Deutsches Institut für Ernährungsforschung
Abteilung Biochemie der Mikronährstoffe

Vitamin E

Elucidation of the Mechanism of Side Chain Degradation and Gene Regulatory Functions

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„By three methods we may learn wisdom: First by reflection, which is noblest; second by imitation, which is easiest; and third by experience, which is the bitterest."

Confucius

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Abbreviations

^{32}P	phosphorus 32
ApoE	apolipoprotein E
ABCA	ATP-binding cassette transporter
Bcl2	B-cell leukemia/lymphoma 2
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
Camk2b	calcium/calmodulin-dependent kinase
CAT	chloramphenicol acetyltransferase
CEHC	carboxyethyl hydroxychroman
CIAP	calf intestinal alkaline phosphatase
CMBHC	carboxymethyl hydroxychroman
CMHHC	carboxymethylhexyl hydroxychroman
COX	cyclooxygenase
dpc	days postcoitum
CPHC	carboxypropyl hydroxychroman
CTGF	connective tissue growth factor
CYP	cytochrome P450
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FDR	false discovery rate
γ-GCS	γ-glutamylcysteine synthetase
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
ICAM	intercellular adhesion molecule
LDL	low density lipoprotein
LXR	liver X receptor
MCP	monocyte chemoattractant protein
MMP	matrix metalloproteinase
MOPS	3-[N-morpholino]propanesulfonic acid

MTT	[1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
Munc18-1	syntaxin binding protein 1
Nsf	N-ethylmaleimide-sensitive fusion protein
ONPG	o-nitrophenyl-β-D-galactoside
PBS	phosphate buffered saline
PKC	protein kinase C
PPAR	peroxisome proliferator associated receptor
PXR	pregnane X receptor
ROR	retinoic acid receptor-related orphan receptor
RPMI	Roswell Park Memorial Institute
RXR	retinoid X receptor
Snap25	synaptosomal-associated protein, 25 kDa
SNARE	soluble NSF-attachment protein receptors
SR	scavenger receptor
TAP	tocopherol associated protein
TBME	*tert*-butyl methyl ether
TEAH	tetraethylammonium hydroxide
TGF	transforming growth factor
TTP	tocopherol transfer protein
VAMP2	vesicle-associated membrane protein 2, synaptobrevin
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoproteins

1 Introduction

Vitamin E is one of the four lipophilic vitamins and comprises 8 naturally occurring isoforms. Although discovered 80 years ago as an essential nutrient for normal reproduction in rats, the knowledge about vitamin E, in particular its biological functions, is far from complete. With the detection of vitamin E´s antioxidative property in the 1930s, vitamin E research was mainly focused on this function, most probably underestimating the biological potency of vitamin E. The antioxidative function of vitamin E alone cannot explain, e.g., vitamin E´s importance for fertility. Neither can the antioxidative function explain why in human adults, neurological degeneration display when vitamin E is deficient. However, during the last decade, several specific cellular functions, especially for α-tocopherol, have been described that are independent from the radical scavenging ability. These so-called novel functions cover, e.g., interference with cellular signalling via the inhibition of the protein kinase C, reduction of the uptake of oxidized lipids via down-regulation of the CD36 scavenger receptor or inhibition of the expression of adhesion molecules.

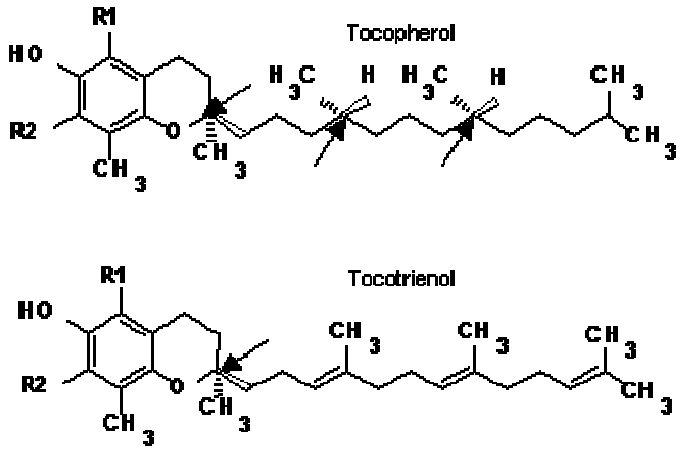
Most recently, a degradation pathway of vitamin E was proposed with an initial oxidation by the drug metabolising cytochrome P450 system. The pathway is supposed to be identical for all tocopherols and tocotrienols, but the amount of excreted metabolites differs markedly between individual vitamin E forms. In general, only sparse amounts of metabolites are found from α-tocopherol whereas high amounts of tocotrienol-derived metabolites are found. Apparently, tocotrienols are eliminated very fast by the human body. This may explain the sometimes observed difference of *in vitro* and *in vivo* effects of different vitamin E forms. It is, therefore, essential to understand how the different degrees in metabolism are regulated.

The present work is focussed on two aspects of vitamin E: metabolism and function. It shows that vitamin E is degraded by cytochrome P450 (CYP) enzymes and that it can induce CYP3A4 in HepG2 cells, and finally, this work raises the question of a possible interaction of vitamin E with the metabolism of drugs, especially with regard to over the counter medication. It further documents that vitamin E influences the expression of other genes *in vivo,* which provides new insights that might explain vitamin E´s essentiality for normal neurological function.

2 Brief review of literature

2.1 Chemistry and dietary sources of vitamin E

The term vitamin E comprises a group of plant derived lipid-soluble vitamers. Their molecular structure is based on a chromanol ring with a side chain at the C2 position. In case of tocopherols, the phytyl side chain is saturated. In tocotrienols the side chain is unsaturated. There are 8 naturally occurring isoforms: 4 tocopherols and 4 tocotrienols, which are designated α-, β-, γ- and δ-, respectively, depending on the number and position of methyl groups at the chromanol ring (Fig. 2.1).



isoform	R1	R2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Figure 2.1 Chemical structure of vitamin E: Shown are the basic molecules for tocopherols and tocotrienols. The number and position of methyl groups at the chromanol ring for corresponding isoforms is given in the table. The chiral centers are indicated with an arrow.

Tocopherols possess three chiral centers in their side chain (at positions 2, 4´, 8´) and tocotrienols one (at position 2). Vitamin E from natural sources occurs only in *RRR*-configuration (tocopherols) and *R*-configuration (tocotrienols), whereas vitamin E of synthetic origin is a racemic mixture (termed *all-rac* vitamin E) of all possible configurations (for tocopherols: *RRR, RSR*, *RRS*, *RSS*, *SRR*, *SSR*, *SRS*, *SSS*). *RRR*-α-Tocopherol was formerly also designated as d-α-tocopherol or (+)-α-tocopherol.

Vitamin E is widely distributed amongst the plant-derived oils. High amounts of α-tocopherol are found in wheat germ oil (~1.6 g α-tocopherol / kg) or sunflower oil (~0.6 g α-tocopherol / kg), whereas soybean oil (~0.7 g

γ-tocopherol / kg) and corn oil (~0.7 g γ-tocopherol / kg) are rich in γ-tocopherol. Tocotrienol concentrations are generally lower compared to tocopherols and are mainly found in palm oil or corn oil (Souci *et al.* 2000).

2.2 Vitamin E-specific binding proteins

As a fat-soluble vitamin, vitamin E is poorly soluble in water-based liquids like plasma or extra- and intracellular fluids. Therefore, vitamin E transport depends on the existence of transport molecules. A vitamin E-specific binding protein was first identified in rat liver cytosol in 1975 (Catignani 1975). The later so-called α-tocopherol transfer protein (α-TTP) is highly expressed in rat and human liver (Sato *et al.* 1991; Arita *et al.* 1995), but also found in pregnant mouse uterus and placenta (Jishage *et al.* 2001; Kaempf-Rotzoll *et al.* 2002) and in human placenta (Müller-Schmehl *et al.* 2004). α-TTP mRNA was found in the brain of humans (Copp *et al.* 1999) and in rat brain, spleen, lung and kidney (Hosomi *et al.* 1998).

α-TTP binds tocopherols and tocotrienols with a high specificity for α-tocopherol with an *R* configuration at position 2 in the chroman ring (Hosomi *et al.* 1997; reviewed in Traber and Arai 1999). Liver α-TTP plays an essential role in the distribution of sufficient α-tocopherol plasma concentrations and in the maintenance of other tissues (see also section below). Patients lacking functional α-TTP possess extremely low plasma α-tocopherol concentrations. Several mutations in the α-TTP gene have been analyzed (Cavalier *et al.* 1998). Mutations in the α-TTP gene are associated with a characteristic syndrome called ataxia with vitamin E deficiency (AVED). The syndrome is characterized by neurological disorders. Studies reveal that α-TTP-deficient patients with very low plasma vitamin E concentrations may suffer from cerebellar ataxia, myopathies, dysarthria, absence of deep tendon reflexes, vibratory and proprioceptive sensory loss (Laplante *et al.* 1984; Krendel *et al.* 1987; Stumpf *et al.* 1987; Larnaout *et al.* 1997; Yokota *et al.* 1997).

How many vitamin E specific binding proteins exist apart from α-TTP is unclear so far. Zimmer *et al*. (2000) identified a tocopherol binding protein (TAP) which is capable of binding α-tocopherol with a higher affinity than the other

tocopherols and was proposed as an intracellular transporter for α-tocopherol (Yamauchi *et al.* 2001).

2.3 Absorption and distribution of vitamin E

Vitamin E is absorbed by passive diffusion in the proximal small intestine together with dietary fat via micelles. In the enterocyte, vitamin E is incorporated into chylomicrons. Vitamin E is transported via the lymphatic pathway and the systemic circulation and is then taken up by chylomicron remnants into parenchymal cells in the liver (reviewed in Herrera and Barbas 2001). In the liver, α-TTP mediates the incorporation of α-tocopherol into nascending VLDL and its release into the circulation system (Hosomi *et al.* 1997). The pathway is illustrated in Fig. 2.2.All non-α-tocopherol vitamin E forms are transferred to a lesser extent by α-TTP, and therefore, α-tocopherol is primarily found in plasma and tissues even if other vitamin E forms predominate in the diet.

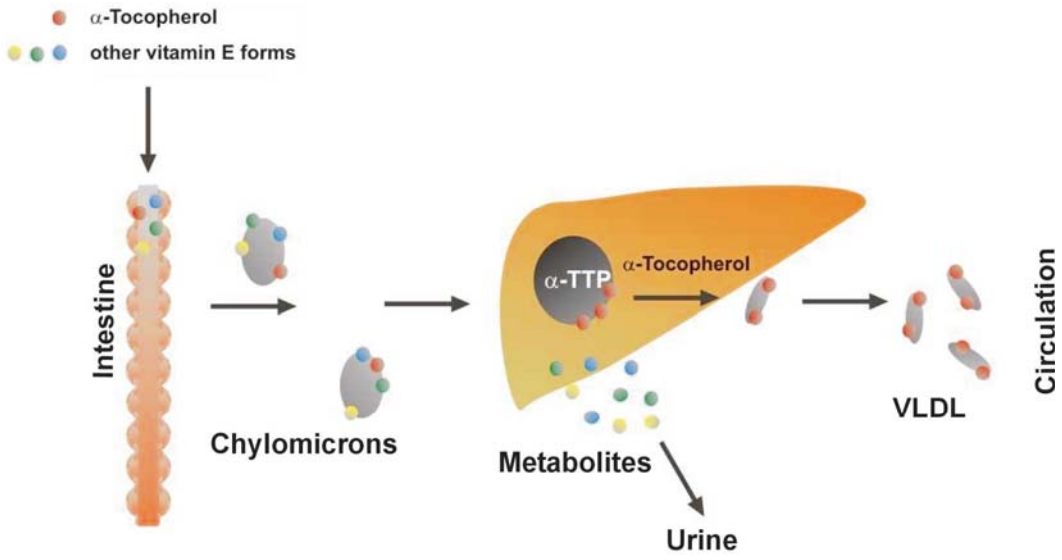


Figure 2.2 Absorption and distribution of vitamin E. For details see text.

Some parts of vitamin E can be transferred between circulating lipoproteins without passing through the liver by the action of the phospholipid transfer protein (Kostner *et al.* 1995). Vitamin E accumulates in adipose tissue where 90% of total body vitamin E is found in healthy subjects. This process of distribution is unlikely to be regulated and appears to be related to its lipid soluble properties (reviewed in Blatt *et al.* 2001).

2.4 Metabolism of vitamin E

2.4.1 Side chain degradation

The first described metabolites of vitamin E were the so-called “Simon-metabolites” tocopheronic acid and tocopheronolactone, both having an opened chroman ring indicating that vitamin E has acted as an antioxidant (Simon *et al.* 1956). However, in the 1990s a metabolite with an intact chroman ring was identified in humans after supplementation with α-tocopherol (Schönfeld *et al.* 1993). This metabolite, α-CEHC (α-carboxyethyl hydroxychroman), was analogous to metabolites from δ-tocopherol found in rats (Chiku *et al.* 1984) and to metabolites from γ-tocopherol in humans (Wechter *et al.* 1996). Furthermore, Schultz *et al*. (1995) identified α-tocopherol metabolites with an opened chroman structure as artifacts when samples were kept under oxygenating conditions. Hence α-CEHC was found when care was taken to avoid oxygenation of the sample (i.e. under inert gas) while the respective Simon-metabolites were found in the absence of protection or under oxygenation. Therefore, Simon-metabolites probably are the result of an oxidative splitting of the chroman ring of CEHC during sample preparation. The proposed mechanism of side chain degradation of tocopherols via initial ω-hydroxylation with subsequent β-oxidation was confirmed by the identification of the CEHC precursor CMBHC (carboxymethylbutyl hydroxychromans), α-CMBHC (Pope *et al.* 2000; Schülke *et al.* 2000), γ-CMBHC (Parker and Swanson 2000) and δ-CMBHC (Pope *et al.* 2000; Birringer *et al.* 2001). Additionally, the next upstream step in the proposed pathway of β-oxidation, was verified by the detection of the precursor of α-CMBHC, α-CMHHC (α-carboxymethylhexyl hydroxychroman, Birringer *et al.* 2001).

2.4.2 CYP induced ω-hydroxylation

The initial ω-hydroxylation required for the degradation of long-chain fatty acids via β-oxidation is catalyzed by cytochrome P450 (CYP) enzymes. CYP3A4 and CYP4F2 have been suggested as the most probable candidates for vitamin E degradation as evidenced by the inhibition of the degradation of α-

and γ-tocopherol (Parker *et al.* 2000) by ketoconazole, a specific inhibitor of CYP3A forms. Further evidence comes from the increase in the metabolism of α-tocopherol by rifampicin, an inducer of CYP3A4 (Birringer *et al.* 2001). Alternatively, microsomes selectively expressing CYP4F2 were most effective in hydroxylating γ-tocopherol compared to the activity of other human liver CYPs (Sontag and Parker 2002). α-Tocopherol was metabolized to only a minor degree by CYP3A4 and CYP4F2. Therefore, it might be possible that different CYPs metabolize different forms of vitamin E with different affinity and efficiency. So far, the enzyme(s) which physiologically initiate(s) the first step of degradation is (are) not known. A CYP-mediated or even induced metabolism of vitamin E is of considerable interest because CYPs actually metabolize xenobiotics. The higher metabolism of *all-rac*-α-tocopherol, □-tocopherol or tocotrienols compared to *RRR*-α-tocopherol would therefore suggest that the organism recognizes some vitamin E forms as xenobiotics.

A subset of cytochromes P450 can be induced or inhibited in the liver by a variety of substances. Most strikingly, many CYP substrates are also modulators of CYP expression (Waxman 1999). A transcriptional regulation of cytochromes P450 by vitamin E had already been discussed in the 1970s. Carpenter *et al*. (reviewed 1972) observed a decrease in microsomal hydroxylation from rats that were fed a vitamin E-deficient diet. More recently, Birringer *et al*. (2001) not only suggested that CYP3A4 initiates tocopherol metabolism, but also observed an increase in α-CEHC formation from *all-rac*-α-tocopherol when HepG2 cells were pretreated with *all-rac*-α-tocopherol for 10 days. This supports the idea of an upregulation of CYP3A4 by α-tocopherol itself.

Transcriptionally, CYP3A4 can be regulated by a nuclear receptor, the pregnane X receptor, PXR (also referred as PAR or SXR; Kliewer *et al.* 1998; Lehmann *et al.* 1998). PXR belongs to the family of orphan receptors as the natural ligand was initially unknown (Bertilsson *et al.* 1998; Kliewer *et al.* 1998). By binding to specific DNA *cis* elements, known as hormone response elements (HRE), PXR activates as a heterodimer with RXR the transcription of target genes. Besides CYP3A4, multidrug resistance 1 (MDR1) gene and multidrug resistance associated proteins (MRP) represent prominent target genes of PXR

(Pfrunder *et al.* 2003). The PXR/RXR specific HREs are composed of direct or everted repeats of the consensus sequence AGGTCA and separated by several nucleotides. PXR is abundantly expressed in liver and intestine tissues, in which CYP3A4 is also highly expressed, and plays a pivotal role in intestinal and hepatoprotection.

Interference between the vitamin E degradation and the CYP system could be far-reaching. As CYPs have a broad catalytic selectivity, side effects of supranutritional dosages of vitamin E can not be excluded (reviewed in Brigelius-Flohé *et al.* 2002). This is of special importance in the case of CYP3A4, the most abundant CYP in human liver. CYP3A4 substrates include a wide range of drugs, steroids and carcinogens (reviewed in Guengerich 1999). Therefore, it would be of great interest whether vitamin E itself is able to influence CYP3A expression (reviewed in Brigelius-Flohé 2003).

2.5 Functions of vitamin E

2.5.1 Vitamin E in fertility

Vitamin E was first described in 1922 by Evans and Bishop as an essential nutritional factor for normal development in rats (Evans and Bishop 1922). Feeding a vitamin E-deficient diet resulted in the resorption of fetuses in pregnant rats.

α-TTP knock out mice display very low or even undetectable plasma concentrations of vitamin E (Terasawa *et al.* 2000; Jishage *et al.* 2001; reviewed in Leonard *et al.* 2003). Therefore, impaired fertility caused by vitamin E deficiency can be studied in animals lacking α-TTP. As shown by Jishage *et al*. (2001), the placentas of pregnant α-TTP knockout mice are severely impaired and show a marked reduction of the labyrinthine trophoblasts, which also emphasizes the importance of vitamin E and α-TTP for fertility. As a consequence, embryos die at the time when the labyrinthine region should take over the nutritional supply to the fetus. However, the exact role of vitamin E in fertility is not known. Although α-TTP was detected in human placenta (Müller-Schmehl *et al.* 2004), it is not known whether human patients, lacking functional α-TTP, also have an impaired fertility.

2.5.2 Antioxidant functions of vitamin E

Only a few years after its discovery, the antioxidant property of vitamin E was described in the 1930s (Olcott and Emerson 1937) and since that time vitamin E has been classified as a lipid soluble antioxidant. The antioxidant function of vitamin E is discussed to play an essential role in the prevention of oxidative stress diseases like atherosclerosis or cancer (reviewed in Dutta and Dutta 2003). As an antioxidant, vitamin E is able to protect polyunsaturated fatty acids in membranes or circulating lipoproteins against lipid peroxidation through scavenging reactive oxygen species. The reaction is nonenzymatic and fast (Wang and Quinn 1999). The basic mechanism is shown in Fig. 2.3.

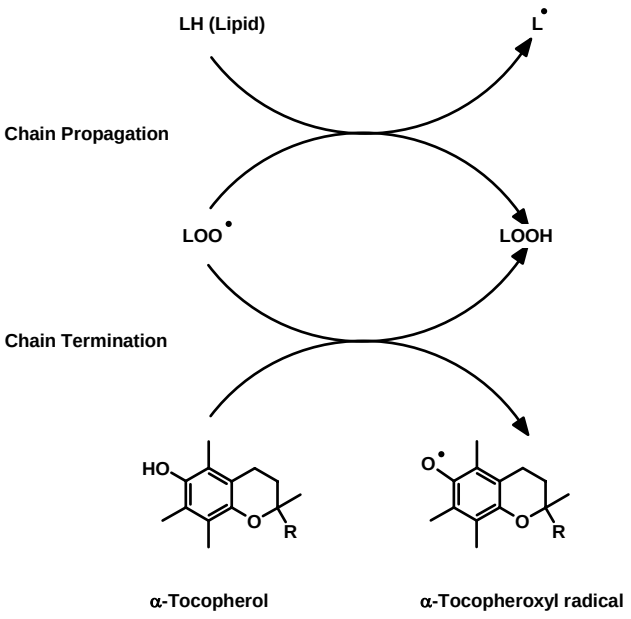


Figure 2.3 Mechanism of inhibition of lipid peroxidation by vitamin E: The scheme is exemplified for α-tocopherol. For details see text.

By reacting with free radicals, lipids ("LH") lose one hydrogen and become themselves radicals ("L•"), which react with free oxygen to produce a peroxyl radical ("LOO•"). This peroxyl radical attacks other lipids and, therefore, initiates a chain reaction by the repetition of this process. Finally, e.g., in cell membranes, this may disturb the structural integrity of the membrane. Vitamin E can interrupt the chain reaction by interaction with the lipid peroxyl radical forming a hydroperoxid ("LOOH"). The resulting tocopheroxyl or tocotrienoxyl radical can be regenerated, e.g., by ascorbate (Constantinescu *et al.* 1993).

2.5.3 Novel functions of vitamin E

By far, not all effects of vitamin E can be assigned to antioxidant properties. Vitamin E has specific molecular functions, e.g., in cellular signaling and gene expression, which are not necessarily related to antioxidative mechanisms (reviewed in Zingg and Azzi 2004). Furthermore, these novel functions appear to be of more relevance regarding vitamin E's essentiality than the antioxidant property.

In 1988, the pioneering discovery of the inhibition of the protein kinase C (PKC) activity by vitamin E in smooth muscle cells (Mahoney and Azzi 1988) was the beginning of a renaissance in vitamin E research. It was suggested by Azzi´s group (Boscoboinik *et al.* 1991) that via the inhibition of the PKC, which is an important factor in cell signaling and a modulator of gene expression during cell growth, proliferation and differentiation, vitamin E may inhibit cell proliferation. The inhibition of PKC is specific to α-tocopherol and occurs via the activation of protein phosphatase type 2A which dephosphorylates PKC thereby inhibiting its activity (Ricciarelli *et al.* 1998). As a confirmation of a non-antioxidant mechanism, β-tocopherol had no effect, despite sharing a similar antioxidant property with α-tocopherol. In addition, by the inhibition of PKC, α-tocopherol inhibits the NADPH oxidase assembly and, therefore, diminishes the production of superoxide anions (reviewed in Jialal *et al.* 2001). The inhibition of PKC also results in a decreased adhesion of monocytes to human endothelium (reviewed in Jialal *et al.* 2001) and a diminished aggregation of human platelets (Freedman *et al.* 1996).

The modulation of gene expression by vitamin E has been confirmed in a number of independent experiments (Tab. 2.1). At the transcriptional level, vitamin E regulates the expression of several genes including collagen α1 and α-TTP in the liver (Chojkier *et al.* 1998; Shaw and Huang 1998), collagenase in skin (Ricciarelli *et al.* 1999), vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1) in endothelial cells (Zapolska-Downar *et al.* 2000), different integrins in erythroleukemia cells (Breyer and Azzi 2001), α-tropomyosin in smooth muscle cells (Aratri *et al.* 1999), and scavenger

receptors class A (SR-A) and CD36 in macrophages and smooth muscle cells (Teupser *et al.* 1999; Ricciarelli *et al.* 2000).

Table 2.1 Novel functions of vitamin E

Object	Experimental model	Protein Expression	mRNA Expression	Reference
Bcl2-L1	rat liver		↓	(Fischer *et al.* 2001)
CD36	smooth muscle cells	↓	↓	(Ricciarelli *et al.* 2000)
CD95	smooth muscle cells	↓		(Lee and Chau 2001)
c-Jun	human breast cancer cells		↑	(Yu *et al.* 1998)
Collagen alpha 1(I)	Mice		↓	(Chojkier *et al.* 1998)
CTGF	smooth muscle cells	↑	↑	(Villacorta *et al.* 2003)
Cyclin D1 / E1	human prostate carcinoma cells	↓ γ-tocopherol		(Gysin *et al.* 2002)
E-Selectin	HUVEC	↓ α-tocotrienol		(Theriault *et al.* 2002)
Glycoprotein II-2	human erythroleukemia cells		↓	(Chang *et al.* 2000)
ICAM-1	HUVEC	↓ α-tocotrienol		(Theriault *et al.* 2002)
MMP-1	skin fibroblasts	↓		(Ricciarelli *et al.* 1999)
p16, p21, p27	human prostate carcinoma cells	↑ γ-tocopherol		(Gysin *et al.* 2002)
p53	smooth muscle cells	↓		(Lee and Chau 2001)
PPARγ	rat primary hepatocytes	↑		(Davies *et al.* 2002)
PPARγ	colon cancer cell line	↑ α- and γ-tocopherol	↑ α- and γ-tocopherol	(Campbell *et al.* 2003)
SR-A I/II	human monocytes		↓	(Teupser *et al.* 1999)
SR-BI	HepG2		↑	(Witt *et al.* 2000)
TGF-β	Rats		↓	(Chan *et al.* 1998)
α-tropomyosin	rat vascular SMC	↑	↑	(Aratri *et al.* 1999)
VCAM-1	HUVEC	↓ α-tocotrienol		(Theriault *et al.* 2002)
α-TTP	Rats		↑ α- and δ-tocopherol	(Fechner *et al.* 1998)
γ-GCS	HaCaT keratinocytes		↑	(Masaki *et al.* 2002)

Arrows indicate the effects under the influence of vitamin E (α-tocopherol, if not otherwise indicated) on protein and mRNA expression, respectively, in different experimental models.

Down-regulation of genes involved in the inhibition of apoptosis (defender against cell death 1 protein, Bcl2-L1), cell cycle (G1/S-specific cyclin

D1) and up-regulation of the γ-glutamylcysteine synthetase catalytic subunit can be also caused by tocopherols (Fischer *et al.* 2001; Gysin *et al.* 2002; Masaki *et al.* 2002).

The tocopherol-associated protein TAP was described as an intracellular vehicle for tocopherol, e.g., between membrane compartments (Zimmer *et al.* 2000). Noteworthy is that TAP in an artificial system has been shown to modulate transcriptional activity in response to the binding of tocopherol and, therefore, suggesting TAP as having a role as a transcription factor (Yamauchi *et al.* 2001).

At the post-translational level, additional to the inhibition of PKC, α-tocopherol inhibits cyclooxygenase (COX) activity in monocytes thus leading to a decrease in prostaglandin E_2 levels (Wu *et al.* 2001). However, an antioxidant mechanism cannot be excluded for the latter effect since these enzymes need a certain hydroperoxide tone for activity. Also, other vitamin E forms inhibit COX activity with an extent of inhibition varying in proportion to their antioxidant capacity. Potent anti-inflammatory properties of vitamin E may contribute to the inhibition of the release of interleukin-1β via the inhibition of 5-lipoxygenase by α-tocopherol (Devaraj and Jialal 1999). Additionally, γ-tocopherol as well as γ-CEHC were reported to reduce prostaglandin E_2 synthesis by direct inhibition of COX-2 activity (Jiang *et al.* 2000). The latter observation pointed to a specific function also for vitamin E metabolites. γ-CEHC, e.g., was first described as LLU-alpha (Wechter *et al.* 1996), having a natriuretic function, possibly by inhibition of the 70 pS K+ channel in the apical membrane of the thick ascending limb of the kidney.

These newly discovered actions of vitamin E may help to explain the sometimes observed beneficial effects of vitamin E in chronic and degenerative diseases like atherosclerosis or cancer (reviewed in Brigelius-Flohé *et al.* 2002). However, further experimental studies are required to investigate potentially different effects on cell signaling and biological activities among isoforms of vitamin E.

2.6 Aim of this work

This study focussed on investigations regarding the regulation of metabolism and on novel functions of vitamin E. The specific aims were:

- To confirm the proposed pathway of vitamin E side chain degradation in HepG2 cells with individual vitamin E forms via HPLC based analysis of the released metabolites.

- To investigate whether vitamin E can regulate the expression of CYPs and to evaluate a possible role for the PXR. For that purpose, a cell culture based model which allows the transient expression of the PXR and a PXR sensitive reporter gene had to be established. A putative regulation via PXR had to be confirmed also *in vivo*.

- To extend the findings on vitamin E-induced gene regulation *in vivo*. For that purpose mice had to be fed diets differing in the vitamin E content. The analysis of differential gene expression using microarray technology should then identify vitamin E-sensitive genes.

3 Experimental procedures

3.1 Cell culture

The cell line used in this work was HepG2 (ATCC number HB8065). HepG2 cells were grown in flasks and multiwell plates in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mM alanyl-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2. Lipophilic substances (e.g., rifampicin, vitamin E, vitamin K, coenzyme Q_{10}) were applied in FCS by adding the corresponding compound from an ethanolic stock solution to the serum followed by an overnight incubation at 4°C as described for vitamin E (Birringer *et al.* 2001). Respective amounts of ethanol were added to the FCS used for control. Ethanol concentration in the culture medium never exceeded 0.4%.

3.2 Protein determination

Quantification of protein was performed according to Bradford (Bradford 1976) and modified for micro well plates. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

10 µl of protein solution were incubated for 15 min at room temperature with 200 µl brilliant blue G-250 (BioRad, München, Germany). Measurement was done in a MR5000 microplate reader (Dynatech, Denkendorf, Germany) at 630 nm. Protein was quantified using a protein standard curve made with BSA.

3.3 Determination of vitamin E metabolites CEHC and CMBHC in cell culture medium by HPLC

After incubation of cells, the cell culture medium was removed for sample preparation as follows (Birringer *et al.* 2002): α-CPHC (α-carboxypropyl hydroxychroman), an artificial intermediate of the ω-oxidation pathway of tocols with 4 C-atoms in the side chain was used as an internal standard (Birringer *et*

al. 2002). To the 8 ml cell culture medium, 100 µl ascorbate (40 mg/ml H_2O), 20 µl α-CPHC (30 µmol/l in ethanol) and 1.6 ml sodium acetate buffer (0.1 mol/l, pH 4.5) were added. After vortexing, the sample was extracted 3 times with 10 ml TBME/BHT (TBME containing 1 ml/l of a 10 g BHT/l ethanol solution). The combined organic phases were evaporated to dryness and redissolved in 200 ml of HPLC loading buffer (50 ml acetonitrile, 50 ml HPLC buffer (see below), 440.8 mg BHT).

Metabolites were separated in a Summit HPLC-system with an ED 50 electrochemical detector (Dionex, Idstein, Germany) and a 250 x 4 mm, RP-18 end-capped column (Merck, Darmstadt, Germany) with a preceding guard column (4 x 4 mm) containing the same stationary phase. HPLC buffer consisted of TEAH (20% in water)/water/acetonitrile (25:540:430) with 0.63 mmol/l EDTA. The pH was adjusted to 5.5 with acetic acid. The flow was set to 0.6 ml/min with a gradient program of 0% acetonitrile (ACN): 0–34 min; 0–45% ACN: 34–52 min; 45–96% ACN: 52–55 min; 96% ACN: 55–65 min; 96–0% ACN: 65–68 min, and 0% ACN: 68–78 min. For coulometric detection, the analytical cell was set to +0.55 V for all metabolites. The peaks for the internal standard α-CPHC, for α-CEHC, γ-CEHC, α-CMBHC and γ-CMBHC eluted at 13.5 min, 9.9 min, 7.8 min, 30.4 min and 21.3 min respectively. CEHC and CMBHC contents were calculated using peak area and concentration of the internal standard α-CPHC. The response factors for α-CEHC, γ-CEHC and α-CMBHC to the internal standard α-CPHC were determined to be 1.0123, 0.9526 and 0.6923 respectively. For lack of γ-CMBHC standard the response factor of α-CMBHC was used based on the assumption that α-CMBHC and γ-CMBHC have a similar response as it was observed for α-CEHC and γ-CEHC (Birringer *et al.* 2001).

3.4 Reporter gene assays

3.4.1 Transformations

Plasmids used in this work were transferred into *E.coli* strains DH5□, Top10 and JM109, by transformation. Transformations were performed according to Sambrook *et al.* (1989). In brief, *E.coli* were grown overnight at

37°C in 10 ml S1 medium of which 4 ml were transferred into 250 ml fresh SOB medium (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl) and grown at 37°C until an OD_{600} of 0.5. Bacteria were pelleted by centrifugation at 4°C and 5,000 g for 5 min. Supernatant was discarded and the pellet was resuspended in 30 ml of ice-cold $CaCl_2$ (100 mM) and incubated 30 min on ice. After another centrifugation step at 4°C and 5,000 g for 7 min, supernatant was discarded and the pellet was resuspended in 5 ml $CaCl_2$ (100 mM, 15% glycerine). Competent cells were stored at –80°C.

5 ng of plasmid DNA were mixed with competent cells, left on ice for 15 min, incubated at 42°C for 1 min and immediately transferred to ice for 1 min. 450 µl SOC medium (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and cells were incubated for 1 hour at 37°C. Thereafter bacteria were plated on agar plates containing 100 µg/ml ampicillin and grown overnight at 37°C. Clones were transferred to 5 ml S1 medium containing 100 µg/ml ampicillin and incubated 12 hours at 37°C and used for plasmid preparation.

3.4.2 Plasmids

The plasmids pSG5hPXR, pBLCAT2(DR3)$_2$, pSG5hPPARα, pSG5hPPARγ, pPPREx3-tk-Luc, pCMX-hLXRα, TK-LXRE3-LUC, pCMX-RXRα, pSG5 and pCH110 were used in this work. For an overview of the used plasmids see Tab. 3.1. pSG5hPXR and pBLCAT2(DR3)$_2$ were kindly provided by S. A. Kliewer, Glaxo Wellcome, Research Triangle Park, NC, USA. The plasmids pSG5hPPARα, pSG5hPPARγ, pPPREx3-tk-Luc, pCMX-hLXRα, TK-LXRE3-LUC and pCMX-RXRα were kindly provided by R. Rühl, Institute of Nutritional Science, University of Potsdam, Bergholz-Rehbrücke, Germany.

pBLCAT2(DR3)$_2$, containing two copies of the rat CYP3A1 (the equivalent for the human CYP3A4, Nelson 1999) PXR binding site (DR3, 5´-TGA ACT n3 TGA ACT-3´); pPPREx3-tk-Luc, containing three copies of the PPARα/γ binding site (DR1, 5´-AGG ACA n1 AGG TCA-3´); TK-LXRE3-LUC, containing three copies of the LXR binding site (DR4, 5´-GGT TTA n4 AGT TCA-3´) were used as reporter. pCH110, a β-galactosidase expression vector (Amersham Biosciences, Braunschweig, Germany) was used for transfection

control. Plasmids were isolated using mini and midi DNA isolation kits either from Qiagen (Qiagen, Hilden, Germany) or Invitek (Invitek, Berlin, Germany) according to the manufacturer protocol.

Table 3.1 Plasmids used in this work.

Name	Description	Reference
pSG5	eukaryotic expression vector	see www.stratagene.com
pSG5hPXR	expression of PXR	(Lehmann *et al.* 1998)
pSG5hPPARα	expression of PPARα	(Kliewer *et al.* 1995)
pSG5hPPARγ	expression of PPARγ	(Kliewer *et al.* 1995)
pCMX-hLXRα	expression of LXRα	(Willy *et al.* 1995)
pCMX-RXRα	expression of RXRα	(Mangelsdorf *et al.* 1992)
pBLCAT2(DR3)$_2$	PXR reporter (chloramphenicol acetyltransferase, CAT)	(Lehmann *et al.* 1998)
pPPREx3-tk-Luc	PPARα and γ reporter (luciferase)	(Forman *et al.* 1995)
TK-LXRE3-LUC	LXRα reporter (luciferase)	(Willy *et al.* 1995)
pCH110	expression of β-galactosidase for transfection control	see www.amersham.com

3.4.3 Transfection of HepG2 cells

For transfection, cells were grown in 24-well plates to about 80% confluency. In general, transfection mixes contained 0.17 µg expression vector of the respective receptor, 0.17 µg respective reporter plasmid and 0.5 µg pCH110 (for normalizing transfection efficiency). To prove a putative PXR dependency, pSG5 (pSG5hPXR without hPXR) was used (as negative control) instead of pSG5hPXR.

Transfection was performed with TfX-20 from Promega (Promega, Mannheim, Germany) according to manufacturer's instructions with 3 µl TfX reagent per µg DNA to be transfected. After 1 h of incubation at 37°C, medium was added to a final concentration of 10% FCS without vitamin E (control) or enriched with vitamin E, vitamin K or coenzyme Q_{10} or the respective positive controls (e.g., rifampicin, a known PXR activator) and incubation continued for 48 h.

3.4.4 Preparation of cell lysates

For harvest, cells were rinsed with PBS, 120 µl of reporter lysis buffer (Promega, Mannheim, Germany) was added. After 15 min incubation at room temperature, cells were scraped from the plate, transferred to microtubes and stored overnight at –80°C. Cell debris was removed by centrifugation at 13,000 rpm in a microcentrifuge for 5 min. Cell lysates were prepared using Promega's reporter lysis buffer according to the manufacturer's protocol. Cell lysates could be used for chloramphenicol acetyltransferase assay, luciferase assay and β-galactosidase assay as well.

3.4.5 Chloramphenicol acetyltransferase (CAT) activity

For measuring CAT-activity, the FAST CAT Green (deoxy) assay kit (Molecular Probes, Leiden, The Netherlands) was used. Added to 50 µl cell lysate were 10 µl component A (chloramphenicol 1:10) and 10 µl acetyl coenzyme A (7.4 mg/ml H_2O). After 3 h at 37°C, 1 ml ice-cold ethyl acetate was added, mixed and centrifuged at 13,000 rpm in a microcentrifuge for 5 min. 950 µl of the upper phase was evaporated to dryness and redissolved in 100 µl HPLC eluent (55% acetonitrile, 45% H_2O). CAT activity was estimated via the amount of fluorescent-labelled acetylated chloramphenicol quantified by HPLC with fluorescence detection (System 440 and SFM 25, Kontron, Neufahrn, Germany) and a 250 x 3 mm RP18 end caped column (Sepserv, Berlin, Germany). Excitation and emission wavelengths were set to 495 nm and 525 nm, respectively. A calibration curve was made from dilutions of the acetylated chloramphenicol reference standard provided in the FAST CAT kit. It was linear in a range from 1:600 to 1:20,000. The mean of R^2 was 0.997 ± 0.0046 (n=5). The inter-day variance of CAT measurements was less than 7.4%.

3.4.6 Measurement of luciferase activity

For measuring luciferase activity, luciferin reagent and luciferase buffer were mixed by vortexing. 100 µl of the mix were automatically injected to 25 µl

of cell lysate in a 96-well plate. Measurement was 3 s after each injection to ensure equal time between all injections and measures.

Luciferin reagent (aliquots stored at -80°C):

- 5 ml 100 mM Tris-HCl (pH 7.8)
- 6.2 mg coenzyme A
- 4.3 mg luciferin
- 8.8 mg ATP

250 ml luciferase buffer (aliquots stored at -20°C):

- 896 mg N-tris(hydroxymethyl)methylglycine ("Tricine")
- 129.8 mg $(MgCO_3)Mg(OH)_2$ x 5 H_2O
- 9.3 mg EDTA
- simmered for 15 min (beaker closed with parafilm), cooled down overnight
- 164.5 mg $MgSO_4$
- 1284.3 mg DTT
- adjusted to pH 7.8 – 8.0 (with NaOH)
- H_2O to 250 ml

3.4.7 Measurement of β-galactosidase activity

β-Galactosidase activity was used as internal transfection control. The levels of active β-galactosidase expression can be quickly measured by its catalytic hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG) substrate to the bright yellow product o-nitrophenolate. β-Galactosidase was assayed by adding 70 µl β-galactosidase-buffer and 30 µl ONPG solution (Sigma, Deisenhofen, Germany) to 50 µl cell lysate followed by incubation at 37°C for 1 hour. Absorption of o-nitrophenolate was measured at 405 nm using a 96-well microplate reader MR5000 (Dynatech, Denkendorf, Germany).

500 ml 2 x β-galactosidase –buffer (aliquots stored at 4°C):

- 10.7 g Na_2HPO_4 x H_2O
- 5.5 g NaH_2PO_4 x H_2O

- 0.75 g KCl
- 0.2 g MgCl x H_2O
- 3.9 g β-mercaptoethanol
- adjusted to pH 7.3
- H_2O to 500 ml

25 ml ONPG solution (aliquots stored at –20°C):

- 100 mg ONPG
- 267 mg Na_2HPO_4 x 2 H_2O
- adjusted to pH 7.5
- H_2O to 25 ml

3.5 Statistical analysis

If not otherwise stated, indicated data are expressed as mean ± SD and the Student´s two-tailed t-test was used to determine significant differences between treatment and control values. Differences at the $P < 0.05$ level were considered statistically significant.

3.6 Animal experiments and gene expression analyses

3.6.1 Animals and diets

Male C57BL6J mice were housed under a constant 12 h light / 12 h dark cycle and allowed free access to diet and water. All animal studies were carried out in accordance with the principles and procedures of the Federation of European Laboratory Animal Science Associations regulations.

Three groups, each consisting of six weanling male mice (1 month old), were fed a diet based on vitamin E stripped basis diet (sniff, Spezialdiäten GmbH, Soest, Germany) and vitamin E content adjusted to 2 mg, 20 mg and 200 mg *RRR*-α-tocopheryl acetate (T3001 from Sigma, Deisenhofen, Germany) per kg diet respectively for 3 months. The last week each group was divided into 2 subgroups, of which 1 was given once daily 50 µl olive oil and the other group 50 µl olive oil containing 250 µg γ-tocotrienol by administration via a gastric tube. Before the mice were euthanized by decapitation, blood was

collected from anesthetised mice (Forene, Abbot, Wiesbaden, Germany) by retroorbital puncture and plasma was extracted by centrifugation and stored at –80°C. Livers were freeze clamped with aluminium tongs cooled down in liquid nitrogen and immediately stored at -80°C.

3.6.2 Determination of liver vitamin E content

Livers were minced with mortar and pistil under liquid nitrogen and transferred to a 15 ml plastic tube containing 1 ml pyrogallol (6% w/v in ethanol). After adding 4.3 nmol δ-tocopherol (in ethanol) as an internal standard, the sample was saponified with 400 µl 40% potassium hydroxide for 30 min at 70°C. Vitamin E was extracted 2 times with 2.5 ml n-hexane after adding 1.6 ml 2% sodium chloride and evaporated to dryness. Residues were dissolved in 95% methanol. Concentrations of α-tocopherol and γ-tocotrienol were determined in a Summit HPLC-system with an ED 50 electrochemical detector (Dionex, Idstein, Germany) and a 250 x 4 mm, RP-18 end-capped column (Merck, Darmstadt, Germany) with a preceding guard column (4 x 4 mm) containing the same stationary phase. The mobile phase consisted of 95% methanol with 1.02 g/l lithium acetate and 0.05% v/v trifluor acetic acid [A] or 100% propanol [B]. The flow was set to 1.0 ml/min with a gradient program of 0% [B]: 0–35 min; 0–30% [B]: 35–38 min; 30% [B]: 38–105 min; 30–0% [B]: 105–108 min, and 0% [B]: 108–120 min. For coulometric detection, the analytical cell was set to +0.85 V. α-Tocopherol and γ-tocotrienol content was calculated using peak area and concentration of the internal standard δ-tocopherol. The response factors for α-tocopherol and γ-tocotrienol were calculated to 0.438 and 0.439, respectively.

3.6.3 Determination of plasma vitamin E concentration

100 - 200 µl plasma was mixed with 1 ml methanol, 2.5 ml n-hexane and 4.3 nmol δ-tocopherol (in ethanol) as internal standard. Vitamin E was extracted 2 times with 2.5 ml n-hexane and the hexane phases were evaporated to dryness. Residues were dissolved in 95% methanol. Tocopherol and tocotrienol concentrations were determined in a Summit HPLC-system with an ED 50

electrochemical detector (Dionex, Idstein, Germany) and a 250 x 4 mm, RP-18 end-capped column (Merck, Darmstadt, Germany) with a preceding guard column (4 x 4 mm) containing the same stationary phase. The mobile phase consisted of 95% methanol with 1.02 g/l lithium acetate and 0.05% v/v trifluor acetic acid (48 min). The flow was set to 1.0 ml/min. For coulometric detection, the analytical cell was set to +0.85 V. α-Tocopherol and γ-tocotrienol content was calculated using peak area and concentration of the internal standard δ-tocopherol. The response factors for α-tocopherol and γ-tocotrienol were calculated to 0.438 and 0.439 respectively.

3.6.4 Sample preparation, hybridization to cDNA arrays (Clontech) and data analysis

The preparation of the arrays and samples and hybridization of the microarrays was carried out in cooperation with Dr. Helena Sztajer at the Gesellschaft für Biotechnologische Forschung in Braunschweig, Germany. RNA from mouse liver was used to generate samples for hybridization to Atlas mouse toxicology 1.2 membranes from Clontech (Clontech, Palo Alto, USA). Synthesis of cDNA and hybridization was carried out according to the manufacturer protocol using the Atlas gene-specific primer mix by reverse transcription and radioactive labelling with ^{32}P.

Isolation of RNA

During the entire experimental process care was taken to avoid contamination of samples with RNases. RNase-free solutions and material (centrifuge, pipettes, tubes etc.) dedicated solely for work on RNA were used throughout the process. RNase Away (Ambion, Austin, USA) was used to eliminate RNases from the working area. Total RNA from liver was isolated using an RNA isolation kit from Invitek (Invitek, Berlin, Germany) and on-column DNA digestion. For this purpose, 10 – 20 mg liver were homogenized in a rotor stator homogenizer (IKA-Werke, Staufen, Germany) in 700 µl of lysis solution. On-column DNA digestion was carried out using RNase-free DNase (Qiagen, Hilden, Germany). Concentrations of RNA samples were determined photometrically. RNA quality was checked by gelelectrophoresis. Per lane 1 µg

RNA (in 6 µl RNase-free water) with 1.5 µl 5 x RNA-loading buffer was run in a 1.2% agarose gel (1.2 g RNase-free agarose in 100 ml 1 x FA-buffer) in 1 x FA gel-running buffer at 70 V for 10 – 30 min.

10 x FA-buffer:

- 200 mM 3-[N-morpholino]propansulfonic acid (free acid)
- 50 mM sodium acetate
- 10 mM EDTA
- adjusted to pH 7.0 (with NaOH)

1 x FA gel-running buffer:

- 100 ml 10 x FA-buffer
- 20 ml 37% formaldehyde
- 880 ml RNase-free water

5 x RNA-loading buffer (aliquots stored at 4°C):

- 16 µl saturated bromphenol blue solution
- 80 µl 500 mM EDTA, pH 8.0
- 720 µl 37% formaldehyde
- 2 ml 100% glycerol
- 3.084 ml formamide
- 4 ml 10 x FA-buffer
- to 10 ml with RNase-free water
- RNase-free H_2O to 10 ml

Reverse transcription, labelling and column chromatography

For reverse transcription, 4 µg of total RNA in 5 µl H_2O from each mouse liver and 1 µl Clontech´s cDNA synthesis primer were incubated for 2 min at 70ºC followed by 33 min at 50°C after adding 4 µl of 5 x Clontech´s reaction buffer, 1 µl DTT, 2 µl H_2O, 5 µl [α^{32}P]dATP (Amersham, Uppsala, Sweden) 2 µl of 10 x oligo dT and 2 µl MMLV RNase H^- Reverse Transcriptase (Promega, Mannheim, Germany). To purify the labeled cDNA samples from unincorporated [^{32}P]-labeled nucleotides and small (< 0.1 kb) cDNA fragments, Clontech´s BD

Atlas Nucleospin Extraction Kit was used: 176 µl NT2-buffer was added to each sample, mixed by pipetting and transferred to a NucleoSpin Extraction Spin Column which was placed in a 2-ml collection Tube and centrifuged at 14,000 rpm in a microcentrifuge for 1 min. The NucleoSpin column was placed into a fresh 2-ml collection tube, 400 µl buffer NT3 was added and centrifuged at 14,000 rpm in a microcentrifuge for 1 min. This step was repeated twice. NucleoSpin column was transferred to a clean 1.5-ml micro centrifuge, 100 µl Buffer NE was added, soaked for 2 min and centrifuged at 14,000 rpm in a microcentrifuge for 1 min. Radioactivity was checked using 2 µl of the flowthrough in a Beckmann scintillation counter (Beckmann, Fullerton, USA). Samples should have a total of 1–10 x 10^6 cpm.

Hybridization

Clontech´s BD ExpressHyb was prewarmed at 68°C. For each sample, 0.5 mg Sheared salmon testes DNA (D7656, Sigma, Deisenhofen, Germany) was heated at 95–100°C for 5 min, chilled quickly on ice and added to 5.5 ml BD ExpressHyb. The mix was kept at 68°C until use. Hybridization bottles (15 cm, Biometra, Göttingen, Germany) were filled with deionized H_2O. The arrays were wetted by placing them in a dish of deionized H_2O, all the water was poured off from the bottle, then the arrays were placed into the bottle. Air pockets between the array and the bottle were avoided. The prepared 5.5 ml BD ExpressHyb (at 68°C and containing sheared salmon testes DNA, see above) was evenly distributed over the array membrane. The bottles were placed into a OV5 Biometra hybridization oven (Biometra, Göttingen, Germany). Prehybridization with agitation continued for 30 min at 68°C. For hybridization 11 µl 10x denaturating solution (1M NaOH, 10 mM EDTA) was added to 100 µl labeled sample and incubated at 68°C for 20 min. 5 µl C_ot-1 (competitor) DNA and 115 µl 2x neutralizing solution (1 M NaH_2PO_4, pH 7.0) were added and incubated at 68°C for 10 min. The cDNA samples were transferred to the hybridization bottles into the prehybridization solution and hybridized overnight with continuous agitation at 68°C. The next day, both wash solution 1 (2x SSC, 1% SDS) and wash solution 2 (0.1x SSC, 0.5% SDS) were prewarmed to 68°C. The hybridization solution was carefully removed and discarded. The bottle was filled to 80% with prewarmed wash solution 1 and the membrane was washed

for 30 min with continuous agitation at 68°C. This step was repeated three more times. Afterwards, the bottle was filled to 80% with prewarmed wash solution 2 and the membrane was washed for 30 min with continuous agitation at 68°C. Finally, using forceps the arrays were removed from the bottles and placed into dishes with 2x SSC at room temperature. After 5 min, arrays were wrapped in plastic (Severin, Sundern, Germany) and sealed.

1 l 20x saline-sodium citrate-buffer (SSC)

- 175.3 g NaCl
- 88.2 g Na_3 citrate x 2 H_2O
- adjusted to pH 7.0 with 1 M HCl

Scanning and image analysis

After hybridization, the wrapped arrays were placed in exposure cassettes with a storage phosphor screen (Amersham Biosciences, Braunschweig, Germany) at room temperature. The storage phosphor screens were scanned using a phosphoimager Storm 860 (Molecular Dynamics, USA). The raw data obtained from the scanned array images were analyzed and calculated using ArrayVision 8.0 software (Amersham Biosciences, Braunschweig, Germany) and normalized as follows: Background for each membrane was determined by measuring a template in a defined pixel area surrounding all cDNA clusters. Background intensity was subtracted resulting in a net intensity for each spot. This intensity was normalized to the average intensity of all spots on the membrane.

Stripping cDNA samples from the array membranes

To re-use the array after exposure to phosphoimaging, the cDNA was removed from the array by stripping. In a 2 l beaker, 1 l of 0.5% SDS solution was heated to boiling. The plastic wrap was removed from the arrays. The array membranes were placed immediately into the boiling solution. Prolonged exposure of the membrane to air was avoided. Boiling was continued for 10 min. The solution was removed from heat and allowed to cool for 10 min. The array membranes were rinsed in wash solution 1 (2X SSC, 1% SDS). After

washing, the array was removed from the solution, immediately wrapped in plastic and sealed. The efficiency of stripping was checked with a Geiger hand counter and by exposure to phosphoimaging. Arrays were stored at –20°C until needed. Each membrane was stripped not more than two times.

Data analysis

cDNA array data were analyzed for significant changes by using SAM software (for details about SAM software see appendix). Unlogged data were calculated as two class unpaired. Fold change was set to 2, and the delta value was adjusted manually to receive significant results.

3.6.5 Oligonucleotide arrays (Affymetrix)

The preparation of the samples and the hybridization of the microarrays were carried out in the laboratory of Prof. Joe Lunec (Leicester, UK). The protocol was provided by Leicester (see appendix). In principle (see Fig 3.1), labeled cRNA samples derived from the mRNA of the experimental animal are hybridized to nucleic acid probes attached to the array. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented.

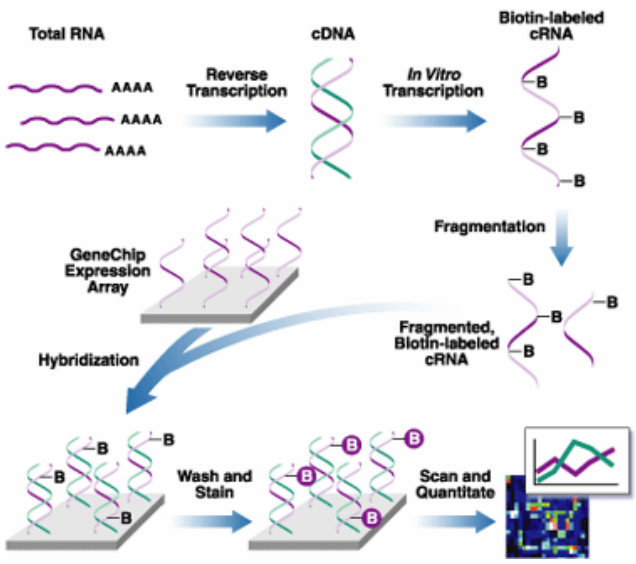


Figure 3.1 Affymetrix experimental procedure (adapted from www.affymetrix.com).

4 Results

4.1 Metabolism of vitamin E

4.1.1 Confirmation of the proposed pathway

For the formation of CEHC from tocopherols, an initial ω-oxidation of the side chain with 5 subsequent steps of β-oxidation was proposed (Birringer *et al.* 2001).

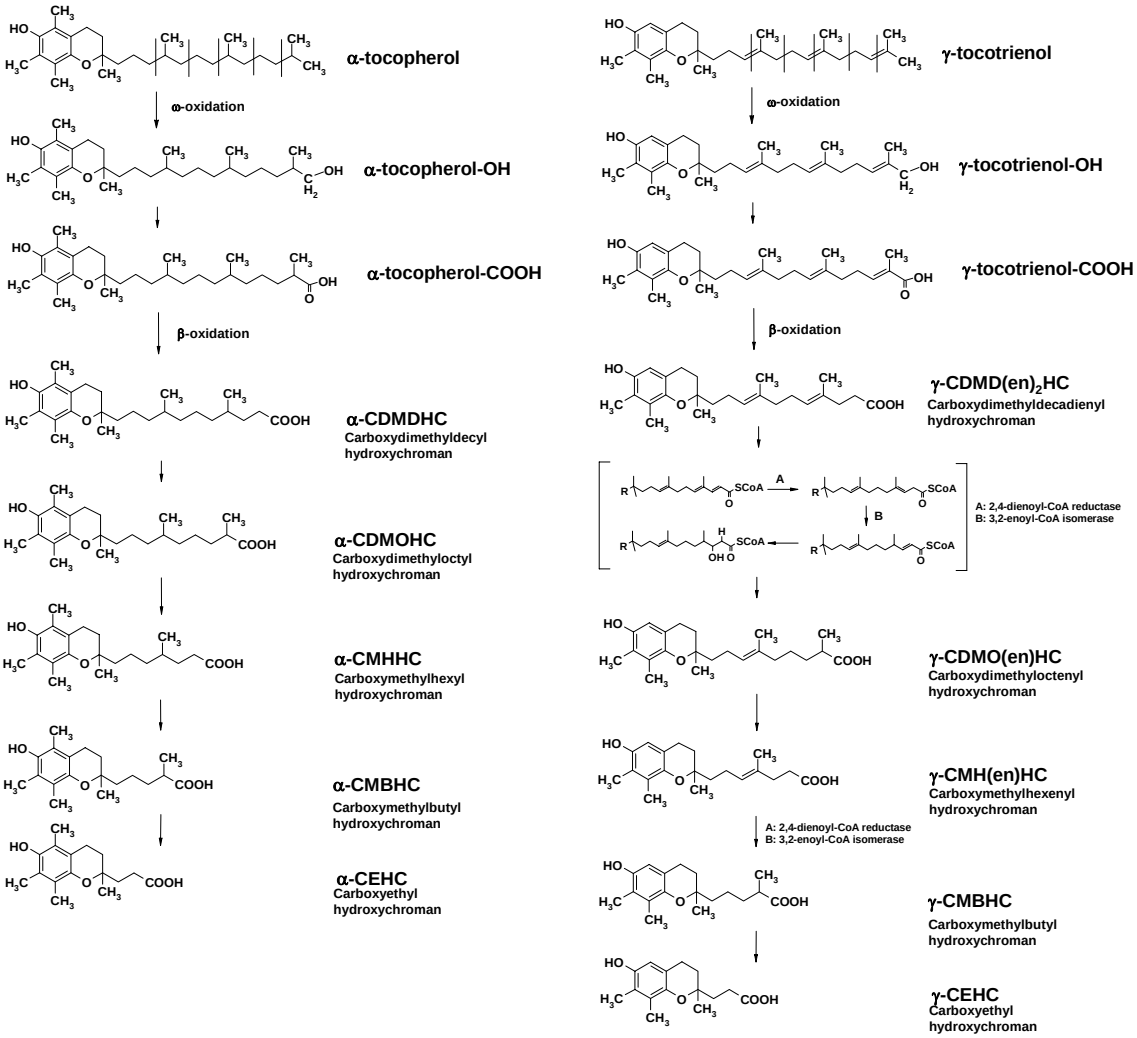


Figure 4.1 Mechanisms of tocopherol and tocotrienol side-chain degradation: Pathway is exemplified for α-tocopherol (left) and γ-tocotrienol (right).Side-chain degradation starts with the initial ω-hydroxylation, followed by five β-oxidation cycles. Auxiliary enzymes required for the degradation of tocotrienol side chain are indicated (step A: 2,4-dienoyl-CoA reductase; step B: 3,2-enoyl-CoA isomerase). The pathway was published in 2002 (Birringer *et al.* 2002) from where the figure is taken.

In HepG2 cells, which were used as a model to study vitamin E metabolism, the pathway was confirmed in a concerted action of the vitamin E group in our lab by the identification of the respective intermediates for tocopherols as well as for tocotrienols which are also degraded to CEHC. Also CMBHC, the precursor of CEHC is the same for tocopherols and tocotrienols. For the tocotrienols this was surprising because of the unsaturated side chain. Instead of CMBHC, a metabolite containing a double bond was expected for the tocotrienol metabolism. The identification of further carboxylic acid intermediates revealed a more complex mechanism of degradation for tocotrienols (Birringer *et al.* 2002). Auxiliary enzymes known in the degradation pathway of unsaturated fatty acids obviously are required for the degradation of tocotrienols (Fig. 4.1). 2,4-Dienoyl-CoA reductase and 3,2-enoyl-CoA isomerase are required for intermediates with two conjugated double bonds (carboxydimethyldecadienyl hydroxychroman and carboxymethylhexenyl hydroxychroman), which are not accepted by the enoyl-CoA hydratase in the β-oxidation pathway. Thus, the side chain of tocotrienols is metabolized the same as unsaturated fatty acids, further confirming the validity of the mechanism.

4.1.2 Metabolism of vitamin E in HepG2 cells

To determine the difference in the metabolic rate between different vitamin E forms, HepG2 cells were seeded to 1.5 x 10^5 cells per well in 6-well plates. In former experiments (Birringer *et al.* 2001) the requirement of a pretreatment of HepG2 cells with α-tocopherol for 10 days to detect metabolites from *all-rac*-α-tocopherol was demonstrated. Hence one half of the plates was cultured in the presence of 100 µM *all-rac*-α-tocopherol for 10 days and designated as “adapted”, the other half was cultured in vitamin E-deficient medium. Thereafter medium was changed to vitamin E-deficient medium in all plates. To ensure that the adapted cells do not release metabolites anymore from *all-rac*-α-tocopherol taken up in the adaption phase, culture medium was analyzed for metabolites until metabolites were no longer detected. This generally lasted 4 days. Thereafter adapted and non-adapted cells were incubated with *RRR*-α-tocopherol, *all-rac*-α-tocopherol, γ-tocopherol,

α̃tocotrienol or γ-tocotrienol at 50 µM each, respectively. The medium was replaced every 24 h over a period of 10 days. The medium from each day and well, respectively, was analyzed for CEHC and CMBHC.

The amount of metabolites released into the cell culture medium derived from the individual vitamin E forms was different. Also differences between adapted and non-adapted HepG2 cells were observed. The release of CEHC was lowest from *RRR*-α-tocopherol followed by α-tocotrienol, γ-tocopherol and was about 100-fold higher after γ-tocotrienol incubation than after α-tocopherol treatment. A preincubation of cells with 100 µM *all-rac*-α-tocopherol tended to cause a slightly higher release of CEHC for all vitamin E forms. The difference between adapted and non-adapted cells was highest for α-tocopherol (with CEHC from adapted cells being 2.5 times higher than from non-adapted cells after 10 days) and only marginal for α-tocotrienol for which the graphs for adapted and non-adapted cells were nearly congruent (Fig. 4.2).

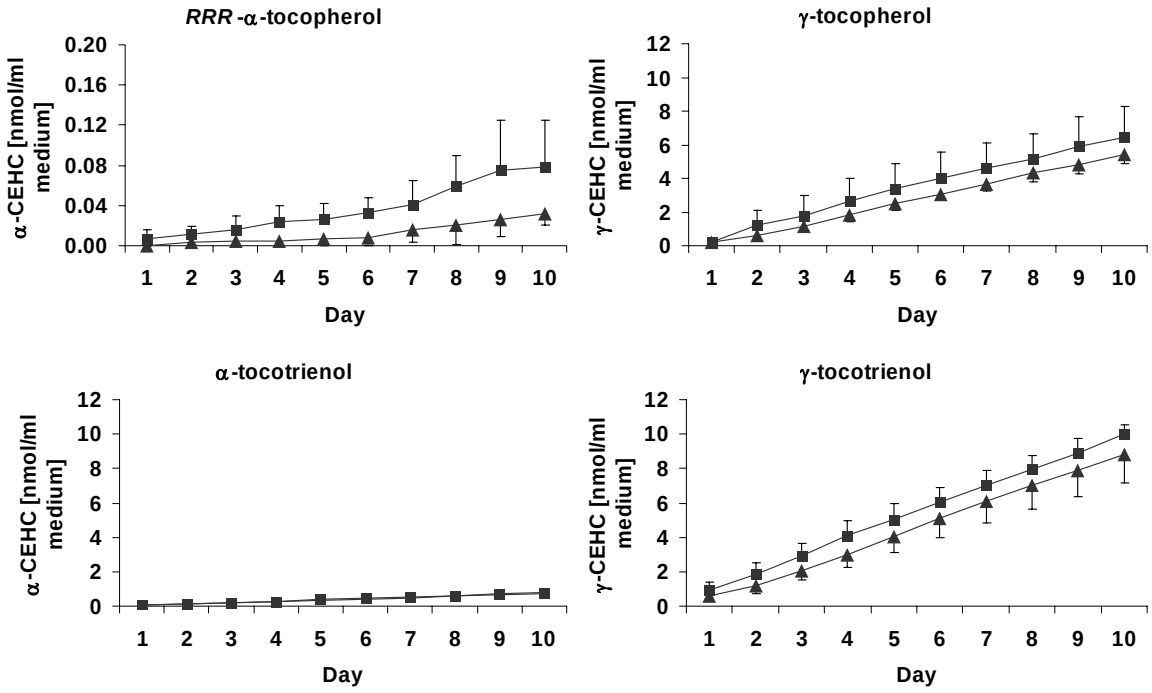


Figure 4.2 CEHC derived from individual forms of vitamin E: CEHC from adapted HepG2 cells (squares) and CEHC from non-adapted HepG2 cells (triangles). Values are means ± SD from three wells. For experimental details see text and experimental procedures. Please note that the ordinate for *RRR*-α-tocopherol is scaled differentially!

Concentrations of CMBHC found in the medium were generally higher (Fig. 4.3) than those of CEHC. α-Tocopherol supply again led to lower metabolite concentrations in the medium compared to the other vitamin E forms. The α-tocotrienol noticeably produced CMBHC amounts comparable to γ-tocotrienol, which was in contrast to the low production of α-CEHC (Fig. 4.2). The release of CMBHC was slightly higher when cells were adapted.

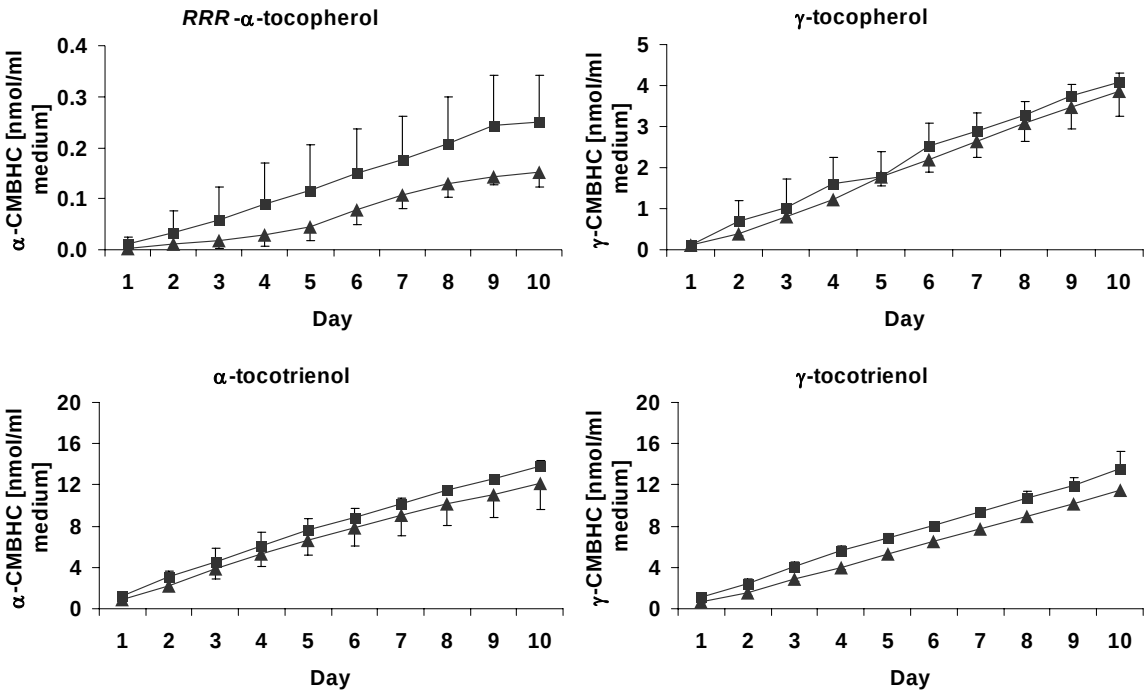


Figure 4.3 CMBHC derived from individual forms of vitamin E: CMBHC from adapted HepG2 cells (squares) and CMBHC from non-adapted HepG2 cells (triangles). Values are means ± SD from three wells. Please note that the ordinates are scaled differentially! For experimental details see text and experimental procedures.

By comparing the release of α-CEHC derived from cells incubated with *RRR*-α-tocopherol or *all-rac*-α-tocopherol marginal differences were observed (Fig. 4.4). The release of α-CEHC was slightly lower for *RRR*-α-tocopherol. Virtually no differences between the two isoforms were observed for α-CMBHC release. Adaptation of the cells resulted in a similarly enhanced release of metabolites of *RRR*-α-tocopherol and *all-rac*-α-tocopherol suggesting an activation of α-tocopherol metabolizing enzymes. An enhanced release of

metabolites as result of the adaptation of the cells was observed neither for γ-tocopherol nor for the tocotrienols.

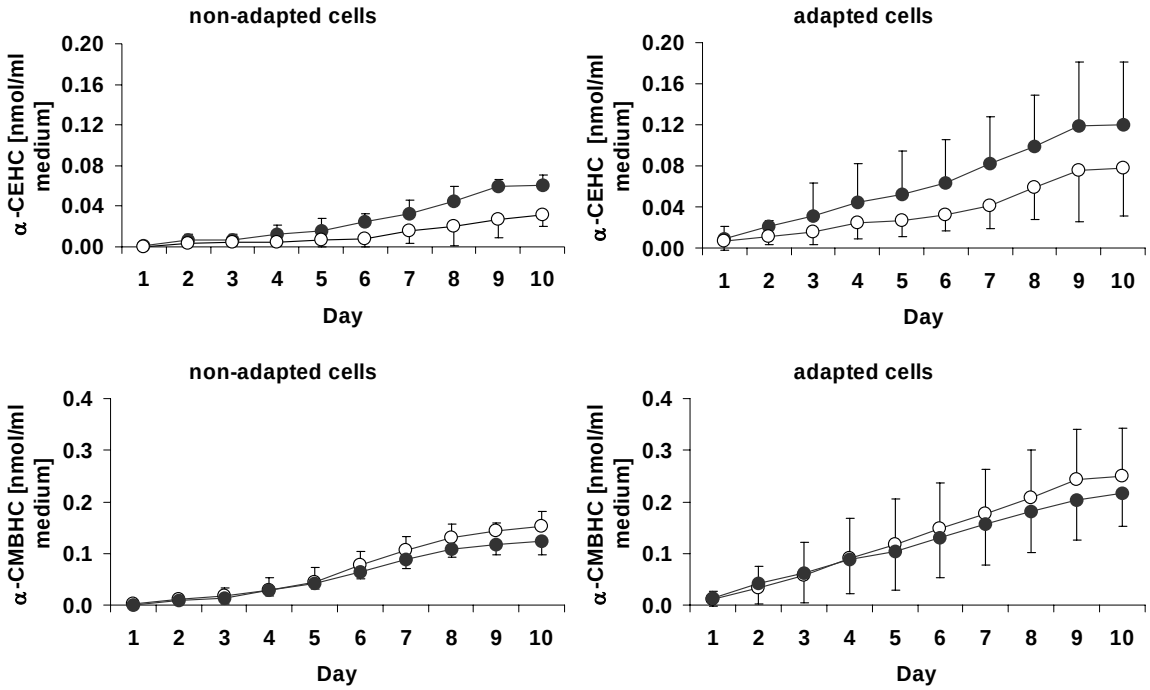


Figure 4.4 Comparison of metabolism of *RRR*-α-tocopherol *vs*. *all-rac*-α-tocopherol: CEHC (top) and CMBHC (bottom) in non-adapted (left) and adapted (right) HepG2 cells derived from *RRR*-α-tocopherol (open circles) or *all-rac*-α-tocopherol (black circles) in HepG2 cells. For experimental details see text and experimental procedures.

The reason for an enhanced metabolism of α-tocopherol after adaptation of the cells could be an activation of metabolising enzymes. An involvement of CYPs was already evidenced by the inhibition of the degradation of α-tocopherol by ketoconazole, a specific inhibitor of the CYP3A family, in HepG2 cells (Parker *et al.* 2000). Additionally, rifampicin, an inducer of CYP3A enzymes, increased the metabolism of α-tocopherol (Birringer *et al.* 2001). Many cytochromes P450 can be induced or inhibited in the liver by their own substrates (Watkins 1999). The molecular mechanism in case of CYP3A is the activation of the pregnane X receptor, PXR. Therefore the effect of vitamin E on the expression of a PXR-regulated reporter gene was investigated.

4.2 Vitamin E-mediated gene activation *in vitro*

4.2.1 Activation of PXR in HepG2 cells

To investigate whether vitamin E activates PXR, a standard cell-based reporter gene assay was used. Since HepG2 cells express PXR and are able to incorporate and metabolize vitamin E, they were used for these experiments. Cells were transiently transfected with an expression vector for human PXR, a reporter plasmid containing two copies of a CYP3A1 PXR binding site upstream of the thymidine kinase promoter and the CAT gene - the rat CYP3A1 represents the equivalent to the human CYP3A4 gene (Nelson 1999). A β-galactosidase expression plasmid was used for transfection control. The cells were incubated with rifampicin as a positive control and different vitamin E forms as indicated (Fig. 4.5). Except for *all-rac*-α-tocopherol, all tested vitamin E forms stimulated CAT activity. Noticeably α- and γ-tocotrienol were the most effective activators of PXR followed by rifampicin > δ-tocopherol > *RRR*-α-tocopherol > γ-tocopherol. α-Tocotrienol and γ-tocotrienol increased CAT-activity 10.1 and 10.6-fold and, thus, caused even a higher activation than the positive control rifampicin. In contrast, CAT activation by *RRR*-α-tocopherol was at 2.6-fold comparably low but significant.

The vitamin E content of the cells was not causative for the different PXR-activating potential. HepG2 cells treated with 50 µM of different vitamin E forms for 48 hours had in fact a higher γ-tocotrienol content than *RRR*-α-tocopherol. However, they showed no difference in *RRR*-α-tocopherol and *all-rac*-α-tocopherol content (data not shown). γ-Tocopherol content was twice as *RRR*-α-tocopherol but γ-tocopherol was a weaker PXR-activator. To ensure the observed increase in CAT activity was indeed PXR-mediated, the experiment was repeated but with the PXR expression plasmid omitted. For this purpose the “empty” pSG5 vector was used instead of pSG5hPXR. In cells transfected this way, the CAT activity was significantly lower than in cells cotransfected with PXR (Fig. 4.6). Nevertheless, also without an overexpression of PXR, a weak activation of the PXR-responsive element was observed, which confirmed the physiological relevance of the system.

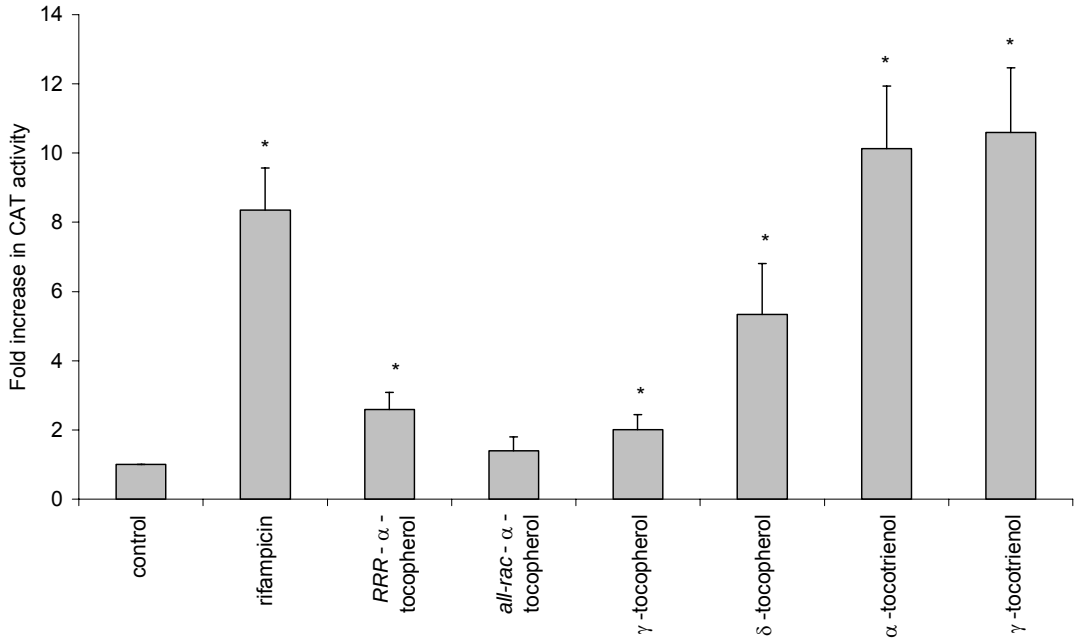


Figure 4.5 Different vitamin E forms induce CAT activity via PXR. HepG2 cells were transfected with the expression plasmid for the human PXR, a PXR-driven CAT reporter gene, and β-galactosidase expression plasmid. Cells were treated with 10 µM rifampicin and 50 µM concentrations of the indicated vitamin E forms. CAT activity was measured and normalized to β-galactosidase activity. Data are plotted as fold induction relative to untreated cells (control) and represent the mean of four independent experiments measured in triplicate ± SD. Asterisk indicates $P < 0.05$ *vs*. untreated control.

For the activation of PXR, RXRα is the essential heterodimerization partner. To exclude that the overexpression of RXRαhas the same effect like PXR overexpression, RXRαwas transfected instead of PXR. However, in cells transfected with RXRαthe CAT activity was as low as in the cells transfected without PXR and without RXRα (Fig. 4.6).

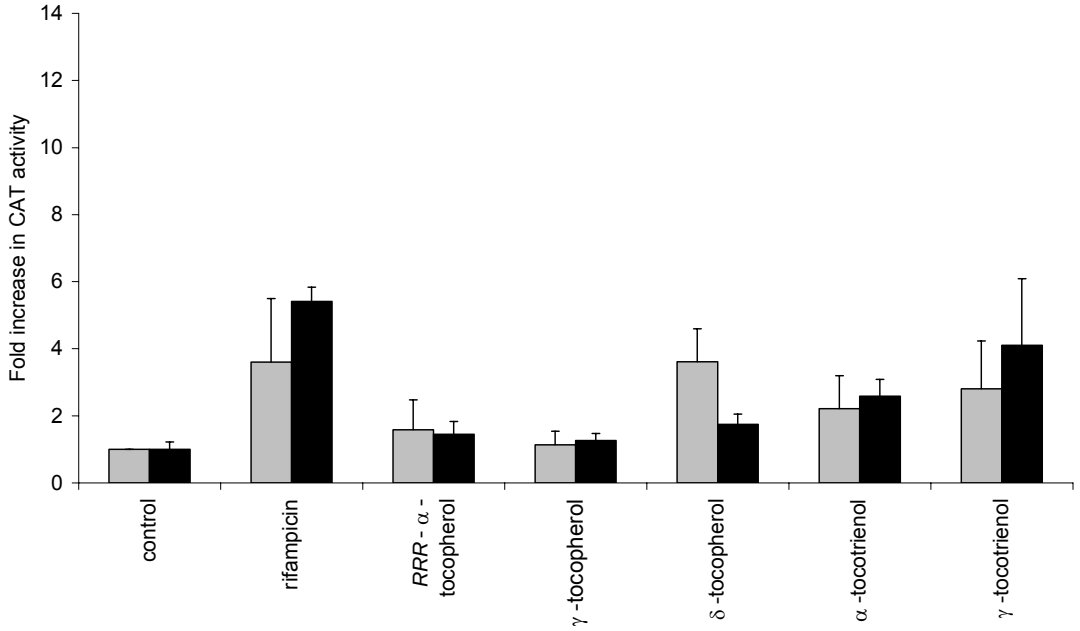


Figure 4.6 Induction of CAT activity by vitamin E is PXR- and not RXR-mediated. HepG2 cells were transfected with the expression plasmid without human PXR (gray) or with the expression plasmid containing the human RXRα (black), a PXR-driven CAT reporter gene and β-galactosidase expression plasmid. Cells were treated with 10 µM rifampicin and 50 µM concentrations of the indicated vitamin E forms. CAT activity was measured and normalized to β-galactosidase activity. Data are plotted as fold induction relative to untreated cells (control) and represent the mean of three independent (gray) and one (black) experiments measured in triplicate ± SD, respectively.

PXR-mediated activation was also dose-dependent. γ-Tocotrienol, the most effective PXR activator, was compared to the positive control rifampicin in a range from 10 nM to 50 µM. Rifampicin showed an increase in CAT activation beyond 0.1 µM, an increase in CAT activation by γ-tocotrienol was observed from 1 µM on (Fig. 4.7). To summarize, the CAT activation by vitamin E under physiological levels of PXR can be increased by overexpression of PXR but not by overexpression of RXRα, meaning that the induction of CAT by vitamin E was essentially PXR dependent.

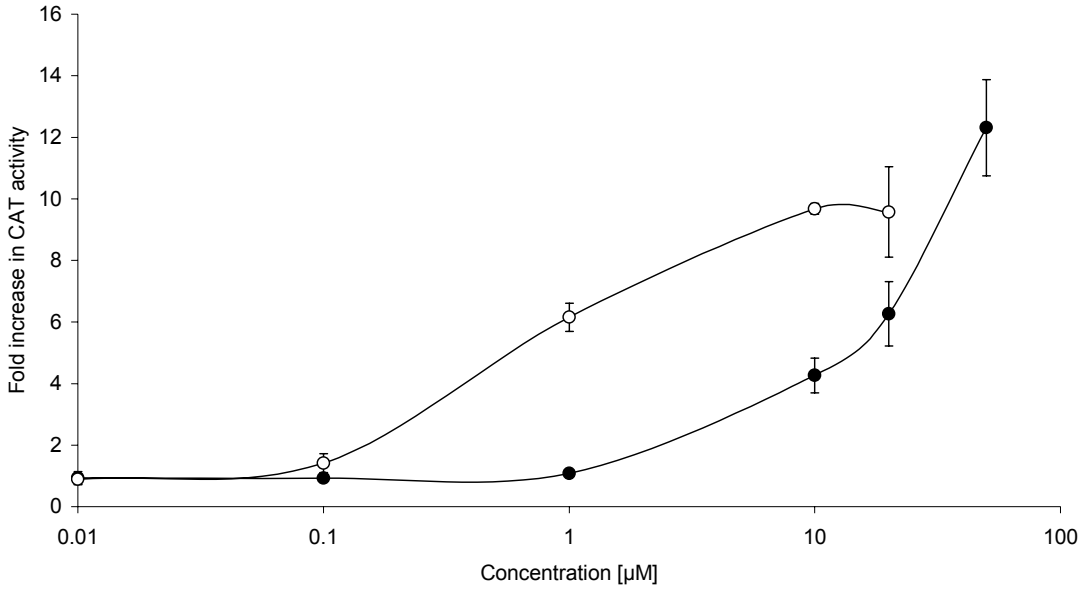


Figure 4.7 Dose response of rifampicin and γ-tocotrienol-mediated PXR-activation. Cells were transfected with the expression plasmid for human PXR, a PXR-driven CAT reporter gene and β-galactosidase expression plasmid and treated with increasing concentrations of rifampicin (open circles) and γ-tocotrienol (black circles). CAT activity was measured and calculated as described in Fig. 4.5. Data are expressed as fold induction relative to untreated cells and represent the mean of three independent experiments measured in triplicate ± SD, respectively.

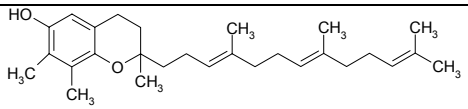
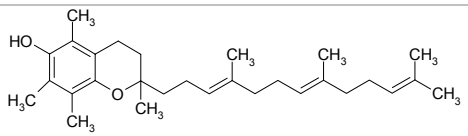
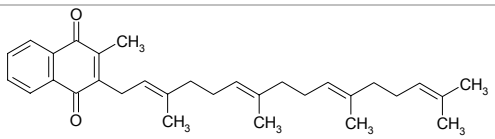
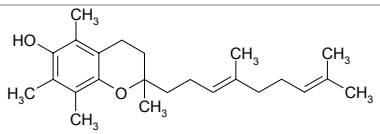
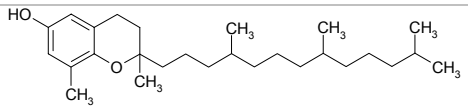
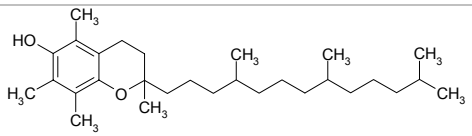
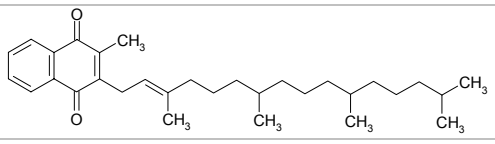
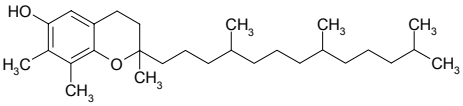
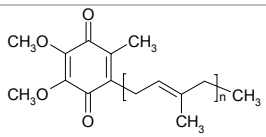
4.2.2 PXR activation by compounds structurally related to vitamin E

There is a high structural similarity of the side chain and the final metabolites of vitamin K and coenzyme Q_{10} with the side chain and the final metabolites of vitamin E. K acid 1 and Q acid 1 obviously are the second final metabolites as are the CMBHCs derived from individual forms of vitamin E, whereas K acid 2 and Q acid 2 correspond to CEHCs, the final products of vitamin E metabolism (Fig. 5.1). This suggests that vitamin E, vitamin K and coenzyme Q_{10} are metabolized via the same mechanism which initiated an additional test as to whether they would be active in the PXR reporter gene assay as vitamin E.

For this purpose, HepG2 cells were transfected with the PXR-reporter system and treated with 10 µM rifampicin and 50 µM concentrations of vitamin K_1, vitamin K_2, 10 µM coenzyme Q_{10} and 5 µM α-tocodienol, an artificial α-tocotrienol derivate lacking one isoprenoid unit (synthesized in our lab by Dr.

Marc Birringer), respectively. Indeed, vitamin K_2 (menaquinone) which has an unsaturated side chain, activated the reporter gene with the same efficiency as tocotrienols, whereas vitamin K_1 (phylloquinone) which has a saturated side chain, was as efficient as α-tocopherol (Tab. 4.1). Coenzyme Q_{10} did not show any effect. The synthetic compound α-tocodienol, which has a similar structure as α-tocotrienol but with a side chain shorter by one isoprenoid group, increased CAT activity up to 8.5-fold at concentrations as low as 5 µM.

Table 4.1 PXR-mediated CAT activities for vitamin E, vitamin K and coenzyme Q_{10}.

Compound	Structure	Fold increase in CAT activity
γ-tocotrienol		10.6
α-tocotrienol		10.1
vitamin K_2		10.7
α-tocodienol		8.5 (at 5 µM)
δ-tocopherol		5.3
α-tocopherol		2.6
vitamin K_1		3.5
γ-tocopherol		2.0
coenzyme Q_{10} (n=10)		1.2 (at 10 µM)

If not other indicated, data are representative for a concentration of 50 µM.

4.2.3 Vitamin E metabolism is not required for PXR activation

Watkins *et al*. (2001) identified polar residues in the hydrophobic ligand-binding cavity of hPXR as critical determinants. This is further confirmed that many PXR activators have a polar structure (Lehmann *et al.* 1998) which would be better met by vitamin E metabolites. Therefore, it was tested whether metabolites are required for PXR activation. The metabolites with shortened side chain, α-CEHC and α-CMBHC, were not able to activate PXR at a concentration of 10 µM (Fig. 4.8).

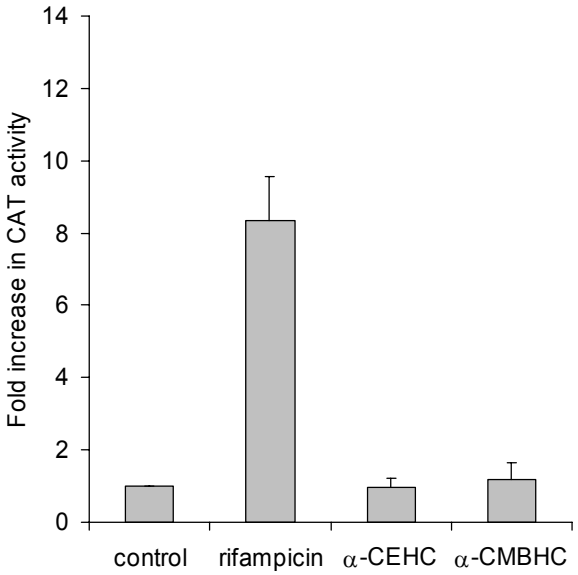


Figure 4.8 Vitamin E metabolites α-CEHC and α-CMBHC do not induce CAT activity via PXR. HepG2 cells were transfected as described in Fig. 4.5 and treated with 10 µM rifampicin and 10 µM concentrations of the indicated vitamin E metabolites. CAT activity was measured and normalized to β-galactosidase activity. Data are expressed as described in Fig. 4.5 and represent the mean of three independent experiments measured in triplicate ± SD.

ω-Oxidation of vitamin E as first step in the metabolism results in more polar groups like hydroxyl- and carboxyl-groups (Fig. 4.1). It would not be unlikely that these metabolites activate PXR. In order to test whether vitamin E metabolism is involved in PXR activation at all, the metabolism was inhibited by specific inhibitors of CYP3A activity. At first, cells were treated with sesamin, a sesame lignane which has markedly inhibited vitamin E metabolism in previous studies (Parker *et al.* 2000).

Neither in the sesamin treated cells nor in the untreated control cells were metabolites of α-tocopherol detected after 48 hours (as already shown in Fig. 4.2). In contrast, metabolites derived from γ-tocopherol, α-tocopherol and γ-tocotrienol as expressed as sum of CEHC and CMBHC were significantly lowered by sesamin to 5%, 14% and 18% respectively (Fig. 4.9).

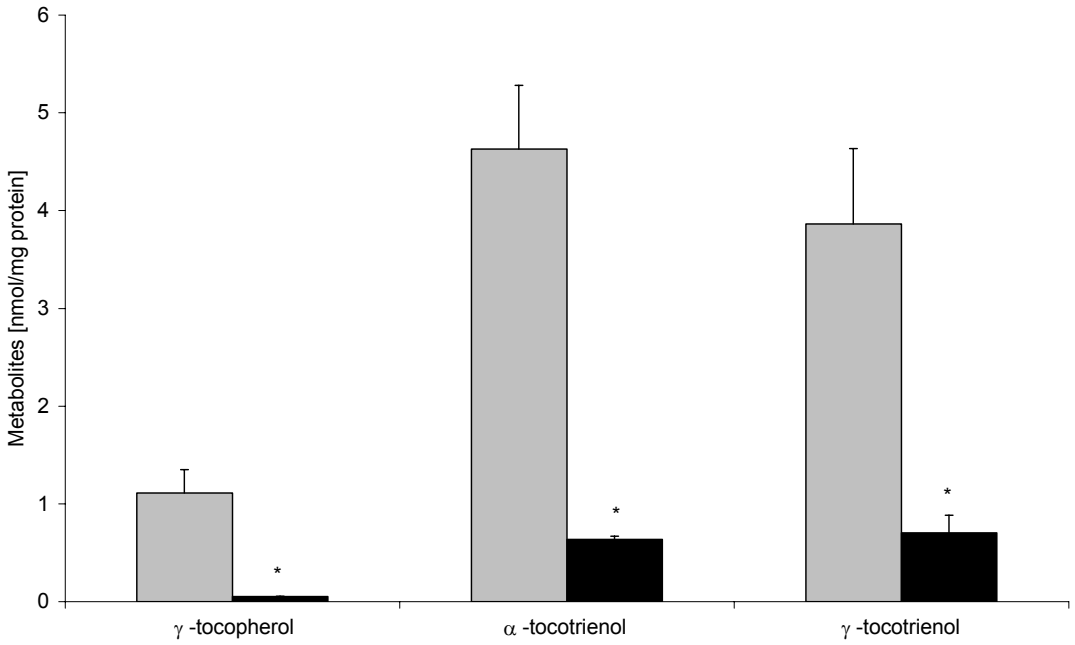


Figure 4.9 Inhibition of vitamin E metabolism in HepG2 cells: Cells were treated with 1 µM sesamin for 1 hour (black bars, untreated controls are gray). After subsequently rinsing with PBS they were incubated with (α-tocopherol, not shown), γ-tocopherol, γ-tocotrienol and α-tocotrienol 50 µM respectively, and in the presence of 1 µM sesamin for 48 hours. Cell culture medium was analyzed for CEHC and CMBHC as described in experimental procedures. Data are expressed as sum of CEHC plus CMBHC related to cellular protein and represent the mean of one experiment measured in triplicate ± SD. Asterisk indicates $P < 0.05$ *vs*. untreated control.

Having verified that sesamin was able to reasonably inhibit vitamin E metabolism, the effect of the inhibition on PXR-mediated CAT activation was investigated. To this end, cells were treated after transfection with 1 µM sesamin for 1 hour. As obvious from Fig. 4.10 sesamin did not significantly change CAT activity that had been induced by rifampicin or by individual forms of vitamin E. This indicates that more likely the tocopherols and tocotrienols themselves rather than their metabolites act on PXR-mediated gene expression.

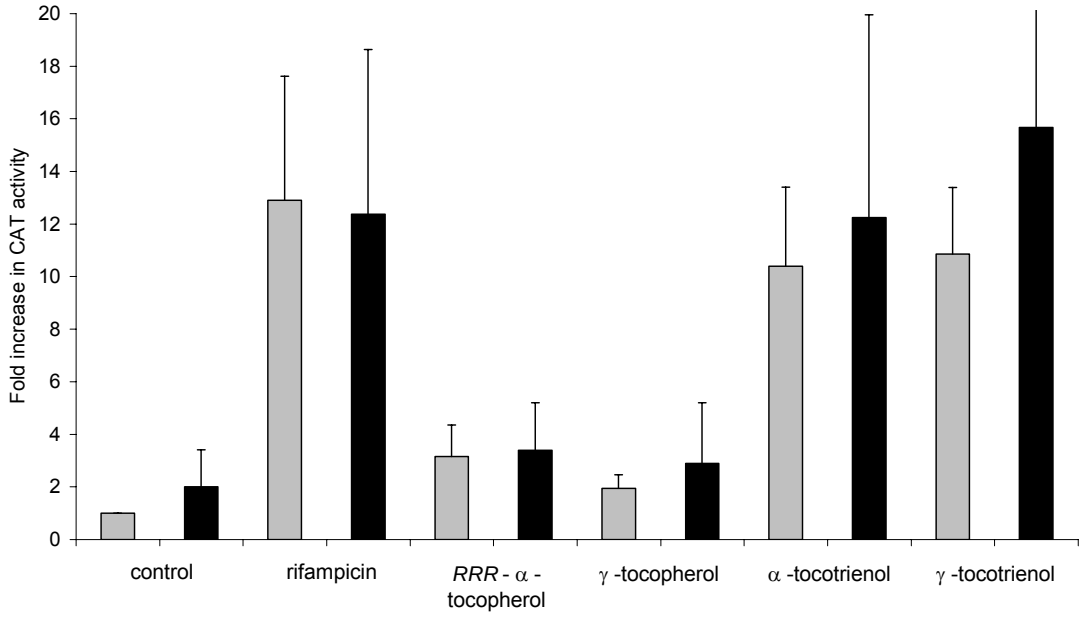
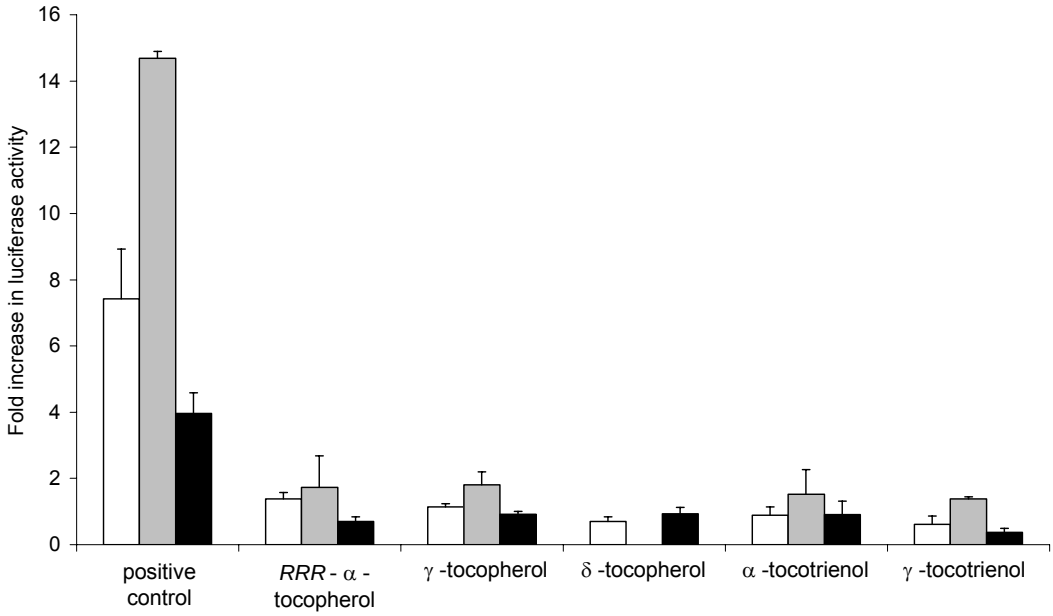


Figure 4.10 Inhibition of vitamin E metabolism does not significantly change PXR activation: HepG2 cells were transfected with expression plasmid for human PXR, a PXR-driven CAT reporter gene and β-galactosidase expression plasmid. Cells were treated with 1 µM sesamin (black) or with ethanol vehicle alone (gray) for 1 hour. The medium was removed and replaced by medium containing 10 µM rifampicin and 50 µM of the respective vitamin E forms (as indicated) plus 1 µM sesamin. After 48 h, CAT activity was measured and normalized to β-galactosidase activity. Data represent the mean of four independent experiments measured in triplicate ± SD, respectively, and are expressed as fold induction relative to untreated cells (control in gray), which was set to 1.

4.2.4 Investigation of vitamin E-mediated gene activation via other nuclear receptors

Several studies suggest the involvement of nuclear receptors in vitamin E effects. α-Tocopherol, e.g., increased the expression of CTGF (connective tissue growth factor) in smooth muscle cells (Villacorta *et al.* 2003) and reduced the expression of CD36 (Ricciarelli *et al.* 2000). Villacorta *et al*. (2003) speculated that both effects may be mediated via PPARγ. An involvement of the liver X receptor (LXR) in vitamin E metabolism can be proposed from LXR´s function in controlling the metabolism of cholesterol and fatty acids (reviewed in Edwards *et al.* 2002). LXR is a regulator of lipoproteinlipase and the ATP-binding cassette transporter sub-family A, member 1 (ABCA1). In an artificial

system lipoprotein lipase was shown to mediate the transfer of tocopherol from chylomicrons to cells (Traber *et al.* 1985). ABCA1 mediates the secretion of cellular α-tocopherol to HDL and thereby ABCA1 probably facilitates α-tocopherol transport between tissues (Oram *et al.* 2001). Therefore, lipoproteinlipase as well as ABCA1 are discussed to play an important role in



the transfer and efflux of vitamin E.

Figure 4.11 Vitamin E does not activate a reporter gene regulated by LXRα, PPARα or PPARγ: HepG2 cells were transfected with expression plasmids for either LXRα(white bars),PPARα (gray bars) or PPARγ (black bars)and the luciferase reporter genes with the corresponding response elements and the β-galactosidase expression plasmid. Cells were treated with the respective ligands as positive control and different vitamin E forms at 50 µM, respectively, for 48 hours. Positive controls were 10 µM 22-(R)-hydroxycholesterol for LXRα, 1 µM GW7647 for PPARαand 1 µM GW7845 for PPARγ. Luciferase activity was normalized to β-galactosidase activity. Data are expressed as fold induction relative to untreated cells and represent one experiment measured in triplicate ± SD.

To investigate whether vitamin E has a direct effect on gene expression via PPARs and/or LXR, PPARα, PPARγ and LXRα were tested in a reporter gene system. The system consisted of expression plasmids for PPARα, PPARγ or LXRα and reporter constructs comprising a respective responsive element coupled to a firefly luciferase gene. For normalization, the β-galactosidase

expression plasmid was used. After transfection, HepG2 cells were incubated with 1 µM GW7647, 1 µM GW7845 and 10 µM 22-(R)-hydroxycholesterol as a positive control for the activation of PPARα, PPARγ and LXRα, respectively, or with individual forms of vitamin E. However, none of the vitamin E forms tested was able to activate the reporter gene regulated by one of the three receptors and the respective response element (Fig. 4.11).

4.3 Differential gene expression in mouse liver

Having found that specific forms of vitamin E were able to activate PXR and, therefore, are able to regulate gene expression in principle, it was necessary to investigate whether vitamin E influenced gene activity also *in vivo*. Using cDNA and oligonucleotide microarrays, the gene expression profiles were compared from mice that were fed diets different in vitamin E content.

4.3.1 Study design

Male C57BL6 mice were fed diets with 3 different α-tocopherol contents for 13 weeks. The diets were adjusted to 2 mg, 20 mg and 200 mg *RRR*-α-tocopheryl acetate per kg diet, respectively. During the last 7 days before the mice were euthanized, 250 µg γ-tocotrienol was applied to half of each diet group daily (Tab. 4.2). γ-Tocotrienol was chosen because it was a strong PXR activator in the reporter gene assay. The day before sacrifice, 3 mice from each group were placed individually into a metabolic cage and urine was collected over a 24 h period. Blood was collected and the livers were frozen in liquid nitrogen.

Table 4.2 Food regime of mice: Male mice were divided into 6 different diet groups. Indications used for each diet group are shown in squared brackets.

	2 mg α-Tocopheryl acetate / kg diet "Deficient"	**20 mg α-Tocopheryl acetate / kg diet "Adequate"**	**200 mg α-Tocopheryl acetate / kg diet "Supranutritional"**
Control	6 mice [2]	6 mice [20]	6 mice [200]
250 µg/d γ-Tocotrienol	6 mice [2+]	6 mice [20+]	6 mice [200+]

Animals showed normal growth and behavior without differences between animals from the different diets. Mice weights rose from 7-11 g to 22-25 g during the 13 weeks.

4.3.2 Vitamin E levels in mouse plasma and liver tissue

Feeding α-tocopheryl acetate led to an increase of α-tocopherol concentrations in plasma and liver. Plasma α-tocopherol concentrations of the supranutritional increased about 4-fold and the adequate group 2-fold compared to the deficient group (Fig. 4.12 A).

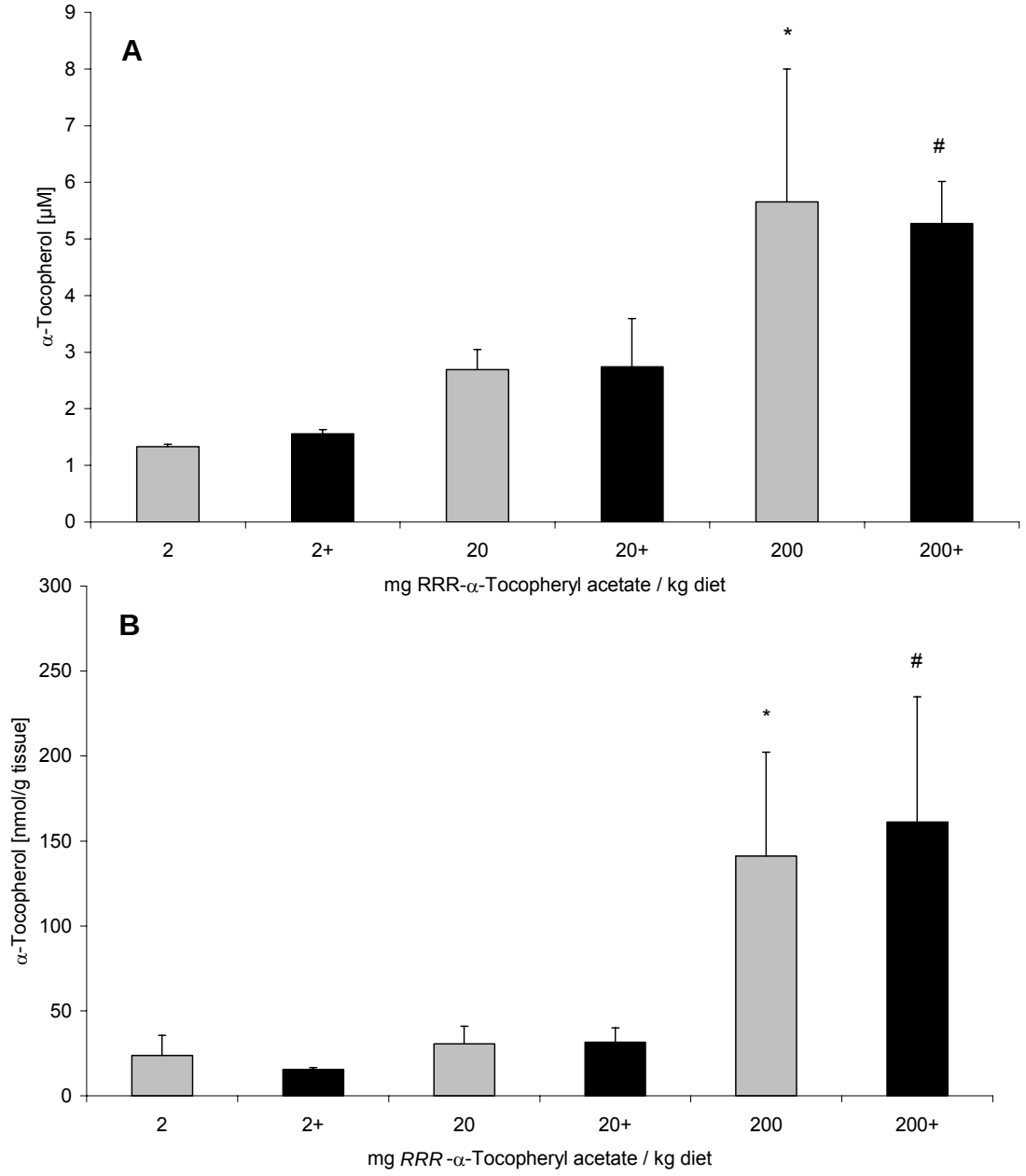


Figure 4.12 α-Tocopherol concentrations in mouse plasma (A) and liver (B): α-Tocopherol content in animals fed different diets (for specifications see Tab. 4.2) was determined by HPLC-ECD. Values are means ± SD from at least three animals. For experimental details see experimental procedures. Data were analyzed by two way ANOVA with the Bonferroni post hoc test. *$p < 0.05$ versus the deficient control group indicated as "2"; $^{\#}p < 0.05$ versus "2+"

Liver α-tocopherol concentration was about 20 nmol/g wet weight in the deficient group. It increased to about 30 nmol/g wet weight in the adequate group and to about 150 nmol/g wet weight in the supranutritional (Fig. 4.12 B). Plasma and liver α-tocopherol concentrations were not influenced by additional feeding of γ-tocotrienol (Fig. 4.12). The content of γ-tocotrienol was close to the detection limit in all samples and was not increased in liver or plasma 24 h after the last feeding. The reason might be a very fast metabolism as concluded from a 20-25-fold increase of urinary γ-CEHC excretion in γ-tocotrienol supplemented mice (Kluth *et al.* 2005).

4.3.3 cDNA arrays

Since the degradation of vitamin E by the CYP-system as well as its ability to activate PXR *in vitro* points to a role of vitamin E in the expression of genes involved in the metabolism of xenobiotics, the effect of vitamin E on differential gene expression was investigated by using the Clontech Mouse Toxicology 1.2 Arrays. Total RNA from 3 mouse livers of each diet group and ^{32}P-labeled cDNA for membrane hybridization was prepared according to manufacturers protocol. Each diet group was represented by three independent hybridizations. 1,176 liver genes were monitored simultaneously on each membrane (Fig. 4.13).

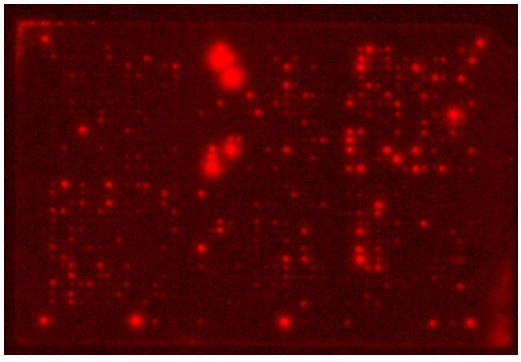


Figure 4.13 Gene expression pattern monitored on the cDNA Mouse Toxicology Array. Shown is a hybridization example from mouse liver mRNA treated with 2 mg α-tocopheryl acetate / kg diet. Labeling and hybridization of cDNA was performed as described in experimental procedures.

Signals from the Clontech Arrays were normalized to the average of all of the gene signals on the membrane. The obtained data were analyzed using a method adapted specifically for microarrays, the Significance Analysis of Microarray (SAM, Tusher *et al.* 2001). SAM is a nonparametric method that decides how to indicate genes as significant, and what the multiple testing error

measure is for each significance region. The error measure is the expected proportion of false-positives among all genes called significant, called the false discovery rate (FDR). The output criteria for vitamin E sensitive genes selected for SAM included 2-fold or greater change in expression in the different diet groups, and a significance threshold expected to produce a median FDR of less than 5%.

However, none of the comparisons was able to meet the criterion of an FDR of less than 5%. For completeness, after disregarding the FDR, up to 411 genes were detected to be regulated between two groups, but with low statistical significance. FDR was high for all comparisons (Tab. 4.3). The lowest FDR (20%) was observed for the supranutritional group plus γ-tocotrienol versus the supranutritional group, the highest for the deficient group plus γ-tocotrienol versus the deficient group (76%). An α-tocopherol effect should be seen most probably between the supranutritional group and the deficient group. A correlation analysis in the gene expression levels between these groups is shown in Fig. 4.14.

Table 4.3 Significant genes among diet groups calculated using SAM.

Clontech Toxicology Array		**Number of genes called significant**			
Effect of	**Comparison**	**Down-regulated**	**Up-regulated**	**Falsely significant**	**FDR [%]**
α-tocopherol	200 *vs*. 2	3	14	8	46
	20 *vs*. 2	0	411	285	69
	200 *vs*. 20	0	10	4	40
α-tocopherol / γ-tocotrienol	200+ *vs*. 2+	0	49	23	46
	20+ *vs*. 2+	30	0	11	36
	200+ *vs*. 20+	0	48	13	26
γ-tocotrienol	2+ *vs*. 2	310	0	236	76
	20+ *vs*. 20	3	1	1	25
	200+ *vs*. 200	0	5	1	20

For designations of the comparisons see Tab. 4.2.

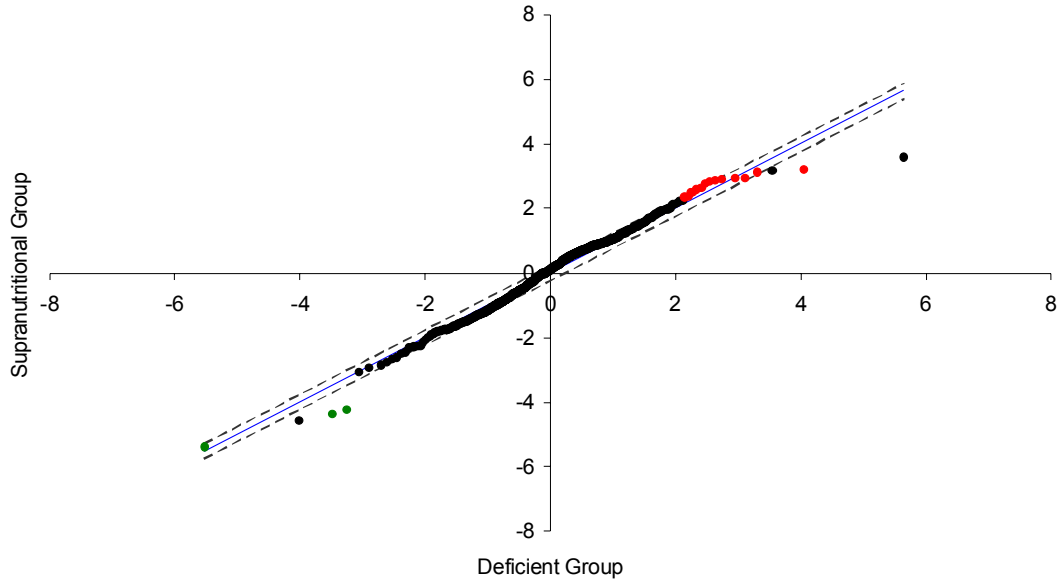


Figure 4.14 Genes with significant changes in expression between the deficient and the supranutritional group. Scatter plot of a total of 1176 genes of the "*Supranutritional Group*" versus the "*Deficient Group*". Significantly up-regulated and down-regulated genes are shown in red and green, respectively. Non-significant genes are shown in black. Shown are 17 significant genes wherefrom 8 are false positive.

Table 4.4 Genes regulated in the supranutritional group ("200") compared to the deficient group ("2").

Gene ID	Gene name	Fold change
M32489	interferon concensus sequence binding protein	-17.5
X84311	cyclin A1	-5.0
M76763	ribosomal protein S18	-2.8
X52803	peptidylprolyl isomerase A	2.7
V00743	alpha fetoprotein	3.1
X65493	intercellular adhesion molecule 2	3.4
J04115	Jun oncogene	3.5
M65255	H2-K region expressed gene 2	3.6
L33406	uromodulin	3.8
AF033261	synuclein, alpha	5.6
AB028272	DnaJ (Hsp40) homolog, subfamily B, member 1	5.9
L26489	proprotein convertase subtilisin/kexin type 3	6.4
X83601	pentaxin related gene	6.6
X01023	myelocytomatosis oncogene	7.1
V00727	FBJ osteosarcoma oncogene	7.3
D16141	lethal giant larvae homolog	7.7
M57697	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	8.0

Of the genes evaluated, the expression level of 17 genes displayed a greater than 2-fold change between the deficient and the supranutritional group whereof 14 were up- and 3 were down-regulated, respectively (Tab. 4.4).

Two analyses ("20 *vs*. 2" and "2+ *vs*. 2") showed a FDR even greater than 50% (Tab. 4.3). An FDR of 50% means that 50% of the genes which were designated as significant, were significant by chance, and thereby, represents a very low statistical significance. Hence, in Tab. 4.4 – 4.10 only the genes from comparisons with an FDR lower than 50% are shown.

Table 4.5 Genes regulated in the supranutritional group ("200") compared to the adequate group ("20").

Gene ID	Gene name	Fold change
AF006688	palmitoyl acyl-coenzyme A oxidase 1	2.0
M35525	hemolytic complement	2.1
AJ011413	albumin 1	2.5
X59421	Friend leukemia integration 1	2.9
M90364	catenin beta	3.4
M36829	heat shock protein, 84 kDa 1	3.5
U41751	etoposide induced 2.4 mRNA	3.7
D16141	lethal giant larvae homolog	5.5
X74760	Notch gene homolog 3 (Drosophila)	12.9
M57697	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	37.6

Table 4.6 Genes regulated in the supranutritional group plus γ-tocotrienol ("200+") compared to the deficient group plus γ-tocotrienol ("2+").

Gene ID	Gene name	Fold change
U95607	DnaJ (Hsp40) homolog, subfamily B, member 3	2.0
NM_013484	complement component 2 (within H-2S)	2.1
L10656	v-abl Abelson murine leukemia oncogene 1	2.1
D90225	pleiotrophin	2.1
U92794	protein kinase C substrate 80K-H	2.3
D14340	tight junction protein 1	2.3
U37775	tuberous sclerosis 2	2.3
X83971	fos-like antigen 2	2.5
D63359	pancreatitis-associated protein	2.5
X00479	cytochrome P450, 1a2, aromatic compound inducible	2.7
X01023	myelocytomatosis oncogene	2.7
X70472	myeloblastosis oncogene-like 2	2.8
X65493	intercellular adhesion molecule 2	2.9
U85610	Indian hedgehog homolog (Drosophila)	2.9
X13664	neuroblastoma ras oncogene	3.0
Z17804	catenin src	3.0
M63848	leukotriene A4 hydrolase	3.1
NM_013650	calcium binding protein A8 (calgranulin A)	3.2
NM_019641	leukemia-associated gene	3.6
S65038	GLI-Kruppel family member GLI	3.8
L28116	peroxisome proliferator activator receptor delta	3.8
AF079458	N-acetyl galactosaminidase, alpha	3.9
M80456	Notch gene homolog 4 (Drosophila)	4.1
U19854	ubiquitin-conjugating enzyme E2H	4.5

U38981	RIKEN cDNA 0610009H04 gene	4.7
D25281	catenin alpha 2	4.8
X05010	colony stimulating factor 1 (macrophage)	4.8
U68058	frizzled-related protein	5.1
U13705	glutathione peroxidase 3	5.1
X53028	chromogranin B	5.5
M84324	matrix metalloproteinase 2	5.7
AF094520	neuroepithelial cell transforming gene 1	6.2
D63902	tripartite motif protein 25	7.2
AF060246	zinc finger protein 106	8.6
AF031956	T-cell lymphoma breakpoint 1	8.7
U12570	von Hippel-Lindau syndrome homolog	11.4
Z67747	zinc finger protein 62	11.4
M16449	myeloblastosis oncogene	11.8
NM_013755	glycogenin 1	12.2
D00659	cytochrome P450, 19, aromatase	14.7
U20344	Kruppel-like factor 4 (gut)	20.9
X60165	pore forming protein	22.0
D63361	regenerating islet-derived 3 gamma	27.1
J04549	proteoglycan, secretory granule	27.6
Z35294	mature T-cell proliferation 1	29.5
L11316	ect2 oncogene	63.9
U23921	osmotic stress protein 94 kDa	172.0
M93128	even skipped homeotic gene 2 homolog	↑
X56045	RAN binding protein 1	↑

↑ gene signal at background level in the deficient group plus γ-tocotrienol

Table 4.7 Genes regulated in the adequate group plus γ-tocotrienol ("20+") compared to the deficient group plus γ-tocotrienol ("2+").

Gene ID	Gene name	Fold change
X68273	CD68 antigen	↓
D30743	wee 1 homolog (S. pombe)	↓
D43804	stromal cell derived factor 1	-82.2
X96767	U1 small nuclear ribonucleoprotein 1C	-22.7
AF041054	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	-15.3
U04807	FMS-like tyrosine kinase 3 ligand	-13.9
X98369	SRY-box containing gene 15	-12.9
L35049	Bcl2-like	-10.0
D78354	phospholipid scramblase 1	-9.4
U50734	maternal inhibition of differentiation	-9.0
X63023	cytochrome P450, steroid inducible 3a13	-8.3
Z31553	chaperonin subunit 2 (beta)	-6.3
U12961	NAD(P)H dehydrogenase, quinone 1	-5.7
M20157	early growth response 1	-5.3
X61800	CCAAT/enhancer binding protein (C/EBP), delta	-5.2
U22339	interleukin 15 receptor, alpha chain	-5.0
S74520	caudal type homeo box 2	-5.0
Z31557	chaperonin subunit 6a (zeta)	-4.8
X61753	heat shock factor 1	-4.8
D17407	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF)	-4.6
M33212	nucleophosmin 1	-4.4

U43184	Fas (TNFRSF6)-associated via death domain	-3.7
AF013632	neutral sphingomyelinase (N-SMase) activation associated factor	-3.7
AJ224740	CASP2 and RIPK1 domain containing adaptor with death domain	-3.6
X14309	solute carrier family 3, member 2	-3.4
U64828	nuclear receptor coactivator 1	-2.9
AF049606	nuclear factor of activated T-cells, cytoplasmic 1	-2.9
AF064448	feminization 1 homolog b (C. elegans)	-2.9
U35741	thiosulfate sulfurtransferase, mitochondrial	-2.8
U78085	ribosomal protein S5	-2.1

↓ gene signal at background level in the adequate group plus γ-tocotrienol

Table 4.8 Genes regulated in the supranutritional group plus γ-tocotrienol ("200+") compared to the adequate group plus γ-tocotrienol ("20+").

Gene ID	Gene name	Fold change
NM_019641	leukemia-associated gene	2.1
D63361	regenerating islet-derived 3 gamma	2.2
AF014371	ras homolog A2	2.3
U76425	K-ras type A/B	2.3
M84324	matrix metalloproteinase 2	2.5
X01023	myelocytomatosis oncogene	2.6
L11316	ect2 oncogene	2.7
Z31557	chaperonin subunit 6a (zeta)	2.7
X13664	neuroblastoma ras oncogene	2.8
X05010	colony stimulating factor 1 (macrophage)	2.9
U85610	Indian hedgehog homolog (Drosophila)	3.0
L33406	uromodulin	3.7
NM_013650	calcium binding protein A8 (calgranulin A)	4.3
X14309	solute carrier family 3 member 2	4.4
X53028	chromogranin B	4.7
D49482	osmotic stress protein 94 kDa	4.8
X61753	heat shock factor 1	4.8
U95607	DnaJ (Hsp40) homolog, subfamily B, member 3	5.2
M26391	retinoblastoma 1	5.4
AF033011	distal-less homeobox 5	5.4
M63848	leukotriene A4 hydrolase	5.4
X78936	parathyroid hormone receptor	5.6
D14572	core binding factor beta	6.0
L28177	growth arrest and DNA-damage-inducible 45 alpha	6.1
U36799	retinoblastoma-like 2	6.6
D17407	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF)	7.1
X78684	B-cell receptor-associated protein 29	7.3
U66827	hydroxysteroid 17-beta dehydrogenase 3	10.1
NM_009769	Kruppel-like factor 5	12.2
L28116	peroxisome proliferator activator receptor delta	12.7
X58876	transformed mouse 3T3 cell double minute 2	13.6
AF012251	casein kinase II, alpha 2, polypeptide	14.7
U50734	maternal inhibition of differentiation	15.0
L12693	cellular nucleic acid binding protein	18.9
U23921	osmotic stress protein 94 kDa	37.7
U12236	integrin, alpha E, epithelial-associated	49.7
AB013874	low density lipoprotein receptor-related protein 4	↑

U08215	heat shock protein, 70 kDa 4	↑
X74760	Notch gene homolog 3 (Drosophila)	↑
U68058	frizzled-related protein	↑
X06746	early growth response 2	↑
X69618	inhibin alpha	↑
U70622	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	↑
X56045	RAN binding protein 1	↑
U59761	ATPase, Na+/K+ transporting, beta 3 polypeptide	↑
U19118	activating transcription factor 3	↑
J04549	proteoglycan, secretory granule	↑
X57277	RAS-related C3 botulinum substrate 1	↑

↑ gene signal at background level in the adequate group plus γ-tocotrienol

The comparisons with additional γ-tocotrienol ("200+ *vs*. 2+","20+ *vs*. 2+" and "200+ *vs*. 20+") are presented in Tab. 4.6 - 4.8.

Among the comparisons, in which the effect of additional 250 µg γ-tocotrienol per day on different α-tocopherol diets was investigated ("2+ *vs*. 2","20+ *vs*. 20" and "200+ *vs*. 200"), the highest number of significant genes was in the deficient group, but the FDR was also the highest among all comparisons (76%, Tab. 4.3). These groups with additional γ-tocotrienol were compared with respect to the identification of genes, which were only regulated in the presence ofγ-tocotrienol. However, γ-tocotrienol regulated genes were not identical in the deficient, adequate and supranutritional groups plus γ-tocotrienol (Tab. 4.9 and 4.10), suggesting, that these genes were not regulated independently of α-tocopherol.

Table 4.9 Genes regulated by additional γ-tocotrienol in the adequate group ("20+ *vs*. 20").

Gene ID	Gene name	Fold change
L28116	peroxisome proliferator activator receptor delta	-12.5
X81443	nuclear distribution gene C homolog (Aspergillus)	-6.9
X61432	calmodulin 1	-4.9
AF006688	palmitoyl acyl-coenzyme A oxidase 1	2.5

Table 4.10 Genes regulated by additional γ-tocotrienol in the supranutritional group ("200+ *vs*. 200").

Gene ID	Gene name	Fold change
U51167	isocitrate dehydrogenase 2 (NADP+), mitochondrial	2.7
U92794	protein kinase C substrate 80K-H	3.2
X61753	heat shock factor 1	8.2
L03529	coagulation factor II (thrombin) receptor	8.6
U19854	ubiquitin-conjugating enzyme E2H	11.1

A gene of special interest was Cyp3a11, the mouse homolog of the human CYP3A4 (Nelson 1999), which is expressed under the control of PXR. In our lab we showed an increase of the human CYP3A4 in HepG2 cells by γ-tocotrienol treatment (Landes *et al.* 2003). The Clontech Array data also revealed an increase in Cyp3a11 expression under the control of α-tocopherol, however, it was not significant. The expression of Cyp3a11 was increased about 2.7-fold from the deficient to the supranutritional group (Fig. 4.16).

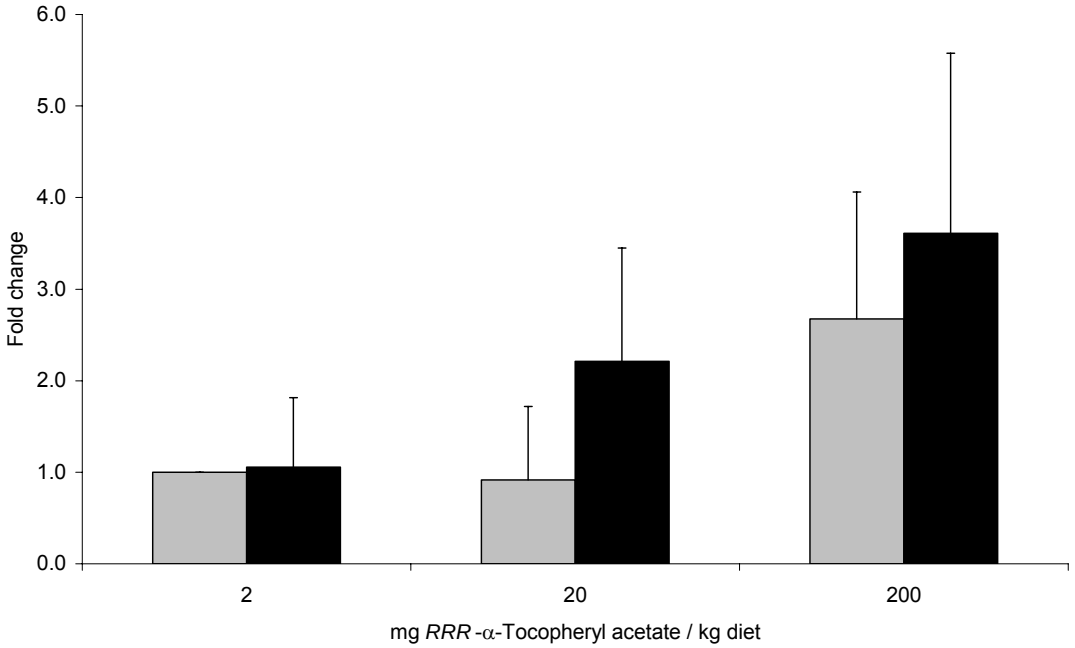


Figure 4.15 Cyp3a11 mRNA expression in mouse liver by using Clontech Arrays in the diet groups with α-tocopheryl acetate alone (gray) and α-tocopheryl acetate with additional γ-tocotrienol (black). Bars are means of 3 animals ± SD and are shown as fold change relative to the deficient group without additional γ-tocotrienol ("2", gray).

The Array data were confirmed in our lab (Kluth *et al.* 2005) by quantitative real time PCR using Cyp3a11 specific primers. The Cyp3a11 PCR products were normalized to hypoxanthine guanine phosphoribosyl transferase and quantified by the comparative method. Liver mRNA of Cyp3a11 was induced by a factor of 2.5 in mice fed the adequate ("20") or supranutritional ("200") α-tocopherol diet compared to the deficient group ("2"). Additional γ-tocotrienol in the diet did not enhance mRNA of Cyp3a11 contents.

Although several genes were present in more than one comparison (e.g., ICAM-2, lethal giant larvae homolog, Yamaguchi sarcoma viral oncogene homolog, heat shock factor 1, myelocytomatosis oncogene, peroxisome proliferator activator receptor delta, osmotic stress protein 94 kDa), the Clontech cDNA Arrays showed only weak significance when at all. In consideration of the criterion of a low false discovery rate, no genes were identified. Moreover, this type of microarray is limited to investigating the expression profile of only a few genes. Currently this means ~1,200 genes that are interesting from a toxicological point of view. Therefore, to extend the gene profiling analysis, another microarray approach had to be taken into consideration. In order to find genes that are regulated in a vitamin E dependent manner, an expression analysis using Affymetrix Arrays was used. Instead of a cDNA, the genes on Affymetrix Arrays are represented by oligonucleotides. This type of microarray technology allowed the detection of ~14,000 genes.

4.3.4 Oligonucleotide arrays

Total RNA from livers from 2 mice per diet group was prepared and transcribed into cRNA according to manufacturer´s protocol. Biotin labeled cRNA was hybridized to Affymetrix Array Mouse Genome 430A. The experiment was performed in duplicate. Two samples from each group were used to perform two independent hybridizations. The raw data were analyzed using SAM (see appendix) and dChip. The dChip software was used to normalize the raw data to the whole expression level and to perform comparative analyses (Li and Wong 2001). A difference in gene expression between diet groups was considered as significant at a threshold of at least two. This data modeling approach allowed to discriminate from the initial large gene lists of differentially expressed genes, many of which may be due to culture or experimental artifact, to a biologically relevant list of candidate genes that may be under the control of vitamin E.

Although the majority of the ~14,000 genes remained unchanged, an increase in α-tocopheryl acetate in the diet resulted in an alteration of the expression profile (Tab. 4.11). Depending on the groups compared, the expression of about 80 – 900 genes was changed. In contrast to the SAM

analyses from the cDNA arrays, the SAM analyses from the Affymetrix Arrays showed FDRs less than 0.6% and the number of false significant genes was not higher than 1.

Table 4.11 Significant genes among diet groups calculated using SAM and dChip.

Affymetrix 430A Array		**dChip analysis Number of genes called significant**		**SAM analysis**	
Effect of	**Comparison**	**Down-regulated**	**Up-regulated**	**Falsely significant**	**FDR [%]**
α-tocopherol	200 *vs*. 2	330	338	0.8	0.4
	20 *vs*. 2	10	72	0	0
	200 *vs*. 20	85	87	0	0
α-tocopherol / γ-tocotrienol	200+ *vs*. 2+	149	124	0.8	0.2
	20+ *vs*. 2+	504	108	0.8	5.6
	200+ *vs*. 20+	116	184	0	0
γ-tocotrienol	2+ *vs*. 2	144	224	0.5	0.6
	20+ *vs*. 20	509	59	0	0
	200+ *vs*. 200	639	264	0.7	0.4

For designations of the comparisons see Tab. 4.2.

Depending on the α-tocopheryl acetate content in the diet, both an up- and down-regulation of genes was observed (Tab. 4.12). The extend of the fold change varied mostly between a factor of 2 and 10 (up or down). Sometimes the signal obtained with certain genes was close to the background signal, meaning that they were absent or expressed only at a very low level. An arrow in the tables instead of a factor indicates this situation and shows only whether the genes were up- or down-regulated compared to the reference group. The identified genes were categorized using the mouse genome informatics data base at http://www.informatics.jax.org/.

Table 4.12 α-Tocopherol related change in gene expression ("200 *vs*. 2").

Gene ID	**Gene name**	**Fold change**	**Function/process**
AK018713	cytochrome b-245, alpha	↓	superoxide metabolism / part of the NADPH oxidase
NM_007663	cadherin 16	↓	cell adhesion
NM_023455	camello-like 4	↓	negative regulation of cell adhesion

AI790558	insulin-like growth factor binding protein 7 *	-10.8	regulates IGF availability
BC013542	membrane-associated protein 17 *	-9.5	up-regulated in kidney, lung, colon and breast cancer
AK006269	PDZ domain containing 1	-8.6	intracellular signal cascade
NM_027884	Tensin *	-5.8	cell migration, actin binding
BC012707	glutathione S-transferase, theta 2 *	-5.4	glutathione metabolism, detoxification
BE307351	CD36 antigen	-4.9	scavenger receptor
L27424	tissue inhibitor of metalloproteinase 3 *	-4.8	angiogenesis inhibitor
NM_009263	secreted phosphoprotein 1 *	-4.7	cell adhesion
AI790290	N-myc downstream regulated 1*	-4.4	mast cell activation
NM_011314	serum amyloid A 2	-4.2	acute phase response
NM_009117	serum amyloid A 1	-4.1	acute phase response
NM_008087	growth arrest specific 2 *	-4.0	apoptosis, cell cycle
NM_011794	bisphosphate 3'-nucleotidase 1	-3.7	regulation of transcription
NM_009150	selenium binding protein 1	-3.5	selenium binding
NM_008180	glutathione synthetase *	-3.3	glutathione biosynthesis
NM_009883	CCAAT/enhancer binding protein (C/EBP), beta	-3.2	apoptosis, neuron differentiation
NM_007631	cyclin D1 *	-3.2	cell cycle
BC006622	glypican 4	-3.2	development
BF580781	hepatic nuclear factor 4	-2.4	regulation of transcription
BC022110	aminolevulinic acid synthase 1	-2.4	heme biosynthesis
NM_023160	camello-like 1	-2.3	negative regulation of cell adhesion
BF682469	CD8 antigen, beta chain	-2.2	immune response
AF014010	polycystic kidney disease 2 *	-2.0	cell cycle arrest
NM_007984	fascin homolog 1, actin bundling protein (Strongylocentrotus) purpuratus)	2.1	actin binding
BI687658	protein kinase, cAMP dependent regulatory, type I, alpha *	2.4	cell proliferation, mesoderm formation, organogenesis
NM_008778	p21 (CDKN1A)-activated kinase 3	2.4	cell cycle control
BE986602	internexin neuronal inter-mediate filament protein, alpha	2.4	cytoskeleton organization, neurogenesis
NM_008445	kinesin family member 3C	2.5	microtubule based process
BE991142	Bcl2-like 2 *	2.6	apoptosis
NM_013798	actin-like	2.7	acts together with profilin
NM_021278	thymosin, beta 4, X chromosome *	2.8	cytoskeleton organization and biogenesis
NM_133769	p53-inducible protein	3.2	present in synaptosomal extracts
AB015790	sortilin-related receptor, LDLR class A repeats-containing *	3.2	cholesterol metabolism, development
D87968	protein tyrosine phosphatase, non-receptor type substrate 1	3.3	cell-matrix adhesion, cytoskeleton organization
NM_007835	dynactin 1	3.3	cytoskeleton
BC006606	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform *	3.5	cell proliferation/differentiation
BC005490	amyloid beta (A4) precursor protein	3.7	apoptosis
NM_011655	tubulin, beta 5 *	3.7	microtubule

AI323543	phosphoprotein enriched in astrocytes 15 *	3.8	apoptosis, intracellular signaling cascade
AV006937	dynein, cytoplasmic, light chain 1 *	3.8	actin filament organization
AF483486	cystatin C *	3.9	cysteine protease inhibitor
BC016208	hypothetical protein MGC27683	4.0	Hdac 11, regulation of transcription
NM_021515	adenylate kinase 1	4.1	ATP metabolism, cell cycle arrest
BI109632	kinesin family member 1B *	4.3	microtubule based processes
NM_008447	kinesin family member 5A	4.3	microtubule based processes
BI684958	erythrocyte protein band 4.1-like 1 *	4.4	actin cytoskeleton organization
BB560492	profilin 2 *	5.4	actin cytoskeleton organization
M35131	neurofilament, heavy polypeptide	5.5	intermediate filament cytoskeleton organization and biogenesis
NM_013670	small nuclear ribonucleo-protein N *	5.8	splicing
NM_008747	neurotensin receptor 2	6.0	electron transport
BC024935	ADP-ribosylation factor 3 *	6.1	signal transduction
BC008117	tubulin, alpha 1 *	6.5	microtubule
BF124540	cysteine rich protein 1	8	cytoskeleton biogenesis
BC006894	gap junction membrane channel protein alpha 1	8.4	cell communication/signaling
AF071549	chemokine (C-X3-C motif) ligand 1 *	8.6	cell adhesion, immune response
NM_008691	neurofilament 3, medium	9.2	intermediate filament cytoskeleton
BC010712	CREBBP/EP300 inhibitory protein 1	9.6	negative regulation of transcription
BC003475	tubulin, beta 2	10.0	microtubule
BB464434	selenoprotein W, muscle 1 *	10.8	selenoprotein
BB251922	cyclic nucleotide phosphodiesterase 1	12.2	myelin marker
BC010581	stathmin 1 *	14.3	axonogenesis, regulating cell proliferation, differentiation
X59274	protein kinase C, beta *	↑	signal transduction
BB458655	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	↑	possible tumor suppressor
AK011545	brain abundant, membrane attached signal protein 1	↑	regulation of transcription
NM_021881	Quaking	↑	vasculogenesis
NM_007906	eukaryotic translation elongation factor 1 alpha 2	↑	anti-apoptosis, translational elongation
AB006361	prostaglandin D2 synthase (21 kDa, brain)	↑	prostaglandin biosynthesis
D50366	kinesin-associated protein 3 *	↑	microtubule-based process
BM120053	ectodermal-neural cortex 1 *	↑	development

↓ gene signal at background level in the supranutritional group but present in the deficient group; ↑ gene signal at background level in the deficient group but present in the supranutritional group; * gene is present and regulated in the same direction in the comparison between the supranutritional plus γ-tocotrienol group ("200+") and the deficient plus γ-tocotrienol group ("2+")

α-Tocopherol changed the expression of several genes for proteins which are essential for glycolysis or for the metabolism of fatty acids and xenobiotics (Tab. 4.13). From these genes, more were down- than up-

regulated, e.g., genes coding for β-oxidation proteins such as enoyl coenzyme A hydratase 1, L-3-hydroxyacyl-coenzyme A dehydrogenase and acetyl-coenzyme A dehydrogenase. Up-regulated were, e.g., genes encoding proteins involved in glycolytic processes like aldolase 3 and 1 and phosphofructokinase. However, the Affymetrix Arrays did not show a signifikant change in Cyp3a11 expression.

Table 4.13 α-Tocopherol induced change in expression of genes involved in intermediary metabolism ("200 *vs*. 2").

Gene ID	Gene name	Fold change	Function/process
AI118428	UDP-glucuronosyltransferase 2 family, member 5 *	↓	metabolism of lipophilic xenobiotics
NM_007823	cytochrome P450, subfamily IV B, polypeptide 1 *	↓	fatty acid metabolism
AB022340	SA rat hypertension-associated homolog	↓	fatty acid metabolism
BC015290	carboxylesterase 2	-7.3	metabolism of xenobiotics
BC002148	fatty acid binding protein 4, adipocyte *	-6.5	fatty acid transport
BM119915	low density lipoprotein receptor-related protein 2	-6.4	receptor mediated endocytosis
BC010747	cytochrome P450, 4a10	-6.4	fatty acid metabolism
NM_007812	cytochrome P450, 2a5 *	-5.1	drug and steroid metabolism
BC013442	solute carrier family 27 (fatty acid transporter), member 2 *	-5.0	fatty acid metabolism
NM_008820	peptidase 4 *	-4.6	collagen catabolism
NM_008509	lipoprotein lipase *	-4.6	lipid catabolism
NM_010007	cytochrome P450, 2j5 *	-4.1	fatty acid metabolism
U93702	neuraminidase 1 *	-3.8	glycoconjugate metabolism
NM_009949	carnitine palmitoyltransferase 2	-3.6	fatty acid transport
NM_008212	L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain *	-3.6	mitochondrial β-oxidation of short chain fatty acids
NM_007382	acetyl-coenzyme A dehydrogenase, medium chain *	-3.4	catalyzes β-oxidation
NM_009108	nuclear receptor subfamily 1, group H, member 4 (FXR) *	-3.2	nuclear receptor regulating genes involved in bile acid metabolism
NM_015763	lipin 1	-3.2	lipid metabolism
NM_008194	glycerol kinase *	-3.1	carbohydrate metabolism, glycerol metabolism
BB546344	hydroxysteroid (17-beta) dehydrogenase 11	-3.1	steroid metabolism
NM_008322	isocitrate dehydrogenase 2 (NADP+), mitochondrial *	-3.0	carbohydrate metabolism
NM_054094	butyryl coenzyme A synthetase 1	-2.9	fatty acid biosynthesis
BC027198	isovaleryl coenzyme A dehydrogenase *	-2.9	leucine catabolism
NM_023523	peroxisomal trans-2-enoyl-CoA reductase	-2.9	fatty acid elongation
AK010384	leucine aminopeptidase 3	-2.8	proteolysis and peptidolysis
BB703752	aldehyde dehydrogenase 9, subfamily A1	-2.7	carnitine metabolism

BG070487	acetyl-coenzyme A acetyltransferase 1	-2.5	isoleucine catabolism
NM_025337	aflatoxin B1 aldehyde reductase *	-2.4	aldehyde metabolism
NM_016772	enoyl coenzyme A hydratase 1, peroxisomal	-2.4	peroxisomal fatty acid metabolism
NM_007381	acetyl-coenzyme A dehydrogenase, long-chain	-2.4	fatty acid metabolism
BC024112	aldolase 2, B isoform	-2.4	glycolysis
NM_030686	dehydrogenase/reductase (SDR family) member 4 (Dhrs4)	-2.1	peroxisomal retinal metabolism
BI111416	enoyl coenzyme A hydratase, short chain, 1, mitochondrial	-2.0	fatty acid metabolism
NM_015729	acyl-coenzyme A oxidase 1, palmitoyl	-2.0	fatty acid metabolism
NM_007824	cytochrome P450, 7a1	-2.0	bile acid biosynthesis
BC026667	aldehyde dehydrogenase family 1, subfamily A3	2.1	lipid metabolism
BB204486	3-phosphoglycerate dehydrogenase *	2.4	L-serine biosynthesis
NM_011116	phospholipase D3	3.0	metabolism
NM_007438	aldolase 1, A isoform	3.1	glycolysis
NM_021514	phosphofructokinase, muscle	3.9	glycolysis
NM_016920	ATPase, H+ transporting lysosomal V0 subunit a isoform 1 *	4.2	proton transport
BF225398	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 *	4.4	mitochondrial transport
NM_133348	brain acyl-CoA hydrolase *	4.4	fatty acid metabolism
NM_011099	pyruvate kinase, muscle *	8.7	deficient in a type of hemolytic anemia
BC008184	aldolase 3, C isoform *	21.8	glycolysis
NM_013509	enolase 2, gamma neuronal *	↑	glycolysis

↓ gene signal at background level in the supranutritional group but present in the deficient group; ↑ gene signal at background level in the deficient group but present in the supranutritional group; * gene is present and regulated in the same direction in the comparison between the supranutritional plus γ-tocotrienol group (“200+”) and the deficient plus γ-tocotrienol group (“2+”)

Several genes encoding calcium-dependent proteins or proteins which play a role in the homeostasis of calcium were up-regulated in an α-tocopherol dependent manner (Tab. 4.14). Calmodulin 1, 2, 3 and visinin-like 1, e.g., were up-regulated 3- to 5-fold by α-tocopherol in the supranutritional group compared to the deficient group. Calcium/calmodulin-dependent protein kinase II, beta was up-regulated by a factor of 3.9, necdin by 7.2 and calsyntenin by 10.4, respectively.

Table 4.14 Change in expression of calcium associated genes by α-tocopherol ("200 *vs*. 2").

Gene ID	Gene name	Fold change	Function/process
NM_010585	inositol 1,4,5-triphosphate receptor 1	2.6	calcium ion transport
AU079514	calmodulin 1 *	2.8	cell cycle, G-protein coupled receptor protein signaling pathway
BC021347	calmodulin 2 *	3.4	cell cycle, G-protein coupled receptor protein signaling pathway
AK011566	alpha-spectrin 2, brain *	3.5	calmodulin binding
NM_007595	calcium/calmodulin-dependent protein kinase II, beta (Camk2b)	3.9	G1/S transition of mitotic cell cycle
BB396904	calmodulin 3 *	4.8	cell cycle, G-protein coupled receptor protein signaling pathway
NM_054072	protocadherin alpha 1	5.2	calcium ion binding
NM_012038	visinin-like 1 *	5.3	calcium ion binding
BF148071	protein phosphatase 3, regulatory subunit B, alpha isoform (calcineurin B, type I) *	5.7	calcium ion binding
NM_010308	guanine nucleotide binding protein, alpha o *	6.8	calcium channel regulation
AI313926	protein phosphatase 3, catalytic subunit, alpha isoform *	7.1	calmodulin binding
AV124445	necdin	7.2	calcium homeostasis
BG065300	calsyntenin 1 *	10.4	calcium homeostasis, cell adhesion
NM_009722	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	12.2	calcium ion transport, regulation of muscle contraction

* gene is present and regulated in the same direction in the comparison between the supranutritional plus γ-tocotrienol group ("200+") and the deficient plus γ-tocotrienol group ("2+")

Because the investigated mRNA was from liver origin, an unexpected observation was the up-regulation of several genes which participate in vesicular transport in neurological processes (Tab. 4.15).

Table 4.15 Genes associated with vesicular transport and neuronal function were regulated by α-tocopherol ("200 *vs*. 2").

Gene ID	Gene name	Fold change	Function/process
BM222025	adaptor-related protein complex AP-3, beta 1 subunit *	-2.6	vesicle mediated transport
NM_016800	vesicle transport through interaction with t-SNAREs 1B homolog	2.1	intracellular transport
AK018789	neurotrophic tyrosine kinase, receptor, type 2	2.3	neurogenesis
AK003859	reticulon 4 *	2.8	neurogenesis
AI850720	RAS protein-specific guanine nucleotide-releasing factor 1	3.4	cell proliferation, intracellular signaling cascade, regulation of synaptic plasticity
AK004874	cAMP-regulated guanine nucleotide exchange factor II *	3.5	insulin secretion, exocytosis, cAMP mediated signaling

U60884	bridging integrator 1	3.8	synaptic vesicle endocytosis
NM_016801	syntaxin 1A	4.3	neurotransmitter transport
BB311061	nasal embryonic LHRH factor *	4.5	neurophilic migration
NM_011738	tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, eta polypeptide *	5.2	activator of tyrosine and tryptophan hydroxylases
L29457	dynamin *	5.8	responsible for membrane fission
AF326545	syntaxin binding protein 1 (Munc18-1)	6.3	synaptic vesicle maturation
BG871810	vesicle-associated membrane protein 2 (synaptobrevin, VAMP2) *	7.1	regulation of exocytosis
NM_009306	synaptotagmin 1	7.4	transport
NM_008069	gamma-aminobutyric acid (GABA-A) receptor, subunit beta 1	8.3	synaptic transmission
BC022954	synapsin I	8.3	neurotransmitter secretion
NM_009001	RAB3A *	8.7	regulation of exocytosis
BE688087	gamma-aminobutyric acid (GABA-B) receptor, 1	8.8	neurotransmission
NM_019634	transmembrane 4 superfamily member 2 *	10.0	neurite outgrowth
BB400581	N-ethylmaleimide-sensitive fusion protein (Nsf) *	10.8	vesicular trafficing, syntaxin binding
BC025807	ATPase, Na+/K+ transporting, alpha 2 polypeptide *	12.1	neurotransmitter uptake
NM_009305	synaptophysin *	13.5	endocytosis, synaptic transmission
BB348674	glycoprotein m6a *	18.9	surface glycoprotein expressed on neurons
BC014803	complexin 1 *	↑	uptake and exocytosis system of synaptic vesicle traffic
NM_013540	glutamate receptor, ionotropic, (alpha 2), AMPA2*	↑	synaptic transmission
NM_010777	myelin basic protein *	↑	myelination
AI385669	glutamate receptor, ionotropic, (zeta 1), NMDA1	↑	synaptic transmission
BC018249	synaptosomal-associated protein, 25 kDa (Snap25) *	↑	intracellular protein transport
AV028402	thymus cell antigen 1, theta	↑	glycoproteins constituents of thymocytes and neurons
BM115022	stathmin-like 2 *	↑	intracellular signaling cascade,

↑ gene signal at background level in the deficient group but present in the supranutritional group; * gene is present and regulated in the same direction in the comparison between the supranutritional plus γ-tocotrienol group ("200+") and the deficient plus γ-tocotrienol group ("2+")

The gene expression for numerous synaptic proteins was changed. Vesicle-associated membrane protein 2 (VAMP2, synonym: synaptobrevin), synapsin 1, synaptotagmin, syntaxin 1A, syntaxin binding protein 1 (synonym: Munc18-1), synaptophysin, N-ethylmaleimide sensitive fusion protein (Nsf), RAB3A, complexin 1, synaptosomal-associated protein, 25 kDa (Snap25), glutamate receptor, ionotropic (AMPA2), glutamate receptor, ionotropic

(NMDA1), e.g., encode proteins that are important for vesicle-mediated release of hormones and neurotransmitters and revealed a significant up-regulation in the supranutritional group compared to the deficient group.

Table 4.16 Genes dose dependently regulated by α-tocopherol.

Gene ID	Gene name	Fold change		
		20 *vs*. 2	**200 *vs*. 20**	**200 *vs*. 2**
BC024935	ADP-ribosylation factor 3	2.4	2.5	6.1
BC008184	aldolase 3, C isoform	10.1	2.2	21.8
NM_009722	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	5.3	2.3	12.2
BG065300	calsyntenin 1	4.3	2.4	10.4
AF071549	chemokine (C-X3-C motif) ligand 1	4.0	2.2	8.6
BB251922	cyclic nucleotide phosphodiesterase 1	3.6	3.4	12.2
L29457	dynamin	2.5	2.3	5.8
BB400581	N-ethylmaleimide sensitive fusion protein (Nsf)	3.9	2.8	10.8
AB006361	prostaglandin D2 synthase (21 kDa, brain)	↑	4.1	↑
BB464434	selenoprotein W, muscle 1	4.1	2.6	10.8
NM_013670	small nuclear ribonucleoprotein N	2.9	2.0	5.8
BC010581	stathmin 1	6.8	2.1	↑
NM_009305	synaptophysin	4.5	3.0	13.5
BC008117	tubulin, alpha 1	2.0	3.2	6.5
NM_011738	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	2.0	2.6	5.2
AI118428	UDP-glucuronosyltransferase 2 family, member 5	-2.4	↓	↓

↓/↑ gene signal at background level in one group but present in the reference group

Among the genes significantly changed by α-tocopherol several candidates were regulated between the adequate and the deficient group (“20 *vs*. 2”) as well between the supranutritional and the adequate group (“200 *vs*. 20”). These genes (Tab. 4.16), e.g., N-ethylmaleimide sensitive fusion protein, synaptophysin and calsyntenin 1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, were considered to be dose dependently regulated by α-tocopherol.

Genes which were regulated in the same manner between the supranutrional and the deficient group without (“200 *vs*. 2”) as well as with additional γ-tocotrienol (“200+ *vs*. 2+”), respectively, were indicated by an asterisk in Tab. 4.12 – 4.15.

To identify the effect of γ-tocotrienol on differential gene expression, genes were sought which were deemed significant with the same direction of change in all three of the groups ”2+ *vs*. 2”, “20+ *vs*. 20” and “200+ *vs*. 200”. No gene which met this criterion could be identified. However, genes which were regulated howsoever by additional γ-tocotrienol in all of the three groups are listed in Tab. 4.17. Noticeable, the effect of γ-tocotrienol was always contrariwise in one of the three groups compared to the others. The expression of cytochrome P450 4a10 and L-3-hydroxyacyl-coenzyme A dehydrogenase was also influenced (down-regulated) by α-tocopherol between the supranutritional and the deficient group (“200 *vs*. 2”, Tab. 4.13). The effect of α-tocopherol on the expression of both genes in the supranutritional group was inverted by γ-tocotrienol.

Table 4.17 Genes regulated by additional γ-tocotrienol in the deficient, the adequate and the supranutritional group.

Gene ID	Gene name	Fold change in[#]		
		2+	**20+**	**200+**
BC010747	cytochrome P450, 4a10	-2.0	-5.0	3.2
AB016602	G protein-coupled receptor 37-like 1	3.7	2.0	-2.2
NM_008212	L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	-2.0	-2.2	2.6
BE952082	olfactomedin 1	↑	2.4	↓
AI591480	phospholipid transfer protein	2.6	-3.5	-11.7
NM_018760	solute carrier family 4 (anion exchanger), member 4	-2.7	-3.1	5.1
BB747266	uromodulin	↓	2.9	↑

[#] fold change in expression in the deficient, the adequate and the supranutritional group with additional γ-tocotrienol (“2+”, “20+” and “200+”), respectively, compared to the groups without γ-tocotrienol (“2”, “20” and “200”); ↓/↑ gene signal at background level in one group but present in the reference group

5 Discussion

5.1 Metabolism of vitamin E

The identification of vitamin E metabolites - carboxyethyl hydroxychromans (CEHC) - with an intact chroman ring led to the proposed pathway of vitamin E degradation via initial ω-hydroxylation with subsequent β-oxidation. The final metabolite CEHC, is excreted in the urine by humans and other mammals, after conjugation with glucuronic acid (reviewed in Brigelius-Flohé *et al.* 2002). CEHC is not the only metabolite found in human urine. In addition to α-CEHC, the urinary excretion of the precursor α-CMBHC (α-carboxymethylbutyl hydroxychroman) was also described (Schülke *et al.* 2000), which further confirmed the pathway. The excretion of α-CEHC and α-CMBHC together did not exceed 5% of the supplemented α-tocopherol in α-TTP-deficient patients as well as in healthy subjects. Metabolites with longer side chain and, therefore, greater hydrophobicity may be found in plasma or in the bile or are excreted via faeces. This has yet to be investigated. Therefore, the identification of metabolites with longer side chain *in vitro* (see Fig. 4.1, Birringer *et al.* 2002; Sontag and Parker 2002) was not unexpected.

Although the CYP-mediated initial degradation of vitamin E is generally accepted, the responsible cytochrome P450 isoform is not yet identified. In the present work, the incubation of HepG2 cells released highly different amounts of CEHC and CMBHC from different vitamin E forms, in general demonstrating that all non-α-tocopherol forms are degraded preferentially. Interestingly, Birringer *et al*. (2001) observed an increase in α-CEHC formation from α-tocopherol after treatment with rifampicin, a known inducer of CYP3A-type cytochromes and so α-tocopherol was suggested to be a CYP3A substrate. From this and the observation made in the present work that also α-tocopherol pretreatment induces α-CEHC formation, it can be concluded that probably α-tocopherol itself induces CYP3A. This is known for multiple CYP substrates which can induce the expression of their own metabolizing CYP as a ligand of nuclear receptors (reviewed in Edwards *et al.* 2002 and Francis *et al.* 2003). Hence, the remarkable differences in the formation of metabolites from the individual tocopherols and tocotrienols may be the result of a differentiated

degradation through a differential regulation of CYPs by the vitamin E forms itself. Therefore, the effect of vitamin E on the pregnane X receptor (PXR) was investigated. PXR is activated by a wide variety of compounds and also regulates the expression of CYP3A4.

5.2 Vitamin E activates the pregnane X receptor

Vitamin E indeed activates the PXR-mediated expression of genes in an *in vitro* reporter gene assay. The pattern of the PXR-activating potential varied between the different vitamin E forms. α-Tocotrienol and γ-tocotrienol revealed the highest efficacy followed by δ-tocopherol > *RRR*-α-tocopherol > γ-tocopherol. The induction of the PXR reporter gene CAT (chloramphenicol acetyltransferase) was dose-dependent, and after omitting PXR transfection, the CAT induction was decreased but not abolished. Both reflect that the increase in CAT activity is essentially PXR dependent and that the expression of endogenous PXR in HepG2 cells is still sufficient for CAT induction. As further proof, the direct binding of α- and γ-tocotrienol as well as of α- and γ-tocopherol to PXR was observed in our lab (Pfluger *et al.* 2004). Also Zhou *et al*. (2004) found that vitamin E binds and activates PXR. In their work only tocotrienols but not tocopherols interact with PXR. However, it should be noted, that Zhou *et al*. only used the racemic mixtures of the individual vitamin E forms which they did not discuss. In the present work, the racemic mixture of α-tocopherol, *all-rac*-α-tocopherol, also did not activate PXR. Therefore, it can be supposed that the chirality of the respective vitamin E forms may be important for the interaction with the PXR binding cavitiy.

The potential of PXR-activation was not related to the differences in metabolism. In HepG2 cells as shown in the present work, the release of metabolites is highest for tocotrienols and lowest for α-tocopherol. In humans, the excretion of α-CEHC from *RRR*-α-tocopherol increases after a threshold plasma level of 7-9 µmol α-tocopherol/g total plasma lipids is exceeded. Thus, α-CEHC was suggested as a marker for α-tocopherol supply (Schultz *et al.* 1995). However, this approach failed as AVED patients excrete high amounts of α-CEHC in spite of low plasma levels of α-tocopherol (Schülke *et al.* 2000). This

means that more likely the action of α-TTP than the α-tocopherol plasma level determines the degradation of α-tocopherol. Furthermore, in healthy subjects a physiological variation of the α-TTP capacity and therefore a varying α-CEHC excretion cannot be excluded. Whether α-TTP protects individual vitamin E from metabolism in HepG2 cells remains to be clarified. The regulation of hepatic vitamin E metabolism via binding of vitamin E to PXR with a subsequent induction of oxidation systems, conjugation systems and transporters resulting in the production, conjugation and excretion of the respective CEHCs is appealing (reviewed in Traber 2004). In the present work the formation of metabolites was strongest from tocotrienols, which were also the strongest PXR activators. However, γ-tocopherol was a weaker PXR activator than α-tocopherol but showed higher metabolite formation. *All-rac*-α-tocopherol, which did not stimulate PXR, showed a release of metabolites comparable to *RRR*-α-tocopherol. Thus, the PXR-activating capacity of vitamin E does not necessarily reflect the metabolic rate of individual forms of vitamin E.

5.3 Vitamin K is degraded like vitamin E and also activates the PXR

Vitamin K covers several vitamers, phylloquinone (vitamin K_1) and the menaquinones (vitamin K_2). Phylloquinone (2-methyl-3-phytyl-1,4-naphtoquinone) is synthesized in plants and is the only natural vitamin K available for therapeutic use. Vitamin K_2 represents a series of compounds in which the phytyl side chain of phylloquinone has been replaced by a side chain built up of 2 to 13 isopentenyl units. Animals can synthesize menaquinone-4, the form with a side chain of 4 isopentenyl units linked to menadione (2-methyl-1,4-naphtoquinone, or vitamin K_3) and is considered to be one of the physiological active forms, also in humans. Metabolism of vitamin K has been studied in humans and rats. Administration of radio labeled phylloquinone to healthy male volunteers led to the detection of urinary metabolites 2-methyl-3-(3´,3´-carboxymethylpropyl)-1,4-naphtoquinone later called K acid 1, and 2-methyl-3-(5´-carboxymethylpropyl)-1,4-naphtoquinone later called K acid 2 (Shearer and Barkhan 1973). Rats fed a diet containing [^{14}C] labeled menaquinone-4 excreted glucuronides of K acid 1 and K acid 2 (Tadano *et al.* 1989). In both

studies it was suggested that the metabolites had been formed by the side chain degradation via ω- and β-oxidation.

Coenzyme Q (ubiquinone) is an essential component of the respiratory chain. Electrons from complex I and complex II are transferred to coenzyme Q, which carries them to complex III. Coenzyme Q is the only electron carrier in the mitochondrial electron transport system, which is not permanently bound or attached to a protein. It is a quinone derivative with a long side chain composed of a varying number of isoprene units - the most common form in mammals contains 10 units (coenzyme Q_{10}). The long isoprenoid tail makes coenzyme Q_{10} highly unpolar, a prerequisite for its rapid diffusion in the inner mitochondrial membrane. Apart from these vital functions, coenzyme Q_{10} has antioxidative capacities and thus, has been suggested as being able to prevent diseases associated with oxidative stress (reviewed in Thomas *et al.* 1999; Witting *et al.* 2000). The metabolism of coenzyme Q_{10} has been studied in guinea pigs to which ^{14}C-coenzyme Q_{10} was applied intravenously (Nakamura *et al.* 1999). Two major metabolites were found: Q acid 1 which was preferentially excreted in the bile and Q acid 2, the main urinary metabolite (Nakamura *et al.* 1999). The structural similarity of the side chain and the final metabolites of vitamin K and coenzyme Q with the side chain and the final metabolites of vitamin E (Fig. 5.1) motivated to test whether they would be active in the PXR reporter gene assay as vitamin E. It was found that, indeed, vitamin K_2 (menaquinone), which has an unsaturated side chain, activated the reporter gene with the same efficiency as tocotrienols, whereas vitamin K_1 (phylloquinone), which has a phytyl side chain with only one double bond, was as efficient as α-tocopherol. Most active was menaquinone, which induced CAT activity 8.4-fold, whereas phylloquinone was less active (2.8-fold induction). The weakest activation was exerted with coenzyme Q_{10} (1.2-fold). The activation of PXR by menaquinone in the meantime was confirmed by another study (Tabb *et al.* 2003). Here, a ranking of all these forms of vitamins tested for their PXR-activating properties resulted in tocotrienols (α- and γ-) as being the most potent activators followed by menaquinone. The induction by these compounds, which are all equipped with an unsaturated side chain, was similar to that obtained with rifampicin. Although still active, components with a saturated side chain were less efficient in activating PXR.

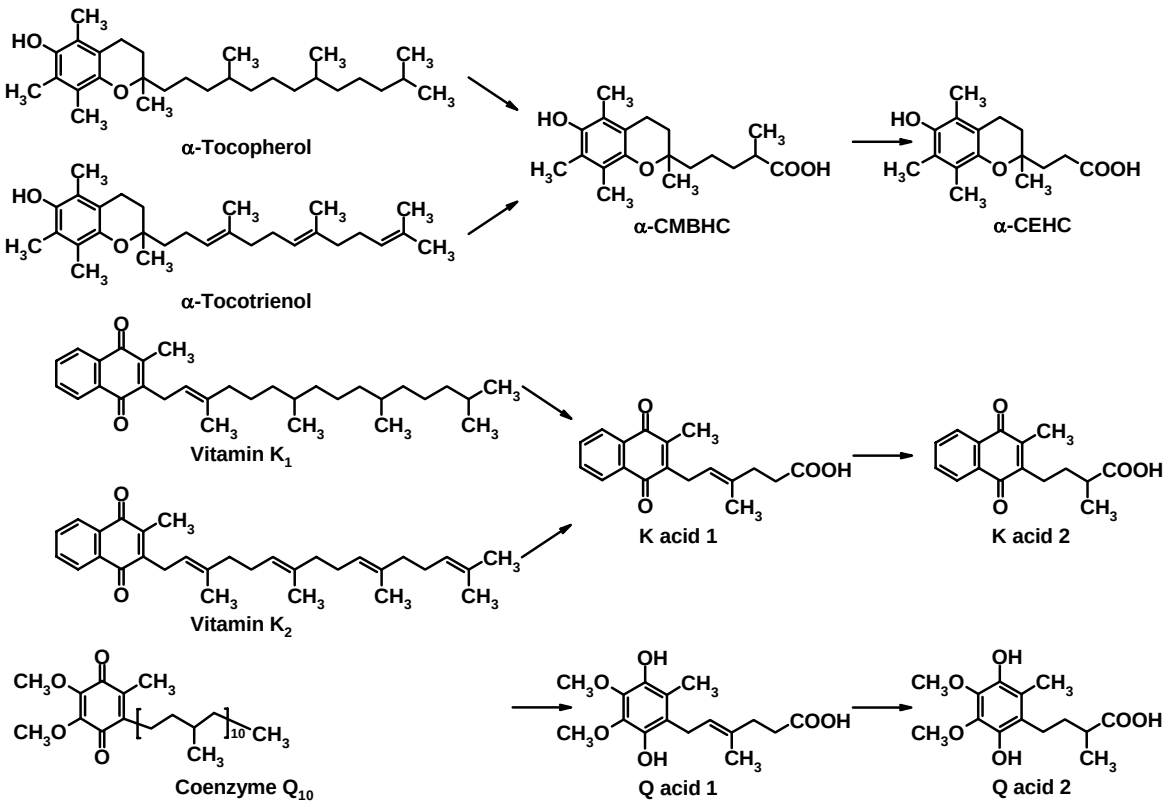


Figure 5.1 Final metabolites of vitamin E, K and coenzyme Q_{10} and their immediate precursors (Shearer and Barkhan 1973; Tadano *et al.* 1989; Nakamura *et al.* 1999; Birringer *et al.* 2002). The final metabolites of tocotrienols and vitamin K do not contain the double bond present in the side chain of the precursors which demonstrates that the respective side chains are degraded by a mechanism known for fatty acids (Birringer *et al.* 2002).

In summary, fat-soluble vitamins and compounds with an isoprenoid or phytyl side chain obviously are metabolized by side chain degradation via an initial ω-hydroxylation and subsequent β-oxidation. ω-Hydroxylation of vitamin E is catalyzed by cytochrome P450 enzymes and this is most likely also for vitamin K and coenzyme Q_{10}, although this needs to be demonstrated. Thus, the CYPs responsible for the elimination of xenobiotics apparently also remove lipophilic vitamins when administered beyond physiological needs. The activation of PXR may reflect a response to supranutritional dosage just to eliminate superficial amounts and one might even argue that the organism handles such supranutritional dosages of vitamins similar to potentially harmful xenobiotics. Additionally, the ability of the lipophilic vitamins E and K to activate a nuclear receptor, at least in an artificial system, points to the possibility that

both, like vitamin A and D, may exert biological functions via an “own” receptor which sporadically has already been proposed (reviewed in Carlberg 1999) but which has yet to be proven.

5.4 Vitamin E and drug interactions

The present observation of PXR activation by vitamin E supports the idea of a possible interference of vitamin E with the metabolism of drugs. Vitamin E-mediated adverse effects during drug therapy with PXR sensitive drugs cannot be excluded. More than 35 million people in the U.S. (Traber 2004) and approximately 4 million people in Germany (Beitz *et al.* 2002), e.g., are using supplemental vitamin E. The remarkable PXR activation of tocotrienols has to be emphasized as they are of particular pharmacological interest. Apart from their antioxidant function, tocotrienols share unique properties in contrast to α-tocopherol. Tocotrienols display anti-tumor activity and have been shown to reduce atherogenic plasma markers such as cholesterol (via a down-regulation of HMG-CoA reductase activity), apolipoprotein B and lipoprotein(a) (Theriault *et al.* 1999). α-Tocotrienol also reduces VCAM-1, ICAM-1 and E-selectin expression in human umbilical vein endothelial cells, whereas γ-tocotrienol stimulated apoB degradation (Theriault *et al.* 1999; Theriault *et al.* 2002). Protective properties of tocotrienols regarding atherosclerotic events were also demonstrated *in vivo*. In hypercholesterolemic swine, tocotrienols reduced various markers (e.g., serum- and LDL-cholesterol, triglycerides, apolipoprotein B) consequently raising hopes for a potent cardioprotective agent (Qureshi *et al.* 2001).

The pregnane X receptor serves as a key regulator of cytochrome P450 3A expression. Thus vitamin E should be able to influence CYP3A expression, which was shown in our lab, as γ-tocotrienol induces mRNA expression of CYP3A forms in non-transfected HepG2 cells (Landes *et al.* 2003). Additionally, in the present work the cDNA array showed an α-tocopherol but not γ-tocotrienol induced increase in Cyp3a11 (the murine homolog to human CYP3A4) expression also *in vivo*. Albeit the significance for the cDNA array results were low and there was no change in the oligonucleotide array, the upregulation of Cyp3a11 expression could be confirmed by real-time PCR

(Kluth *et al.* 2005). The weak response of Cyp3a11 to γ-tocotrienol can be explained by its high degradation rate, which does not allow a sufficient accumulation in the liver. It may also be due to differences in the response properties of the PXR from different species. In humans, rifampicin induces CYP3A type enzymes via PXR, but it does so to a much lesser degree than in rodents (reviewed in Jones *et al.* 2000). In the present work, γ-tocotrienol was a stronger activator of the human PXR in HepG2 cells than α-tocopherol, but the activation of the murine PXR by individual vitamin E forms has not been investigated so far.

Supplementation with high dosages of α-tocopherol might, nevertheless, influence the drug metabolizing system in humans. However, whether the up-regulation of CYP3A is sufficient enough to result in harmful side effects has to be evaluated. PXR activation by vitamin E, and thereby induction of the respective enzymes, is insofar worth considering as these enzymes are involved in the metabolism of various drugs (reviewed in Honkakoski *et al.* 2003). Such effects are described in another natural occurring compound, St. John's wort. This herbal remedy is widely used for the treatment of depression. Recent clinical studies demonstrate that hypericum extracts increase the metabolism of various drugs, including combined oral contraceptives, cyclosporin, and indinavir. It could be shown that hyperforin, a constituent of St. John's wort is a potent activator for PXR. This finding provided the molecular mechanism for the described and also suggested interactions of St. John's wort with the many drugs that are metabolized by CYP3A4 (Moore *et al.* 2000). Therefore, more detailed research, e.g., in clinical studies, to clarify the interaction between vitamin E and xenobiotic metabolism are urgently needed.

5.5 Vitamin E and gene expression *in vivo*

The proof of vitamin E-mediated gene regulation *in vitro* necessitated the confirmation *in vivo*. Because of the manifold potential target genes, the microarray technology displayed a useful approach. To investigate potential effects on the drug metabolising system, the Clontech Mouse Toxicology Array was chosen. The statistical significance for the genes, identified in this

experiment, was however, very low. The results obtained from the Clontech Mouse Toxicology Array were, therefore, anything but convincing. Fischer *et al*. (2001) also used a Clontech Toxicology Array and investigated the effect of vitamin E deficiency on differential gene expression in rats and found only two genes which are regulated only by vitamin E. They merely identified a down-regulation of the SPI-3 serine protease inhibitor and alpha 1 acid glycoprotein by vitamin E supplementation. *Vice versa*, the number of genes regulated by α-tocopherol, as found by the Affymetrix Arrays in the present work, appeared to be overwhelming. Indeed, the Affymetrix Arrays represented about ~14,000 genes whereas the Clontech Array represented only ~1,200 genes. The differences in reliability of the applied array technologies suggest that the design and possibly the quality of synthesized oligos or methods which were used to create oligos are important factors for obtaining accurate results. Also others described differences between cDNA arrays (like from Clontech) and oligonucleotide arrays (like from Affymetrix). The length of the cDNA probe and the GC content are considered problematic when using cDNA arrays (Kuo *et al.* 2002; Li *et al.* 2002). Affymetrix oligonucleotide arrays, in contrast, allow the design of oligomers which uniquely represent the desired cognate gene and make it easier to avoid secondary structures (www.affymetrix.com).

However, the more or less lack of a convincing effect of γ-tocotrienol in both types of microarray experiments in the present work is obviously caused by a rapid metabolism as concluded from the high amount of γ-CEHC found in the 24 h urine of the mice (Kluth *et al.* 2005). Whether this is a mechanism to protect the body from side effects via the *in vitro* observed activation of PXR has to be investigated.

5.6 Vitamin E and synaptic transmitter release

α-Tocopherol changed the expression of several genes, some of them also in a dose-dependent manner. A dose-dependently up-regulated gene, e.g., was the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, which is a Camk2 dependent activator of tyrosine 3-monooxygenase and tryptophan 5-monooxygenase. Both hydroxylases are the rate-limiting enzymes in the biosynthesis of serotonin and noradrenaline. Additionally, the

expression of several genes important in the intermediary metabolism were changed by α-tocopherol. This includes genes encoding proteins which are involved in fatty acid metabolism, glycolysis or amino acid metabolism. Also the expression of genes encoding xenobiotic metabolizing proteins like CYPs was changed, again underlining a possible interference with drug metabolism (see sections above). However, it was not possible to draw these genes into a common pathway. Therefore, the most striking observation was the α-tocopherol-mediated up-regulation of several genes which participate in vesicular transport mechanisms.

Neurotransmitter release is mediated via exocytotic fusion of synaptic vesicles with the presynaptic plasma membrane. In the present work, several important factors in neurotransmitter release, e.g., synapsin, syntaxin 1A, synaptotagmin, synaptophysin, synaptobrevin, RAB3A, complexin 1, N-ethylmaleimide-sensitive fusion protein and Snap25 were down-regulated in the α-tocopherol-deficient group compared to the supranutritional group (illustrated in Fig. 5.2).

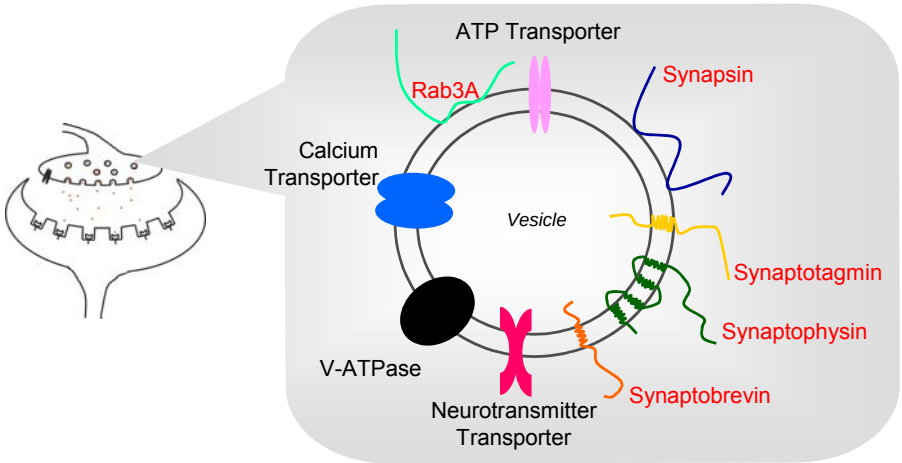


Figure 5.2 Synaptic vesicle proteins: A synapse is illustrated on the left, a presynaptic vesicle is magnified on the right (reviewed in Brunger 2001; Rizo and Südhof 2002 and Li and Chin 2003).α-Tocopherol-regulated genes are highlighted in red.

The specificity of neurotransmitter release requires the localization of both synaptic vesicles and calcium channels to the presynaptic active zone. The life cycle of a vesicle is characterized by three stages: endocytosis, exocytosis

and recycling of the vesicle (reviewed in Brunger 2001). Components of the general fusion machinery that participate in synaptic vesicle exocytosis include soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), N-ethylmaleimide-sensitive factor, Munc-18, Rab3 GTPase, and the exocyst proteins (reviewed in Rizo and Südhof 2002). In addition, synaptic vesicle exocytosis uses a set of unique components, such as synaptotagmin or complexin 1 to meet the special needs of fast calcium-triggered neurotransmitter release.

From sensory perception to learning and memory, this process underlies virtually all functions of the nervous system (reviewed in Li and Chin 2003). The priming step of synaptic vesicle exocytosis (simplified in Fig. 5.3) is thought to require the formation of three neuronal SNAREs, which comprises the proteins synaptobrevin on the synaptic vesicle, SNAP25 and syntaxin on the plasma membrane.

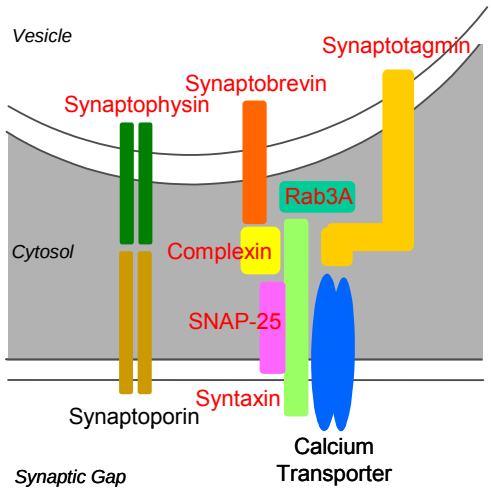


Figure 5.3 Proteins involved in the fusion of synaptic vesicles with the presynaptic membrane (reviewed in Brunger 2001; Rizo and Südhof 2002 and Li and Chin 2003). The respective α-tocopherol-regulated genes are highlighted in red.

The SNARE motifs from these three proteins assemble into a helical bundle to form an extremely stable ternary complex called the SNARE complex. By changing the confirmation of syntaxin, the SNARE complex moves the synaptic vesicle and the plasma membrane towards each other. Complexin 1 displays another essential protein for calcium-triggered neurotransmitter release. Complexin 1 has the ability to bind neuronal SNARE complexes. Most

probably, complexin binds to the central region of the interface between synaptobrevin and syntaxin for stabilization of the SNARE complex (reviewed in Rizo and Südhof 2002). Although Munc-18 has been shown to be absolutely required for synaptic vesicle exocytosis, the exact function of Munc-18 in the fusion process remains unclear so far (reviewed in Rizo and Südhof 2002). It has been suggested that Munc-18 may have a chaperone-like function to regulate the conformational transition of syntaxin 1, thus facilitating SNARE complex formation (reviewed in Li and Chin 2003).

After fusion of the vesicle with the plama membrane, N-ethylmaleimide-sensitive fusion protein (NSF) most likely acts to disassemble the SNARE complexes which were accumulated in the plasma membrane as a result of vesicle fusion. Furthermore, NSF may also recycle SNARE complexes for another round of fusion (reviewed in Li and Chin 2003).

Rab3 has been implicated in regulated exocytosis of neurotransmitters and hormones and is capable of regulating multiple stages of vesicular trafficing, including vesicle budding, motility, docking at the active zone and fusion (reviewed in Li and Chin 2003). Like other small G proteins, Rab3 may function as molecular switch or timer, cycling between the inactive GDP-bound and active GTP-bound form, regulating its effector proteins and downstream targets accordingly (reviewed in Brunger 2001).

The function of synaptophysin is unclear so far. In knockout mice no essential changes in the neurotransmittor release could be observed (McMahon *et al.* 1996). However, by using antisense oligonucleotides complementary to the synaptophysin mRNA and by microinjection of synaptophysin antibodies a reduced calcium-dependent neurotransmitter secretion was demonstrated (Alder *et al.* 1992; Alder *et al.* 1992). Also an interaction between synaptophysin and synaptobrevin was demonstrated, further indicating implications in the control of exocytosis (Edelmann *et al.* 1995).

Glutamate receptors are the predominant excitatory neurotransmitter receptors in mammals and are activated in a variety of normal neurophysiologic processes. In the present work, two glutamate receptors, glutamate receptor, ionotropic alpha 2 (AMPA2) and glutamate receptor, ionotropic zeta 1 (NMDA1) were upregulated by α-tocopherol. The activation of these receptors is essential for basal excitatory synaptic transmission as well as many forms of synaptic

plasticity such as long-term potentiation and long-term depression, which are thought to underlie learning and memory. They are thus also potential targets as therapies for CNS disorders such as epilepsy and Alzheimer's disease (reviewed in Kullmann *et al.* 2000).

In all synapses, calcium triggers neurotransmitter release to initiate signal transmission. Calcium presumably acts by activating synaptic calcium sensors. One of the candidate calcium sensors in release is the synaptic calcium-binding protein synaptotagmin (reviewed in Brunger 2001). Synaptotagmin, an integral membrane protein of synaptic vesicles with two calcium-binding C2 domains is the best-characterized candidate calcium sensor in triggering neurotransmitter release. The binding properties suggest that synaptotagmin may function as a calcium-sensitive, phospholipid-binding machine to promote synaptic vesicle fusion by pulling two membranes together or by helping to release tension in the metastable primed state. In addition to phospholipids, synaptotagmin exhibits calcium-dependent interactions with a variety of other molecules, including syntaxin SNAP-25, the SNARE complex, calcium channels, calmodulin and others (reviewed in Li and Chin 2003). Apart from synaptotagmin, in the present work several other calcium associated genes, e.g., calmodulins and calcium/calmodulin-dependent protein kinase II beta were up-regulated by α-tocopherol. Genes encoding calcium-associated proteins might, therefore, also play an essential role in vitamin E´s importance for normal neurological function.

Although the role of α-tocopherol in synaptic transmitter release has to be confirmed by classical techniques like real time PCR, the concentration of genes which are all involved in the process of neurotransmitter release underscores the significance of α-tocopherol for these genes. Additional strong support also comes from the work of Gohil *et al*. (2003), who used brain cortex from α-TTP-deficient mice to investigate the change in the gene expression profile under vitamin E deficient conditions. They observed a down-regulation of mRNAs encoding synaptic proteins, e.g., complexin 1, vesicle-associated membrane protein, synaptotagmin, visinin-like 1, NMDA or AMPA (Gohil *et al.* 2003) and therefore similar genes as in the present work.

α-Tocopherol may have a role in the prevention and treatment of neurodegenerative diseases like Alzheimer's disease which is supported by

animal and *in vitro* studies. Vitamin E´s function for prevention and treatment of degenerative neurological diseases, however, is often discussed based on its antioxidant properties (reviewed in Berman and Brodaty 2004). With regard to the presented results in this work, it would be interesting whether or not α-tocopherol treatment would be able to enhance neurotransmission and diminish the failure of neurotransmission in Alzheimer's disease patients.

The importance of α-tocopherol to the nervous system is also enhanced by observation from patients deficient in functional α-TTP. Patients display an ataxia, dysarthria, hyporeflexia, and decreased proprioceptive and vibratory sensations (Yokota *et al.* 1997). These patients have to be supplemented with up to 2 g α-tocopherol per day to increase plasma α-tocopherol concentrations and to halt the neurological symptoms (Yokota *et al.* 1997; Schülke *et al.* 2000). It is very unlikely that this beneficial effect can only be attributed to α-tocopherol´s antioxidative property. With the findings of the present and also other works (Gohil *et al.* 2003), alternative mechanisms can now be assumed. Thus, by regulating genes, which are necessary for synaptic neurotransmitter release, α-tocopherol is more essential for neurological signal transduction than it is as a preventing agent to avoid oxidative damage to neurological structures.

5.7 Open questions

An essential missing link is the common regulatory dominator, which could explain how vitamin E is able to regulate gene expression. Gohil *et al*. (2003) searched for identical transcription factor binding sites upstream of α-tocopherol dependent genes and found binding sites for the retinoic acid receptor-related orphan receptor alpha (RORα). ROR-mediated transcriptional activation can be dramatically enhanced by calcium/calmodulin-dependent kinase (CaMK4). This stimulation involves CaMK4-mediated phosphorylation, not of RORs, but likely of specific nuclear cofactors that interact with RORs (reviewed in Jetten *et al.* 2001). In the present work, another calcium/calmodulin-dependent kinase (Camk2b) was up-regulated. Also Camk2b is a prominent kinase in the central nervous system and may function in long-term potentiation and neurotransmitter release. Although widely expressed, in the cerebellum RORα is expressed in the Purkinje cells (reviewed

in Jetten *et al.* 2001). RORα knockout mice exhibit severe cerebellar ataxia due to a defect in Purkinje cell development. In addition, α-TTP is expressed in the Bergmann glial cells, which surround the Purkinje cells. It is suggested that α-TTP supplies vitamin E to Purkinje cells via the Bergmann glial cells (Hosomi *et al.* 1998). Nevertheless, whether α-TTP supplies the Purkinje cells with α-tocopherol to support a calcium/calmodulin-dependent kinase dependent ROR-mediated transcriptional activation of genes encoding synaptic proteins remains highly speculative.

Therefore, further research on RNA and also on protein level is needed to confirm the vitamin E-mediated changes in gene expression validated by gene array technology. In a next step, real time PCRs with RNA from neuronal tissues like brain, western blots and subsequent mechanistical, e.g., transfection and promoter assay experiments will then enable deeper insights into vitamin E´s function and the involved regulatory processes for vesicular transport and neurotransmitter release.

6 Summary

For more than 80 years vitamin E has been in the focus of scientific research. Most of the progress concerning non-antioxidant functions, nevertheless, has only arisen from publications during the last decade.

Most recently, the metabolic pathway of vitamin E has been almost completely elucidated. Vitamin E is metabolized by truncation of its side chain. The initial step of an ω-hydroxylation is carried out by cytochromes P450 (CYPs). This was evidenced by the inhibition of the metabolism of □-tocopherol by ketoconozole, an inhibitor of CYP3A expression, whereas rifampicin, an inducer of CYP3A expression increased the metabolism of □-tocopherol. Although the degradation pathway is identical for all tocopherols and tocotrienols, there is a marked difference in the amount of the release of metabolites from the individual vitamin E forms in cell culture as well as in experimental animals and in humans. Recent findings not only proposed an CYP3A4-mediated degradation of vitamin E but also suggested an induction of the metabolizing enzymes by vitamin E itself.

In order to investigate how vitamin E is able to influence the expression of metabolizing enzymes like CYP3A4, a pregnane X receptor (PXR)-based reporter gene assay was chosen. PXR is a nuclear receptor which regulates the transcription of genes, e.g., CYP3A4, by binding to specific DNA response elements. And indeed, as shown here, vitamin E is able to influence the expression of CYP3A via PXR in an *in vitro* reporter gene assay. Tocotrienols showed the highest activity followed by δ- and α-tocopherol. An up-regulation of Cyp3a11 mRNA, the murine homolog of the human CYP3A4, could also be confirmed in an animal experiment. The PXR-mediated change in gene expression displayed the first evidence of a direct transcriptional activity of vitamin E. PXR regulates the expression of genes involved in xenobiotic detoxification, including oxidation, conjugation, and transport. CYP3A, e.g., is involved in the oxidative metabolism of numerous currently used drugs. This opens a discussion of possible side effects of vitamin E, but the extent to which supranutritional doses of vitamin E modulate these pathways in humans has yet to be determined.

Additionally, as there is arising evidence that vitamin E's essentiality is more likely to be based on gene regulation than on antioxidant functions, it appeared necessary to further investigate the ability of vitamin E to influence gene expression. Mice were divided in three groups with diets (i) deficient in α-tocopherol, (ii) adequate in α-tocopherol supply and (iii) with a supranutritional dosage of α-tocopherol. After three months, half of each group was supplemented via a gastric tube with a supranutritional dosage ọfγ-tocotrienol per day for 7 days. Livers were analyzed for vitamin E content and liver RNA was prepared for hybridization using cDNA array and oligonucleotide array technology. A significant change in gene expression was observed by α-tocopherol but not by γ-tocotrienol and only using the oligonucleotide array but not using the cDNA array. The latter effect is most probably due to the limited number of genes represented on a cDNA array, the lacking γ-tocotrienol effect is obviously caused by a rapid degradation, which might prevent bioefficacy of γ-tocotrienol.

α-Tocopherol changed the expression of various genes. The most striking observation was an up-regulation of genes, which code for proteins involved in synaptic transmitter release and calcium signal transduction. Synapsin, synaptotagmin, synaptophysin, synaptobrevin, RAB3A, complexin 1, Snap25, ionotropic glutamate receptors (alpha 2 and zeta 1) were shown to be up-regulated in the supranutritional group compared to the deficient group. The up-regulation of synaptic genes shown in this work are not only supported by the strong concentration of genes which all are involved in the process of vesicular transport of neurotransmitters, but were also confirmed by a recent publication. However, a confirmation by real time PCR in neuronal tissue like brain is now required to explain the effect of vitamin E on neurological functionality. The change in expression of genes coding for synaptic proteins by vitamin E is of principal interest thus far, since the only human disease directly originating from an inadequate vitamin E status is ataxia with isolated vitamin E deficiency. Therefore, with the results of this work, an explanation for the observed neurological symptoms associated with vitamin E deficiency can be presented for the first time.

7 Zusammenfassung

Chemisch handelt es sich bei Vitamin E um acht lipophile Derivate des 6-Chromanols mit einer Seitenkette. Nach dem Sättigungsgrad der Seitenkette lassen sich die Derivate in die Tocopherole (gesättigte Seitenkette) und die Tocotrienole (ungesättigte Seitenkette mit drei Doppelbindungen) einteilen. Entsprechend der Methylierung des Chromanrings lassen sie sich in α-, β-, γ- und δ-Tocopherol, bzw. Tocotrienol unterscheiden. Davon besitzt α-Tocopherol, das gleichzeitig die im Plasma dominierende Form darstellt, die höchste biologische Aktivität. Aufnahme wie auch der Transport von Vitamin E im Körper sind vergleichsweise gut erforscht. Die Kenntnisse zu Metabolismus und Elimination waren jedoch bis vor kurzem sehr lückenhaft. Lange Zeit waren nur Vitamin E-Metabolite mit geöffnetem Chromanring, die sogenannten Simon-Metabolite Tocopheronsäure und Tocopheronolacton bekannt. Diese Metabolite können nur aus oxidativ gespaltenem Vitamin E entstehen und galten daher auch als Beweis für die antioxidative Wirkung von Vitamin E. Mit verbesserter Analytik wurde vor einigen Jahren gezeigt, dass die Simon-Metabolite größtenteils Isolierungsartefakte sind. Stattdessen wurden Metabolite mit intaktem Chromanring identifiziert. Tocopherole wie auch Tocotrienole werden im Körper durch eine Verkürzung der Seitenkette abgebaut. Die Endprodukte sind in jedem Fall CEHCs (Carboxyethyl Hydroxychromane). Die Seitenkettenverkürzung startet mit einer ω-Hydroxylierung gefolgt von 5 Schritteṇβ-Oxidation. Die ω-Hydroxylierung der Seitenkette durch Cytochrom P450 (CYP) Enzyme wurde indirekt bestätigt. CYP3A4 gilt dabei als eines der wahrscheinlichsten Enzyme im Abbau von Vitamin E, die Beteiligung weiterer CYPs wird jedoch gleichfalls angenommen. Auffällig ist, dass nicht alle Vitamin E-Formen in gleichem Ausmaß abgebaut werden. Die Ausscheidung von CEHCs aus α-Tocopherol ist, verglichen zu andern Vitamin E-Formen, in kultivierten Zellen wie auch *in vivo* sehr gering. Die Art der Seitenkettenverkürzung von Vitamin E spricht für einen Abbau über das Fremdstoff-metabolisierende System, welches auch eine Vielzahl von Medikamenten verstoffwechselt.

Im ersten Teil der vorliegenden Arbeit konnte mittels Reportergenassay in HepG2 Zellen gezeigt werden, dass Vitamin E einen nukleären Rezeptor,

den Pregnan X Rezeptor (PXR), zu aktivieren und die Expression von PXR-regulierten Genen zu beeinflussen vermag. PXR reguliert eine Reihe von Genen für Fremdstoff-metabolisierende Enzyme wie z.B. Cytochrom P450 3A4 durch Bindung an sein responsives Element im Promotor der Zielgene. Die untersuchten Vitamin E-Formen unterschieden sich deutlich hinsichtlich ihrer PXR-Aktivierung. Die Tocotrienole zeigten die höchste PXR-Aktivierung - vergleichbar mit Rifampicin, einem bekannt guten PXR-Aktivator - gefolgt von δ-, α- und γ-Tocopherol. Im Tierversuch an Mäusen konnte die erhöhte Expression von Cyp3a11, dem Homolog des humanen CYP3A4 in Abhängigkeit von der α-Tocopherol-Zufuhr bestätigt werden. Somit konnte erstmals gezeigt werden, dass Vitamin E die Expression von Genen direkt beeinflussen kann. Darüber hinaus unterstreicht diese Beobachtung die Möglichkeit einer Wechselwirkung von pharmakologischen Dosen Vitamin E mit dem Abbau von Medikamenten.

Eine genregulatorische Funktion von Vitamin E ist auf den ersten Blick überraschend. Denn wenngleich Vitamin E vor über 80 Jahren als Fertilitätsfaktor bei Ratten entdeckt wurde, steht die erst später beschriebene antioxidative Eigenschaft von Vitamin E bis heute im Fokus der meisten Publikationen. Die molekularen Mechanismen der Essentialität von Vitamin E wurden dagegen wenig untersucht. Erst in den letzten Jahren finden Funktionen von Vitamin E Interesse, die über seine antioxidative Wirkung hinausgehen. Dabei konnte gezeigt werden, dass Vitamin E *in vitro* die Expression von Genen wie dem Scavenger Rezeptor CD36, dem *Connective Tissue Growth Factor* oder dem Peroxisomen-Proliferator aktivierten Rezeptor gamma beeinflussen kann.

Um weitere Zielgene von Vitamin E *in vivo* identifizieren zu können, wurden im zweiten Teil der vorliegenden Arbeit Mäuse in drei Fütterungsgruppen mit einer a) defizientem b) adäquatem sowie c) mit einer supranutritiven α-Tocopherol-Versorgung über 3 Monate gefüttert. Zusätzlich erhielt die Hälfte der Tiere aus jeder Gruppe während der letzten Lebenswoche eine supranutritive Dosis γ-Tocotrienol pro Tag. Aus den Lebern der Tiere wurde die RNA präpariert und die differentielle Genexpression mittels a) cDNA und b) Oligonukleotide enthaltenden GenChips analysiert.

Eine signifikante Änderung in der Genexpression zwischen den verschiedenen Fütterungsgruppen fand sich jedoch nur in den Analysen der Oligonukleotid GenChips. Dies kann auf die begrenzte Anzahl von Genen zurückzuführen sein, die auf den cDNA GenChips repräsentiert waren. Auch ein signifikanter Effekt von γ-Tocotrienol auf die Genexpression konnte nicht beobachtet werden. Wahrscheinlich ist die hohe Ausscheidung von γ-CEHC, dem Abbauprodukt von γ-Tocotrienol, die im Urin der Tiere gemessen wurde und die damit womöglich verringerte Bioverfügbarkeit von γ-Tocotrienol dafür verantwortlich.

Mit Hilfe der Oligonukleotid GenChips konnte jedoch ein signifikanter Effekt von α-Tocopherol auf die Expression einer Vielzahl von Genen beobachtet werden. Herausstechend war dabei die erhöhte Expression von für den vesikulären Transport essentiellen Genen, die für den synaptischen Signaltransfer benötigt werden. So wurden z.B. Synapsin, Synaptotagmin, Synaptophysin, Synaptobrevin, RAB3A, Complexin 1, Snap25, die ionotrophen Glutamat Rezeptoren alpha 2 und zeta 1 in Abhängigkeit von der α-Tocopherol-Versorgung über die Diät erhöht exprimiert. Die Beobachtung, dass Vitamin E bei neurologischen Prozessen eine Rolle zu spielen scheint ist jedoch nicht neu. Bei Patienten mit einem Mangel an funktionellem α-Tocopherol-Transfer-Protein (α-TTP) kann es zu stark verringerten Plasmakonzentrationen an Vitamin E kommen, da α-TTP eine zentrale Rolle in der Aufnahme und Verteilung von Vitamin E im Körper einnimmt. An diesen Patienten können charakteristische Vitamin E-Mangelzustände beobachtet, die durch eine Reihe von neurologischen Störungen wie Ataxien, Hyporeflexie sowie eine verringerte propriozeptive und vibratorische Sensitivität gekennzeichnet sind. Mit den vorliegenden Ergebnissen kann nun erstmals eine mechanistische Erklärung für diese Symptome diskutiert werden. Eine Bestätigung der vorliegenden Ergebnisse via RT-PCR und Western Blot, z.B. in neuronalem Gewebe wie dem Gehirn, sowie anschließende funktionellen Untersuchungen ist daher dringend geboten.

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9 Appendix

The following procedures of sample preparation, hybridization to the Affymetrix Arrays and the data analysis were carried out by the laboratory of Prof. Joe Lunec, University of Leicester (Leicester, UK).

Isolation of RNA

Total RNA was isolated from liver tissue using TRI Reagent (Sigma, Dorset, UK) following the manufacturer's instructions. Briefly, liver tissue was homogenized in 1 ml TRI Reagent using disposable pellet pestles (Amersham, Uppsala, Sweden). After the cells were lysed, chloroform extraction was performed and total RNA was precipitated using isopropanol. The total RNA pellet was washed twice in 75% ethanol and once in 100% ethanol, dried at room temperature then resuspended in 40 µl of diethyl pyrocarbonate (DEPC)-treated water. Finally, 1 µl of resuspended total RNA was diluted 200 times in DEPC-treated water and then quantified spectrophotometrically.

To assess the quality of the total RNA before using it in downstream experiments, the integrity of the extracted total RNA was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, USA). Briefly, and following the manufacturer's instructions, 1 µl of the extract was loaded into the LabChip cartridge where the pin-electrodes are used to create electrokinetic forces that electrophorese RNA molecules through the polyacrylamide gel-filled micro-channels of the glass chip. The resulting electrophoregram was monitored and visualized by the Agilent 2100 Bioanalyzer software (Agilent Technologies, Palo Alto, USA).

Synthesis of cDNA

The messenger RNA fraction of the total RNA was converted to cDNA by reverse transcription using the SuperScript kit (Invitrogen, Paisly, UK) in combination with a T7-$(dT)_{24}$ oligomer (5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-$(dT)_{24}$-3') (Genset Oligos, Paris, France). For first-strand cDNA synthesis, 6.2 µg of total RNA and 100 pmol of T7-$(dT)_{24}$ were incubated at 70°C for 10 min and placed on ice. First strand buffer (50 mM

TrisHCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol (DDT) and 10 mM dNTPs were then added and the mixture was incubated at 42°C for 2 min. Finally, 200 U of Superscript II Reverse Transcriptase (Promega, Southampton, UK) was added in a reaction volume of 20 µl and incubated at 42°C for 1 h. For second-strand synthesis, second strand buffer (20 mM Tris HCl pH 6.9, 90 mM KCl, 4.6 $MgCl_2$, 0.15 mM β–NAD^+, 10 mM $[NH_4]SO_4$), 10 mM dNTPs, 10 U of *E.coli* DNA ligase, 40 U of *E.coli* DNA polymerase I, 2 U RNase H and DEPC-treated water were added to the first strand reactions in a final volume of 150 µl and incubated at 16°C for 2 hours. Following this incubation, 10 U of T4 DNA polymerase were added and the mixture incubated at 16°C for 5 min. The reaction was stopped by adding 10 µl of 0.5 M EDTA and the double-stranded cDNA was extracted, once with phenol-chloroform (1:1) and once with chloroform. A volume of 320 µl of ethanol, 0.1 volume of sodium acetate and 2 µl of PelletPaint (Novagen, Madison, USA) were added to the aqueous phase, vortexed and precipitated cDNA spun down at 15,000 g for 10 min. The pellets were then washed twice with 80% ethanol and once with 100% ethanol. Finally, dry cDNA pellet was resuspended in 12 µl of DEPC-treated water and 1 µl was run in a 1% agarose gel at 7 V/cm for 1 hour. The blue dye loading buffer (MBI Fermentas, St. Leon-Rot, Germany) used contained bromide ethidium and the GeneRuler 100bp DNA Ladder Plus size marker (MBI Fermentas, St. Leon-Rot, Germany) was used to assess size distribution.

Synthesis of biotin-labeled complementary RNA

In accordance with the Affymetrix GeneChip protocol, synthesis of hybridizable biotin-labeled complementary RNA (cRNA) was performed using the BioArray High Yield RNA Transcript Labeling Kit (Enzo, Farmingdale, USA). The T7 RNA polymerase in the reaction performed *in vitro* transcription from the T7 RNA polymerase promoters in the template using both biotin-labeled ribonucleotides (Bio-CTP and Bio-UTP) and non-labeled ribonucleotides. All the remaining cDNA (11 µl) was used as a template and the reaction was performed in a total volume of 40 µl and incubated at 37°C for 4 hours. Gentle mixing of the reactions was performed every 30 min. Following the *in vitro* transcription, cRNA was precipitated using 2.5 volumes of ethanol and

0.1 volume of sodium acetate, the mixture was vortexed and cRNA was spun down at 15,000 g for 10 min, washed twice with 80% ethanol and once with 100% ethanol then dried at room temperature and resuspended in 40 µl of DEPC-treated water. cRNA quantification was performed by spectrophotometry. cRNA size distribution was assessed by running 4 µg of the cRNA in a formaldehyde denaturing 1.2% agarose gel and using the RNA 6000 Nano LabChip kit as described earlier.

Estimation of target quality and labeling efficiency

The GeneChip Test 3 Array (Affymetrix, Santa Clara, USA) was used as an assessment tool for determining target quality and labeling efficiency prior to utilizing the genome GeneChip 430A murine Array (see below) and consisted of 312 probe sets from different species. The GeneChip Test 3 Array also contained prokaryotic probe sets controls to which are hybridized the cRNA controls “bio B”, “bio C”, “bio D” at different known concentrations. 3’ to 5’ ratios close not greater than 3.0 and ideally close to 1 are indicative of minimal sample degradation and efficient double-stranded cDNA synthesis, whereas the presence of all the cRNA controls at different concentrations indicates that the hybridization was efficient.

GeneChip 430A Array

The GeneChip 430A Array (Affymetrix, Santa Clara, USA) allowed the monitoring of the relative abundance of >22,000 probe sets to analyze the expression level of over 14,000 known murine genes. Every transcript was represented by a probe set made up of 11 25-mer oligonucleotide probe pairs, each pair comprised of a perfect match (PM) and a mismatch (MM) probe. The MM probes were identical to their corresponding PM except for a single base mismatch at a central position. The difference in the fluorescence of the 11 PMs and MMs, in each probe set, was used to correct for the contribution of background and cross-hybridization to the measurement of cell intensities.

Hybridization to GeneChip 430A Array

Prior to hybridization, cRNA (50 µg) was fragmented in a buffer containing 40 mM Tris-acetate pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate for 35 min at 94°C and the efficiency of fragmentation was checked in 1% agarose gel. Fragmented cRNA (21.2 µg) was added to the hybridization buffer containing 100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin (BSA), 50 pM control oligonucleotide B2 (for automatic alignment of the grid), the prokaryotic cRNA controls “bio B”, “bio C”, “bio D” and “cre” (1.5, 5, 25, and 100 pM respectively), and DEPC-treated water. The total volume of the hybridization cocktail was 300 µl and this was then heated at 90°C for 5 min, cooled down to 45°C for 5 min and then centrifuged at 15,000 g for 5 min. The GeneChip Test 3 Arrays were brought to room temperature and pre-wet with non-stringent wash buffer A (6x SSPE, 0.01% Tween 20) prior to adding 80 µl of the hybridization solution. Hybridization was performed at 45°C with permanent rotation at 60 rpm for 16 hours in the Hybridization Oven 640 (Affymetrix, Santa Clara, USA).

Whenever the GeneChip Test 3 Arrays were indicative of minimal sample degradation, good hybridization efficiency and low noise of background fluorescence, 200 µl of the hybridization cocktails were hybridized to the GeneChip 430A Arrays the same way as for the GeneChip Test 3 Arrays.

Following hybridization, the solution was removed and the arrays were filled with non-stringent wash buffer A. Array washing and staining was performed in the Fluidics Station 400 (Affymetrix, Santa Clara, USA) according to the corresponding protocol. For the GeneChip Test 3 Array, the protocol named *Micro_1v1* was used and for the GeneChip 430A, the protocol *EukGE-WS2* was used according to Affymetrix protocol. Briefly, after series of washes with non-stringent wash A buffer at 25°C and then with stringent wash B buffer (100 mM MES, 0.1 m [Na^+], 0.01% Tween 20) at 50°C, arrays were then stained with 1mg/ml streptavidin-phycoerythrin conjugate (SAPE, Molecular Probes, Leiden, The Netherlands) in 1x Staining buffer (100 mM MES, 1 M [Na+], 0.05% Tween 20) containing 50 mg/ml BSA in a total volume of 600 µl, at 25°C for 10 min followed by washing with Wash A buffer. The signal was amplified with a solution containing 3 µg/ml biotinylated anti-streptavidin

antibody (Vector Laboratories, Peterborough, UK), 10mg/ml Normal Goat IgG (Sigma, Dorset, UK) and 50 mg/ml BSA in 1x Staining buffer at 25°C for 10 min. Finally, arrays were re-stained with SAPE solution as performed above. These steps ensured a broad dynamic range for detecting and quantifying both rarely and highly expressed transcripts.

Array scanning

After the wash and stain steps, the arrays were then scanned twice at an emission wavelength of 570 nm using the GeneArray laser scanner (Agilent Technologies, Palo Alto, USA). The Microarray Analysis Suite version 5.0 (MAS v5.0) software (Affymetrix, Santa Clara, USA) was used to monitor scanning and to analyze the scanned image, calculate background and noise, determine the averaged difference of fluorescence intensity between the probe sets for each gene, and perform both absolute and comparative statistical expression algorithm analysis of all probe sets in the array.

Comparative analysis and estimation of false discovery rate

In this experiment, proprietary Affymetrix GeneChip microarray analysis software was used to acquire the expression data from the arrays themselves. Harvard Bioinformatics dChip data mining tools (Li and Wong 2001) was used to normalize and perform the comparative analyses.

Statistical analysis of microarray data must take into account both inherent biological and experimental error as well as the huge multiplicity of high throughput analysis (typical of the 10's of thousands of simultaneous measurements), which will inevitably result in a significant false discovery rate (FDR). A method to calculate FDR in genomic data is vitally important to reduce these large datasets and focus on biologically relevant gene expression changes. In this work, Significance Analysis of Microarrays (SAM) v1.13 (http://www-stat.stanford.edu/_tibs/SAM/) software was used to estimate FDR. SAM is a statistical method for accomplishing this task (Tusher *et al.* 2001). SAM calculated an FDR, which is the median percentage of genes from a list that are likely to be identified as differentially expressed by mistake. According to the SAM algorithm, genes were identified as differentially expressed based on

the difference in expression among the sample groups and the consistency of this expression difference. SAM assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM used permutations of the repeated measurements to estimate the percentage of genes identified by chance, again known as the FDR. The SAM procedure was performed on 22,000 probe sets for which signals were measured in the replicate sets of arrays for each of the diet groups.

The .CEL files generated by the Affymetrix Microarray Suite (MAS 5.0) were converted into .DCP files using dChip (www.dCHIP.org), as described previously by Li and Wong (2001). The .DCP files were normalized to the whole expression level, and the raw gene expression data generated was then normalized using the dChip system of model-based analysis (perfect match-mismatch). An additional analysis was performed using SAM v1.13 (http://www-stat.stanford.edu/_tibs/SAM/). SAM software was used to perform 2-class unpaired analysis to compare the different diet groups separately, again using the dChip normalized data from MAS5 generated .CEL files. The output criteria selected for SAM included 2-fold or greater expression in the different diet groups, and a significance threshold expected to produce a median FDR of less than 5%.

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