	1.01	0.89	0.80	0.75	0.78
14	9	0.80	1.04	0.95	0.83	0.45	0.68
15	9	0.95	1.01	0.92	0.80	0.48	0.89
16	9	0.96	1.09	0.97	0.86	0.57	0.84
17	<1	0.90	0.73	0.76	0.97	0.74	1.08
18	<1	0.72	0.50	0.77	0.72	0.68	0.91
19	<1	0.88	0.58	0.80	1.07	0.61	0.96
20	<1	0.81	0.82	0.91	1.00	0.65	0.97
21	<1	1.24	1.51	1.32	1.36	1.15	1.29
22	<1	0.90	1.00	0.78	0.79	1.20	0.83
23	<1	1.21	1.33	1.07	1.06	1.17	0.89
24	<1	0.95	0.98	0.67	0.95	0.62	0.77
25	146	1.18	1.12	0.69	1.04	0.56	0.66
26	146	0.96	1.03	0.93	1.19	0.79	0.80
27	146	1.05	1.14	1.09	1.21	0.64	1.02
28	146	0.76	0.87	0.74	0.82	0.56	0.73
29	146	0.80	0.71	0.90	1.05	0.70	1.12
30	146	0.77	0.58	0.96	0.85	0.48	0.94
31	146	0.82	0.04	0.88	1.05	0.59	1.25
32	146	0.80	0.56	0.97	0.99	0.73	0.97

APPENDIX

Table 7: Antioxidant enzyme activities and glutathione concentrations in the cortex of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks.

Animal	Treatment (mg TE/kg diet)	Protein concentration (mg/mL)	SOD activity (U/mg protein)	Se-GPx activity (mU/mg protein)	GSH concentration (nmol/mg protein)
1	18	58.8	12.2	80.6	21.2
2	18	65.0	12.5	86.1	23.0
3	18	61.7	12.7	79.9	22.2
4	18	48.5	15.5	85.9	24.8
5	18	48.3	15.8	114.6	27.6
6	18	66.6	12.3	80.5	23.1
7	18	56.6	13.9	94.6	23.1
8	18	54.7	13.0	101.1	26.3
9	9	48.3	14.6	95.8	26.1
10	9	46.9	9.9	104.7	27.8
11	9	47.7	16.7	106.4	28.0
12	9	53.5	14.6	117.6	22.6
13	9	47.9	13.5	98.7	26.1
14	9	61.8	11.3	72.1	23.1
15	9	61.1	11.4	80.8	23.7
16	9	56.8	12.0	88.3	23.3
17	<1	55.3	11.5	94.9	23.1
18	<1	45.5	16.4	98.1	29.9
19	<1	62.1	14.0	76.5	23.8
20	<1	61.1	13.1	91.5	21.1
21	<1	65.4	11.6	67.3	20.6
22	<1	43.2	15.7	105.6	29.9
23	<1	65.4	11.5	75.6	22.2
24	<1	44.3	16.4	94.4	28.8
25	146	50.2	15.1	125.5	24.8
26	146	50.6	12.9	79.0	24.6
27	146	50.4	15.4	100.2	27.2
28	146	47.0	11.9	91.4	25.5
29	146	57.4	15.1	87.2	24.9
30	146	56.9	12.2	72.7	25.2
31	146	44.6	15.1	117.4	27.9
32	146	43.8	15.6	110.4	27.8

APPENDIX

Table 8: F₂-isoprostane concentrations in the cortex of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks.

Animal	Treatment (mg TE/kg diet)	F ₂ -isoprostane concentration	
		(pg/mg net weight)	(ng/mg protein)
1	18	26.2	0.399
2	18	20.2	0.304
3	18	19.5	0.286
4	18	21.8	0.292
5	18	27.2	0.358
6	18	27.6	0.430
7	18	30.3	0.427
8	18	25.2	0.379
9	9	27.4	0.388
10	9	16.9	0.251
11	9	23.4	0.329
12	9	23.4	0.305
13	9	31.1	0.387
14	9	25.5	0.420
15	9	36.7	0.507
16	9	23.3	0.357
17	<1	33.7	0.494
18	<1	27.4	0.384
19	<1	23.9	0.317
20	<1	23.8	0.338
21	<1	39.8	0.561
22	<1	42.3	0.561
23	<1	30.3	0.403
24	<1	25.7	0.398
25	146	26.5	0.389
26	146	30.0	0.401
27	146	19.6	0.295
28	146	20.9	0.273
29	146	27.5	0.361
30	146	29.6	0.407
31	146	24.8	0.350
32	146	25.5	0.428

APPENDIX

Table 9: Relative protein concentration of Bcl-2, Bax and β -actin in cortex homogenates of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are the ratio of target protein/ β -actin.

Animal	Treatment (mg TE/kg diet)	Bcl-2	Bax
1	18	0.211	0.188
2	18	0.127	0.192
3	18	0.359	0.573
4	18	0.477	0.594
5	18	1.126	1.805
6	18	1.135	2.703
9	9	0.157	0.186
10	9	0.178	0.215
11	9	0.616	0.573
12	9	0.489	0.627
13	9	1.151	2.011
14	9	1.689	1.553
17	<1	0.162	0.237
18	<1	0.118	0.198
19	<1	0.659	0.513
20	<1	0.409	0.525
21	<1	0.830	1.910
22	<1	0.879	2.016
25	146	0.364	0.283
26	146	0.200	0.276
27	146	0.442	0.526
28	146	0.852	0.774
29	146	1.271	2.430
30	146	0.935	1.625

APPENDIX

Table 10: Relative expression of selective genes in the liver of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are the ratio of target gene/ β -actin.

Animal	Treatment (mg TE/kg diet)	5 α -R1	CD36	γ GCSm	α TTP
1	18	1.01	1.39	0.86	1.25
2	18	0.97	1.30	0.97	1.64
3	18	0.68	0.88	1.36	1.01
4	18	0.86	0.98	1.25	1.33
5	18	0.88	1.42	1.66	1.26
6	18	0.98	2.55	2.03	2.02
7	18	1.32	1.86	1.76	1.27
8	18	1.26	1.20	1.07	1.37
9	9	1.38	1.68	1.18	1.29
10	9	1.07	1.06	1.45	1.42
11	9	1.20	1.87	1.17	1.53
12	9	0.96	1.57	1.10	1.88
13	9	1.30	1.19	1.21	1.57
14	9	0.98	1.44	1.64	1.91
15	9	1.60	1.32	1.80	1.47
16	9	0.81	0.93	1.51	1.23
17	<1	1.82	3.08	0.70	0.79
18	<1	2.12	3.23	0.47	0.93
19	<1	1.36	2.67	0.84	1.22
20	<1	1.19	3.05	0.88	1.19
21	<1	1.56	3.53	0.71	1.15
22	<1	1.25	2.65	0.74	1.15
23	<1	1.56	2.76	0.92	1.25
24	<1	1.30	2.23	0.80	1.25
25	146	0.94	1.15	1.52	1.32
26	146	1.16	1.14	1.24	1.57
27	146	0.75	1.49	1.32	1.54
28	146	0.68	1.27	1.19	1.54
29	146	1.00	1.74	1.10	1.96
30	146	0.80	1.59	1.61	2.04
31	146	0.66	1.39	1.37	1.35
32	146	1.28	1.27	0.96	1.59

APPENDIX

Table 11: Relative expression of miRNA concentrations in the liver of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are ratio of target miRNA/snoRNA.

Animal	Treatment (mg TE/kg diet)	miR-122a	miR-125b
1	18	1.38	1.12
2	18	1.18	1.22
3	18	1.31	1.20
4	18	1.46	1.21
5	18	1.09	1.29
6	18	1.50	1.26
7	18	1.16	1.56
8	18	1.31	1.43
9	9	1.10	1.08
10	9	1.07	1.00
11	9	1.09	1.36
12	9	0.97	1.06
13	9	1.19	1.40
14	9	1.18	1.19
15	9	1.21	1.29
16	9	0.97	1.21
17	<1	0.98	1.12
18	<1	0.96	0.78
19	<1	0.87	1.04
20	<1	1.05	1.11
21	<1	1.28	1.06
22	<1	1.16	1.12
23	<1	1.30	0.98
24	<1	1.47	1.07
25	146	1.10	1.16
26	146	1.11	0.92
27	146	0.93	1.26
28	146	1.09	1.18
29	146	1.20	0.89
30	146	0.98	0.99
31	146	1.11	1.23
32	146	1.14	1.30

APPENDIX

Table 12: Relative protein concentrations of HO-1 in liver homogenates of rats fed diets with <1, 9, 18, and 146 mg TE/kg diet, respectively, for 24 weeks. Values are the ratio of HO-1/ β -actin.

Animal	Treatment (mg TE/kg diet)	HO-1
1	18	0.356
2	18	0.696
3	18	0.569
4	18	0.098
5	18	0.261
7	18	0.629
8	18	0.528
9	9	0.463
10	9	0.575
11	9	0.402
12	9	0.487
14	9	0.630
15	9	0.888
16	9	0.924
17	<1	0.560
18	<1	0.494
19	<1	0.376
20	<1	0.444
21	<1	0.448
22	<1	0.615
23	<1	0.746
24	<1	0.943
25	146	0.400
26	146	0.470
27	146	0.400
28	146	0.304
29	146	0.505
30	146	0.227
31	146	0.616
32	146	0.620

CURRICULUM VITAE

SONJA GAEDICKE

PERSONAL DETAILS

Date of birth: May 30, 1978

Nationality: German

FIELD OF EXPERTISE

Human nutrition with a focus on vitamin E, its gene regulatory and other biological activities, especially with regard to healthy aging and brain health.

EDUCATION

Nov 2008 Ph.D. in Human Nutrition, Christian Albrechts University (CAU), Kiel, Germany

Aug 2005 Master of Science in Human Nutrition and Home Economics, CAU, Kiel, Germany

PROFESSIONAL EXPERIENCE

Oct 2008-present Regulatory and Scientific Affairs Manager Nutrition Ingredients, BASF SE, Ludwigshafen, Germany

Sep 2005-Sep 2008 Ph.D. student, Institute of Human Nutrition and Food Science, CAU, Kiel, Germany
Associated member of the Graduiertenkolleg (graduate school) 820 "Natural Antioxidants – their activities in plants, foods, animals and humans"

Ph.D. project financially supported by UFOP foundation (Union zur Förderung von Oel- und Proteinpflanzen e.V.), Berlin, Germany

Responsible for study design, performance, chemical and data analyses, and publishing of the results of an animal experiment. Teaching of undergraduate students.

Jan 2005-Aug 2005 Master thesis, Institute of Human Nutrition and Food Science, Christian Albrechts University (CAU), Kiel, Germany

Apr 2003-Mar 2005 Student helper at the Institute of Human Nutrition and Food Science, CAU, Kiel, Germany

Oct 2000-Aug 2005 University studies in Human Nutrition and Home Economics, CAU, Kiel, Germany

Mar 2000-Sep 2000 Sales assistant, Voilà – Käse, Wein und mehr (cheese, wine and specialties shop), Hamburg, Germany

Aug 1997-Feb 2000 Trainee in Hotel Business, Hotel Lindtner Hamburg, Harburg, Germany

SKILLS AND EXPERIENCES

Planning, conducting, and chemical and data analysis of animal studies as well as cell culture experiments

Skilled in analytical and biomolecular techniques (HPLC, spectrometry, ELISA, cell culture, real-time PCR [mRNA], TaqMan [miRNA], and others)

Vocational education and training qualifications

Experienced in the organisation of scientific conferences

Preparation of grant applications and report writing for funding organizations

Presenting research to scientific and non-professional audiences

Teacher and laboratory instructor for graduate and undergraduate students

Familiar with Apple Macintosh and Windows operating systems, office applications, bibliographic databases, and statistical software packages (SPSS and InStat)

LANGUAGES

German: Native speaker

English: Fluent

VOLUNTARY SERVICES

*Oct 2003-
Feb 2005* Member of the committee „Graduate-promotion“,
CAU, Kiel, Germany

*Oct 2002-
Feb 2005* Member of the study and examination board for the
*study program in human nutrition and home economics, CAU,
Kiel, Germany*

*Apr 2001-
Aug 2005* Member of the students council of the Faculty of
*Agricultural and Nutritional Sciences, CAU, Kiel,
Germany*

*Apr 2001-
Oct 2002* Mentor for first-year students, CAU, Kiel, Germany

INTERNSHIPS

*Oct 2004-
Dec 2004* Internship in Scientific Marketing at the Division of
Human Nutrition, BASF AG, Ludwigshafen, Germany

GRANTS

Travel grant for 1,350 € from Deutsche Forschungsgemeinschaft (DFG; "German Research Foundation) to present my research at the Oxygen Club of California's World Congress 2008, March 12-15, 2008, Santa Barbara, CA, USA

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

Deutsche Gesellschaft für Ernährung (German Nutrition Society)
Oxygen Club of California
NuGO – The European Nutrigenomics Organisation

PUBLICATIONS

Peer-reviewed scientific articles

Gaedicke S, Zhang X, Hübbe P, Bösch-Saadatmandi C, Lou Y, Wiswedel I, Gardemann A, Frank J, Rimbach G (2008): Dietary vitamin E, brain redox status and expression of Alzheimer's disease relevant genes in rats. *British Journal of Nutrition* (in press)

Gaedicke S, Zhang X, Schmelzer C, Lou Y, Doering F, Frank J & Rimbach G (2008) Vitamin E dependent microRNA regulation in rat liver. *FEBS Letters* 582, 3542-3546

Rimbach G, Gaedicke S, Hübbe P, Bösch-Saadatmandi C (2006): Gene regulatory activity of dietary vitamin E and ginkgo biloba. *Nutrafoods* 5, 7-14. (invited review)

Book chapters

Rimbach G, Gaedicke S, Hübbe P (2005): Neuroprotective effects of dietary vitamin E and Ginkgo biloba. In: Vitamine und Zusatzstoffe in der Ernährung von Mensch und Tier, R. Schubert, G. Flachowsky, G. Jahreis, R. Bitsch (Eds.), 61-70, ISBN 3-86576-014-7.

Poster presentations

Gaedicke S, Rimbach G
„Effects of dietary α -tocopherol from rapeseed oil on redox status and the expression of Alzheimer's disease-relevant genes in the cortex of rats“
Oxygen Club of California 2008 World Congress; Oxidants and Antioxidants in Biology, March 12-15, 2008, Santa Barbara, CA, USA

Gaedicke S, Krupinska K, Rimbach G
"Neuroprotective properties of vitamin E-rich rapeseed oil in rats"
45th Scientific Congress of the German Nutrition Society, March 13-14, 2008, Friedrich-Wilhelms-University Bonn, Germany

Gaedicke S, Krupinska K, Rimbach G
„Neuroprotektive Eigenschaften von Vitamin E-reichem Rapsöl“ (Neuroprotective properties of vitamin E-rich rapeseed oil in rats)
Functional Foods Symposium of the German Agricultural Society (DLG e.V.), June 28-29, 2007, CAU, Kiel, Germany

Gaedicke S, Falk J, Krupinska K, Ohm V, Schwarz K, Rimbach G
"Preparation of Rapeseed Oil with Different Vitamin E Contents for Use in Nutritional Studies"
42th Conference "Antioxidants in plant foods" of the German Society for Quality Research on Plant Foods (Deutsche Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e.V.), March 19-20, 2007, CAU, Kiel, Germany

EIDESSTÄTTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich die vorgelegte Dissertation mit dem Titel

„Effects of Dietary Vitamin E from Rapeseed Oil on Gene Expression in Brain and Liver of Rats“

selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegt habe.

Sonja Gaedicke