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PCR-verified microarray analysis and functional *in vitro* studies indicate a role of α-tocopherol in vesicular transport

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Abstract

Global gene expression profiles of livers from mice, fed diets differing in α-tocopherol content, were compared using DNA microarray technology. Three hundred and eighty nine genes were found to significantly differ in their expression level by a factor of 2 or higher between the high and the low α-tocopherol group. Functional clustering using the EASE software identified 121 genes involved in transport processes. Twenty-one thereof were involved in (synaptic) vesicular trafficking. Up-regulation of syntaxin 1C (*Stx1c*), vesicle-associated membrane protein 1 (*Vamp1*), *N*-ethylmaleimide-sensitive factor (*Nsf*) and syntaxin binding protein 1 (*Stxbp1*, *Munc18-1*) was verified by real time PCR. At a functional level, α-tocopherol increased the secretory response in RBL and PC12 cells. Although here detected in liver, the α-tocopherol-responsive pathways are also relevant to neurotransmission. A role of α-tocopherol in the vesicular transport might not only affect its own absorption and transport but also explain the neural dysfunctions observed in severe α-tocopherol deficiency.

Keywords: *α-tocopherol, endocytosis, gene regulation, vesicular transport, exocytosis*

Introduction

More than 80 years after the discovery of vitamin E and its essentiality for human and animal life [1] its precise biological role remains a mystery. From the eight different forms of vitamin E, only α-tocopherol is retained in the body and the essentiality of vitamin E is based on this vitamer. The preferential retention of α-tocopherol is achieved by the α-tocopherol transfer protein (α-TTP) which in the liver selectively sorts out α-tocopherol for the distribution to peripheral tissues either by incorporation into VLDL, as initially suggested (reviewed in Ref. [2]), or by alternative pathways [3,4]. A second reason for the high bioactivity of α-tocopherol is its comparatively low metabolic degradation (reviewed in Refs. [5–7]).

Symptoms of vitamin E-deficiency depend on the species. Rodents and calves develop myocardial necroses, rodents and poultry encephalomalacia. Also myopathy and *retinopathia pigmentosa* are often observed phenomena. Female infertility was observed in all mammals so far investigated. Fetuses of α-TTP knockout mice die at day 11.5–14.5 p.c. [8]. Thus, vitamin E plays a crucial role in female reproduction. Whereas nutritive vitamin E-deficiency is rare in humans, defects in the gene for α-TTP lead to a severe general vitamin E-deficiency with characteristic neurological disorders, as in *ataxia with isolated*

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vitamin E deficiency (AVED) or *familial isolated vitamin E deficiency* (FIVE) [9,10]. Thus, vitamin E is also pivotal to neuromuscular coordination.

The molecular mechanism by which vitamin E supports these vital functions is not known so far. Since it has antioxidant functions, in defined chemical systems at least, most research has focused on this property during the last decades. It now has become clear that the essentiality of vitamin E cannot be explained by its antioxidant properties only [11,12]. The key observation that initiated the search for novel functions of vitamin E was an inhibition of muscle cell proliferation and protein kinase C activity by α- but not β-tocopherol [13].

Novel functions of vitamin E also comprise the regulation of gene activity. The regulated genes were either individually identified to respond to different vitamers or by global gene expression analyses. Among the genes regulated by α-tocopherol are Cyp3a11, the murine homolog to the human CYP3A4 [14,15] and the ABC transporter p-glycoprotein [16], i.e. genes involved in the metabolism of vitamin E. First microarray data from rat livers [17] or brains [18] revealed a putative role of vitamin E in the regulation of genes involved in cellular defense mechanisms. Two studies investigated the effect of vitamin E-deficiency on the male reproductive tract in rats. In the epididymis, mainly genes encoding oxidative stress-related proteins were regulated [19], whereas in testes vitamin E-deficiency time-dependently up-regulated enzymes involved in testosterone synthesis and cell cycle progression [20]. In liver, α-tocopherol up-regulated glutamate cysteine ligase, which correlated with an increase of liver glutathione. The CD36 scavenger receptor, coagulation factor IX and 5-α-steroid reductase type I were down-regulated, as were related functional parameters [21]. In skeletal muscles of rats, vitamin E induced genes involved in the formation of muscle structure and extracellular matrix [22] and cytoskeleton genes in the lung of male and female mice [23]. Most interesting findings came from gene expression analyses in the brain of α-TTP-deficient mice. In the cortex of these mice, in which the α-tocopherol content was extremely low, genes involved in the myelination and synaptogenesis were much less expressed than in wild-type controls [24]. A hierarchical cluster analysis further suggested that α-TTP might be required for normal functioning of glial cells and oligodendrocytes [25], which would explain the neuromuscular dysfunction in vitamin E-deficiency.

So far, gene expression in livers from mice fed diets with different α-tocopherol contents has not yet been analyzed systematically. We, therefore, analyzed gene expression in mice fed diets presumed to be deficient, adequate or high in vitamin E according to present recommendations. In the high vitamin E group, we found a preferential up-regulation of many genes involved in the vesicular transport. Up-regulation of four representatives of this gene cluster, i.e. syntaxin 1C, vesicle-associated membrane protein 1 (*Vamp1*), *N*-ethylmaleimide-sensitive factor (*Nsf*) and syntaxin binding protein 1 (*Stxbp1*, *Munc18-1*) was verified by real time PCR, and a potential role of vitamin E in secretory processes was corroborated by an increased secretory response in RBL and PC12 cells cultivated with α-tocopherol.

Materials and methods

Diets

Diets were obtained from Ssniff, Spezialdiäten GmbH, Soest, Germany. Vitamin E-poor basal diet consisted of 240 g/kg casein, 500 g/kg starch, 110 g/kg glucose, 30 g/kg coconut fat, 50 g/kg cellulose, 10 g/kg vitamin mixture (poor in vitamin E), and 60 g/kg minerals and trace elements. Metabolizable energy was 15.4 MJ/kg, 60% from carbohydrates, 31% from protein and 7% from fat. The basal diet was enriched with 2 ("low"), 20 ("adequate") and 200 ("high") mg *RRR*-α-tocopheryl acetate (T3001, Sigma) [14]. The α-tocopherol content analyzed in different pellets of the resulting diet accounted to 4.9 ± 2.6 (7.3 IU), 14.7 ± 2.1 (22 IU) and 132.2 ± 27.6 (197 IU) mg/kg diet, respectively, indicating that a certain amount of α-tocopherol was already present in the basal diet and that the added α-tocopheryl acetate did not survive the food processing procedure completely.

Animals

Male C57BL6 mice which previously responded to α-tocopherol with an up-regulation of hepatic Cyp3a11 [14] were used for this study. All animal studies were carried out according to the regulations of the Federation of European Laboratory Animal Science Associations, complied with the Helsinki Declaration as revised in 1983, and were approved by the respective ministry of the Land Brandenburg. Four weeks-old mice were randomly assigned to one of the experimental diets: vitamin E low, 5 mg ($n = 3$), adequate, 15 mg ($n = 6$), or high, 132 mg ($n = 6$). After 3 months, blood was collected from anesthetized mice by retroorbital puncture and the animals killed by decapitation. Plasma was stored at − 80°C. To prepare RNA for array analyses identical parts of the liver were stored in RNA*later*. The rest of the livers were freeze-clamped in liquid nitrogen and stored at − 80°C.

Vitamin E extraction and analysis

Vitamin E in plasma, sera for cell culture, and tissues was determined by HPLC as described previously [14]. For estimation in the diet, 1 g pellets homogenized in a tissue lyser (Qiagen) was mixed with 5 ml

water and 4.3 nmol δ-tocopherol as internal standard and vortexed 30 s. Five millilitre 1% ascorbic acid, 10 ml ethanol and 1.5 ml KOH was added and the sample saponified 30 min at 70°C. Then, 10 ml n-hexane were added and samples incubated overnight at 4°C. Thereafter, vitamin E was extracted two times with hexane and hexane phases were evaporated to dryness. Residues were dissolved in 95% methanol and vitamin E measured by HPLC as above.

Affymetrix microarray expression analysis

Global gene expression in livers from two animals of each group was analyzed with MOE430A microarrays (Affymetrix) according to the manufacturer's instructions. Total RNA was isolated using TRI Reagent (Sigma) from the aliquots stored in RNA*later* and sample quality and quantity were confirmed by using the RNA 6000 Nano LabChip® Kit on an Agilent 2100 Bioanalyzer. RNA was reverse transcribed using oligo-$(dT)_{24}$–T7 primer (SuperScript Kit, Invitrogen). Biotin-labeled cRNA was synthesized according to the Affymetrix GeneChip® protocol using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences). cRNA size distribution was controlled with the RNA 6000 Nano LabChip® Kit. Quality and labeling efficiency were tested with the GeneChip® Test 3 Array (Affymetrix) before hybridization to the MOE430A arrays. The arrays were stained with streptavidin-phycoerythrin conjugate (Molecular Probes) and scanned with the GeneArray laser scanner (Agilent). The Microarray Analysis Suite version 5.0 (MAS vs. 5.0) and the GeneChip® Operating Software (GCOS) from Affymetrix were used to monitor scanning and to analyze the scanned image, calculating background and noise, determining the averaged difference of fluorescence intensity between the probe sets for each gene, and performing both absolute and comparative statistical expression algorithm analyses.

Data evaluation

Data analysis was conducted with GCOS. Fold change assessments were determined by comparing gene expression between the low and the supplemented group. α-Tocopherol-regulated genes had to fulfill three criteria: "present", changed by a factor of 2 or higher, and the change had to be significant ($p < 0.05$). To define the *p*-value cut-offs for the detection call (present, marginal, or absent) in the single array analysis, the significance levels were set to 0.05 (Alpha 1) and 0.065 (Alpha 2). Global scaling was applied to correct for variations between the arrays. In the comparison analysis, two arrays were each compared in order to detect and quantify changes in gene expression. One array of the low group was defined as baseline to which the other arrays (experimental) were compared. According to the Affymetrix "GeneChip®Expression Analysis—Data Analysis Fundamentals", gene expression data were sorted following the metrics detection, change, and signal log ratio (SLR) in order to determine most robust changes. For determining robust increases "absent" probe sets in the experimental sample were eliminated, and only "increase/marginal increase" probe sets with an $SLR \geq 1.0$ were considered. To determine robust decreases all "absent" probe sets in the baseline sample were eliminated, and all "decrease/marginal decrease" probe sets with an $SLR \leq -1.0$ selected. An SLR of 1 equals a fold change of 2.

Functional clustering and overrepresentation analysis of differentially expressed genes

α-Tocopherol-regulated genes were analyzed for significant functional gene clusters using the Expression Analysis Systemic Explorer (EASE) software [26] available at the Database for Annotation, Visualization and Integrated Discovery (DAVID) [27] (http://david.abcc.ncifcrf.gov). EASE rapidly interprets the biological functions of listed gene and provides statistical methods for discovering enriched biological terms within gene lists, generates gene annotation tables and enables automated linking to online analysis tools. It further ranks functional clusters by statistical overrepresentation of individual genes in specific categories relative to all genes in the same category on the array. Significance is calculated by the Fisher exact probability test. Then EASE calculates a score which refers to one-tail Fisher exact probability value and is based on Jackknifing [26]. The Fisher exact/EASE score values are exactly the same meaning/values as *p*-values. A *p*-value < 0.05 is considered significant. The group enrichment score is the geometric mean of the *p*-values of the members in the corresponding annotation cluster given in a -log scale. It is used to rank the significance of the overrepresentation.

Analysis of mRNA expression by real time PCR

Real time PCR was performed with the freeze-clamped liver samples of all animals of the study. Frozen tissue was pulverized with a mortar and pestle under liquid nitrogen. RNA was extracted from 20–30 mg aliquots further homogenized in the tissue lyser with the SV Total RNA Isolation System (Promega) including a DNase I treatment. RNA (3 μg) was reverse transcribed with 150 fmol oligo$(dT)_{15}$ primer and 180 U M-MLV Reverse Transcriptase (Promega) and the concentration estimated (260 nm). Real time PCR was performed with the M × 3005P™ QPCR System (Stratagene). Primers were designed with the PRIMER3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table I). Each reaction

Table I. Primer sequences for real time PCR (5′–3′).

Gene	Accession number	Primer sequence	Product (bp)
Hprt1	NM_013556	fwd GCAGTCCCAGCGTCGTG rev GGCCTCCCATCTCCTTCAT	166
Nsf	NM_008740	fwd ACTCTCTTGGCTCGACAGATTG rev CCACTGTTAGCACCAAGCCT	164
Stx1c	BC057892	fwd CCTCTGGGATCATCATGGACTC rev CTTCAGGACCAGGAGGGAGTG	191
Stxbp1	NM_009295	fwd ACTCCGCTGACTCTTTCCAA rev TGGATCGTCGGCTTTATAGG	209
Vamp1	NM_009496	fwd TGCTGAAGGGACAGAAGGAG rev CCGGTCATCCAGCTCTGAC	181

(total volume 25 μl) contained 66.6–133.3 ng RNA equivalents, 500 nM forward and 500 nM reverse primer, 1 × iQ™ SYBR Green Supermix (Biorad) supplying 0.2 mM of each dNTP, 0.625 U iTaq DNA polymerase, 3 mM $MgCl_2$ and 10 nM fluorescein. Initial denaturation (5 min, 95°C) was followed by 40 cycles of 15 s at 95°C, 10 s at 62°C and 20 s at 72°C, and a final elongation phase of 10 min at 72°C. The reactions were performed in triplicate in Thermo Fast 96 well plates. Respective gene expression was quantified via an external standard curve created by simultaneous amplification of 2 μl with 1×10^3–1×10^7 molecules of the respective PCR fragment. The fragment was obtained by elution from agarose gels with Invisorb Spin DNA extraction Kit (Invitek) (1 μg of a 1000 bp DNA = 1.52 pmol = 9.1×10^{11} molecules). Relative gene expression was normalized to Hprt1.

Cell culture

RBL-2H3 cells (DSMZ, ACC 312) were cultured in DMEM medium containing 10% FCS, 2 mM L-alanyl-L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. PC12 suspension cells (DSMZ, ACC 159) were grown in RPMI 1640 with identical additions except that FCS was replaced by 10% horse serum plus 5% FCS. Ethanolic *RRR*-α-tocopherol (a kind gift of Cognis Deutschland GmbH) was added to FCS and incubated overnight as described [28]. For control, respective amounts of ethanol were applied accordingly. Cell viability was analyzed by the MTT assay [29].

β-Hexosaminidase release assay

The assay was modified from [30,31] and also established for PC12 cells. RBL (60–80% confluent) or PC12 cells (1×10^6) were washed twice in phenol red-free RPMI medium. Exocytosis in RBL cells was triggered with 4 nM PMA/0.4 μM ionomycin for the times indicated and in PC12 cells with 20 nM PMA/2 μM ionomycin for 1 h at 37°C in phenol red-free RPMI medium (Sigma). Unspecific release was measured in cells without PMA/ionomycin treatment and subtracted from the stimulated one. Degranulation in PC12 cells was stopped by placing the samples on ice. Cells were harvested by centrifugation for 5 min at 250*g*. Both cells were lysed in phenol red-free RPMI medium containing 1% Triton X-100. For estimation of β-hexosaminidase activity 20 μl of supernatants and cell lysates were incubated with 50 μl substrate solution (1 mg/ml *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) in 0.05 M citrate buffer, pH 4.5) in 96-well plates for indicated times at 37°C. The reaction was quenched by addition of carbonate buffer (0.2 M NaOH, 0.2 M glycine, pH 10) and the concentration of the product measured at 405 nm. The percentage of released β-hexosaminidase activity was calculated relative to total β-hexosaminidase activity (supernatant plus lysate).

Statistical analyses

Significance was estimated by Student's *t*-test or by Pearson correlation as indicated. Statistics used for evaluation of array data and functional clustering are described there.

Results

Plasma and liver concentrations of α-tocopherol

α-Tocopherol concentrations in plasma of mice increased with increasing α-tocopheryl acetate content in the diet. In liver, however, α-tocopherol levels were not significantly elevated in the 15 mg group compared to the 5 mg group, whereas the level in the 132 mg group increased substantially (Table II) [14]. Obviously, the α-tocopherol content of the diet presumed to be in the adequate range (15 mg/kg) was not high enough to let α-tocopherol accumulate in the liver and incoming α-tocopherol was preferentially transferred to the plasma. The data shows that an increase in α-tocopherol plasma levels does not necessarily reflect the situation in tissues and suggests that both, the low and adequate diet resulted in a similar α-tocopherol status in the liver.

Table II. α-Tocopherol content in plasma and liver from mice fed diets with different amounts of α-tocopherol.

Group α-Tocopherol/kg diet (mg)	α-Tocopherol	
	Plasma (μM)	Liver (nmol/g)
5	1.33 ± 0.004	23.8 ± 11.7
15	2.69 ± 0.36⋆	30.5 ± 10.5
132	5.65 ± 2.35⋆⋆	141.2 ± 61.2⋆⋆

Mice were fed with diets containing the indicated α-tocopherol contents for three months after weaning. α-Tocopherol content was estimated by HPLC (see Methods). Numbers of mice were 3, 6 and 6 in the 5, 15 and 132 mg group, respectively. Values are means ± SD. ⋆$p < 0.05$, ⋆⋆$p < 0.01$ vs. the 5 mg group.

Global gene expression in livers of mice fed diets with different α-tocopherol content

RNA from livers of two mice of each group were analyzed on microarrays. By employing the stringent criteria defined for significant change in gene expression (see Methods), expression did not significantly differ for any gene between the 5 and the 15 mg group. This, however, is in accordance with the similar hepatic α-tocopherol content in both groups. In contrast, 389 genes were either up- or down-regulated in the 132 mg compared to the 5 mg group. The EASE program, which ranks functional clusters by statistical overrepresentation of individual genes in specific categories relative to all genes in the same category [26], identified cluster 1, the "vesicular transport" cluster as the one with the highest overrepresentation score and the highest significance (Table III). This cluster contains a total of 21 α-tocopherol-regulated genes which can be present in more than one term. A sum of 121 genes are listed in cluster 2, "transport/localization", the one with the second high overrepresentation score and significance, again genes can be present in more than one term. All genes from the vesicle cluster belong to "transport/localisation". Cluster 3 reveals a substantial amount of α-tocopherol-regulated genes involved in neurophysiological processes, most of them also in the transport cluster. Thus, genes of the vesicular transport appear to preferentially respond to α-tocopherol. In fact, almost all key players in vesicle formation, fusion of vesicles with target membranes, and in the regulation of these processes were up-regulated in the high α-tocopherol group (Table IV). The signal intensities for these genes were between 38 and 940 at the baseline chip, whereas, e.g. blot clotting factors, lipid and metal transporting proteins, proteins for detoxification, amino acid and carbohydrate metabolism reached values up to 40,000 and higher. This indicates a relatively low expression rate compared to liver-specific genes and probably also compared to brain. Nevertheless, the selective up-regulation of these genes appears remarkable. For a complete list of all regulated genes see supplemental Tables I and II published in the online edition.

Validation of α-tocopherol-regulated genes by real time PCR

Genes for four proteins playing a crucial role in the vesicular transport were selected for validation by real time PCR (Figure 1). For completeness, liver samples from all animals of each group were included.

Table III. Functional clustering of α-tocopherol-regulated genes identifies the vesicle and the transport/localization cluster as the two most significant annotation clusters.

Category	Gene ontology term	Number of genes in the category	Percentage of all α-tocopherol-regulated genes	EASE score *p*-value
Annotation cluster 1	Enrichment score: 5.38			
GOTERM_CC_ALL	Synaptic vesicle	12	3.02	2.80×10^{-7}
GOTERM_CC_ALL	Cytoplasmic vesicle	21	5.28	9.00×10^{-7}
GOTERM_CC_ALL	Vesicle	21	5.28	1.60×10^{-6}
GOTERM_CC_ALL	Cytoplasmic membrane-bound vesicle	19	4.77	6.40×10^{-6}
GOTERM_CC_ALL	Membrane-bound vesicle	19	4.77	1.10×10^{-5}
GOTERM_CC_ALL	Clathrin-coated vesicle	13	3.27	1.40×10^{-5}
GOTERM_CC_ALL	Coated vesicle	13	3.27	4.90×10^{-5}
Annotation cluster 2	Enrichment score: 5.07			
GOTERM_BP_ALL	Localization	117	29.40	9.60×10^{-8}
GOTERM_BP_ALL	Establishment of localization	116	29.15	1.10×10^{-7}
GOTERM_BP_ALL	Transport	106	26.63	2.30×10^{-7}
GOTERM_MF_ALL	Transporter activity	56	14.07	2.00×10^{-3}
SP_PIR_KEYWORDS	Transport	46	11.56	8.50×10^{-3}
Annotation cluster 3	Enrichment score: 4.57			
GOTERM_BP_ALL	Transmission of nerve impulse	21	5.28	3.20×10^{-8}
GOTERM_BP_ALL	Synaptic transmission	19	4.77	8.00×10^{-8}
GOTERM_BP_ALL	Cell–cell signaling	22	5.53	7.70×10^{-6}
GOTERM_BP_ALL	Neurophysiological process organismal physiological	25	6.28	1.60×10^{-3}
GOTERM_BP_ALL	Process	40	10.05	4.30×10^{-1}

BP, biological process; CC, cellular component; MF, molecular function. Details see "Methods".

Table IV. Genes involved in the vesicular transport and in its regulation up-regulated by α-tocopherol.

Gene title	Fold change	Accession number	Function
ADP-ribosylation factor 3	3.7	NM_007478	Protein transport/small GTPase mediated signal transduction
ATPase, Na + /K + transporting, alpha 2 polypeptide	8.3	NM_178405	Neurotransmitter uptake/
ATPase, Na + /K + transporting, beta 2 polypeptide	9.8	NM_013415	Calcium regulation in cardiac cells
Calcium/calmodulin-dependent protein kinase II gamma	3.0	NM_178597	G1/S transition of mitotic cell cycle
Complexin 1	6.0	NM_007756	Exocytosis/neurotransmitter transport/syntaxin binding
Complexin 2	8.2	NM_009946	Neurotransmitter transport/vesicle docking during exocytosis/syntaxin binding
Cyclin-dependent kinase 5, regulatory subunit (p35) 1	12.1	NM_009871	Central in formation of functional synapses/mediator of neurite outgrowth
DnaJ (Hsp40) homolog, subfamily C, member 5	5.6	NM_016775	Presynaptic chaperone that maintains continued synaptic function
Glutamate receptor, ionotropic, AMPA2 (alpha 2)	9.7	NM_013540	Synaptic transmission
Glutamate receptor, ionotropic, NMDA1 (zeta 1)	5.7	NM_008169	Synaptic transmission
N-ethylmaleimide sensitive fusion protein	4.3	NM_008740	Vesicular trafficking/syntaxin binding
***N*-ethylmaleimide sensitive fusion protein attachment protein beta**	5.5	NM_019632	Intracellular protein transport, syntaxin binding
Protein phosphatase 3, catalytic subunit, alpha isoform	7.0	NM_008913	Required for normal intracellular trafficking of AQP2
RAB11B, member RAS oncogene family	3.5	NM_008997	Regulation of exocytosis
RAB3A, member RAS oncogene family	9.5	NM_009001	Regulation of exocytosis
RAB6B, member RAS oncogene family	6.4	NM_173781	Regulation of exocytosis
Synapsin I	3.6	NM_013680	Neurotransmitter secretion
Synapsin II	6.0	NM_013681	Neurotransmitter secretion
Synaptic vesicle glycoprotein 2 a	3.6	NM_022030	Neurotransmitter transport/synaptic transmission
Synaptogyrin 1	5.3	NM_009303/NM_207708	Targeting of synaptophysin to a ubiquitous synaptic vesicle-sized compartment
Synaptophysin	5.2	NM_009305	Endocytosis/synaptic transmission
Synaptotagmin 11	5.2	NM_018804	Synaptic membrane fusion
Synaptotagmin 13	5.6	NM_030725	Synaptic membrane fusion
Syntaxin 1C⋆,†	3.7	NM_016801	Intracellular protein transport/SNARE binding
Syntaxin binding protein 1, Munc18	9.0	NM_009295	Vesicle docking during exocytosis/axon target recognition
Synuclein, alpha	9.1	NM_009221	Synaptic vesicle transport
Synuclein, beta	4.2	NM_033610	Synaptic vesicle transport
Tetratricopeptide repeat domain 3	11.3	NM_009441	Involved in chaperone, cell-cycle, transciption, and protein transport complexes
Vesicle-associated membrane protein 1	6.4	NM_009496	Vesicle-mediated transport
Vesicle-associated membrane protein 2†	3.3	NM_009497	Synaptic vesicle exocytosis/SNARE binding

Genes printed in bold were down-regulated in α-TTP$^{-/-}$ mice except synuclein alpha, which was up-regulated [24]. Grey shaded genes belong to the "transport/localization" cluster (see Table III); ⋆ The array indicated syntaxin 1A, but sequencing of the PCR product revealed that it was syntaxin 1C (see text); † Not all criteria were achieved for significant up-regulation according to the GCOS guidelines (see Methods).

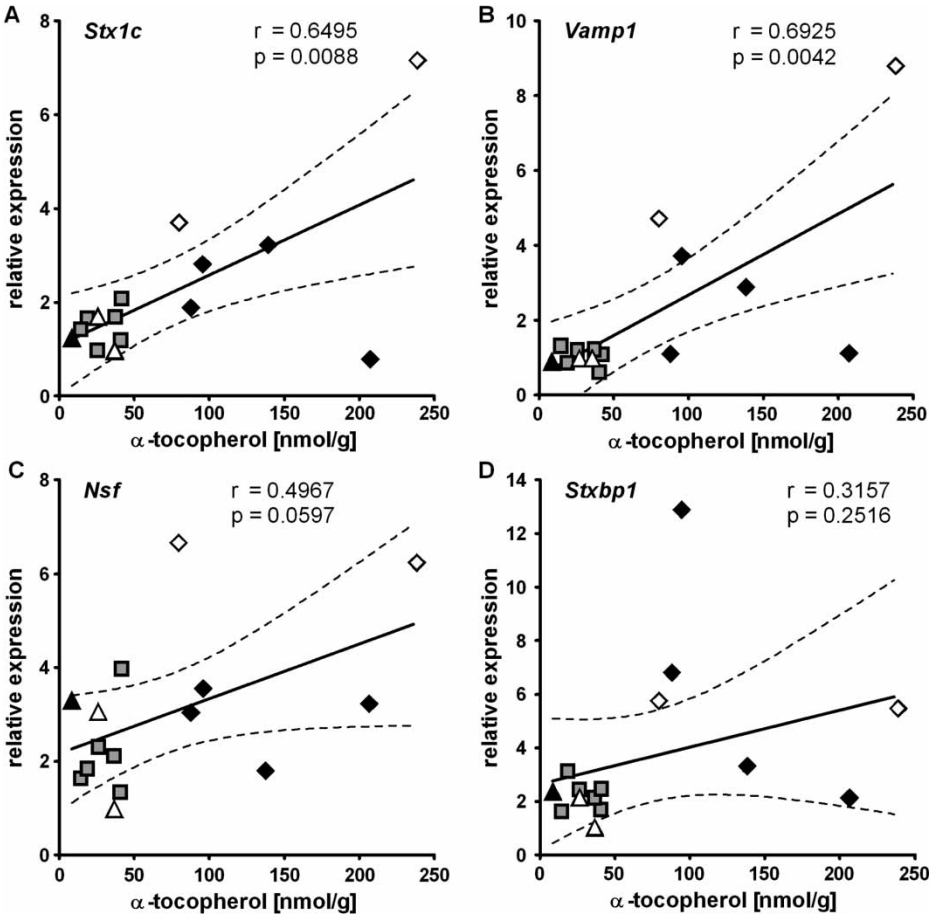

Figure 1. Validation of gene expression microarray data with qPCR. Relative expression of *Stx1c* (A), *Vamp1* (B), *Nsf* (C), and *Stxbp1* (D) in each individual animal of all groups in dependence of hepatic α-tocopherol levels. triangles: 5 mg/kg; squares: 15 mg/kg; diamonds: 132 mg/kg group. Open symbols represent the samples included in the micro array analyses, closed symbols the samples from the remaining individual animals from each group. Pearson's correlation coefficient and *p*-value for the entire data set are indicated. Further details see Methods.

Regarding the syntaxin1A gene (*Stx1a*), the stringent criteria were only reached for one of the animals analyzed in the microarrays. *Stx1a* was nevertheless included into the validation process, since it is essential for the formation of the SNARE complex (see discussion) and array analyses do not always detect a complete set of regulated genes. By sequencing the first PCR products it turned out that it was not *Stx1a* but *Stx1c*. This isoform has been described for humans before [32], and obviously it is also present in mouse liver. *Stx1a* is specific for the brain, whereas *Stx1c* is ubiquitously expressed, therefore the presence of Stx1c only makes sense in the liver (the probe set at the array would have detected both variants). *Stx1c*, *Vamp1*, *Stxbp1* (*Munc18-1*) and *Nsf* were up-regulated in dependence of liver α-tocopherol content (Figure 1(A)–(D)). A correlation analysis revealed that the α-tocopherol-dependent increase was highly significant for *Stx1c* and *Vamp1*, close to significance for *Nsf*, whereas significance was not reached for *Munc18-1* due to high inter-individual variation. Samples analyzed in the microarrays are marked by open symbols and clearly show the up-regulation in the 132 mg group, thus, confirming the microarray data.

Influence of α-tocopherol on vesicular transport at a functional level

For functional studies two cell culture models were chosen. RBL cells are generally used to study stimulated degranulation of lysosomes which can be measured by the release of β-hexosaminidase into the cell culture medium. Growing cells with increasing concentrations of α-tocopherol for 3 days increased cellular α-tocopherol levels from values below 0.03 nmol/mg protein in controls to 14 ± 1, 26 ± 0.2 and 29 ± 2 nmol/mg protein with 50, 100 and 150 μM α-tocopherol, respectively. α-Tocopherol led to a small but significant increase of PMA/ionomycin-stimulated β-hexosaminidase release which was highest at 150 μM α-tocopherol (Figure 2(A)). The second model was the PC12 cell system. The system is widely used to study the stimulated release of transfected human growth hormone [33] which is

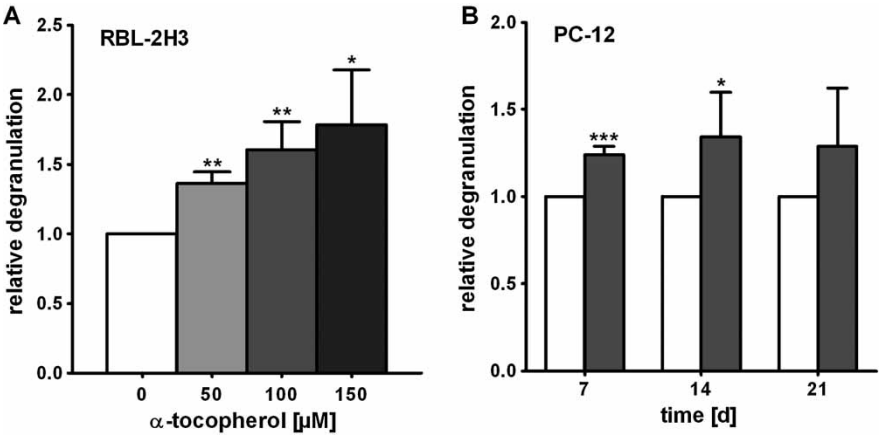

Figure 2. Stimulation of β-hexosaminidase release in RBL-2H3 (A) and PC12 (B) cells by α-tocopherol. RBL cells were incubated with indicated concentrations of α-tocopherol for 3 days. Exocytosis was stimulated with 0.4 μM ionomycin/4 nM PMA for 30 min. PC12 cells were grown in the presence of 100 μM α-tocopherol for 7, 14 and 21 days. Exocytosis was stimulated with 2 μM ionomycin/20 nM PMA for 60 min. Release of β-hexosaminidase from α-tocopherol-treated cells is expressed relative to controls set to 1. Values are means ± SD ($n = 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (*t*-test). Further details see Methods.

stored in similar vesicles as catecholamines. Since transfection efficiency might be influenced by pre-loading of cells with vitamin E, we looked for an assay suitable for the estimation of endogenously packed compounds. The β-hexosaminidase release assay proved to be suitable also for PC12 cells. Incubation of cells with 100 μM *RRR*-α-tocopherol for 7, 14 and 21 days increased the cellular α-tocopherol content from 0.03 nmol/mg protein in controls to a plateau around 15 nmol/protein reached after 3 days. Also in this system, α-tocopherol caused a small but consistent increase in the PMA/ionomycin-stimulated release of β-hexosaminidase (Figure 2(B)). One can argue that compared to α-tocopherol levels in liver tissues, which are around 1 nmol/mg protein (calculated from Table II and an average of 135 mg protein/g liver tissue in our mice) in the 132 mg group, levels in cultured cells are much higher, but obtained with higher α-tocopherol concentrations in the cell culture medium than in plasma. The need for higher concentrations of an effective compound in cultured cells than *in vivo* is a general phenomenon, which underscores the requirement of *in vivo* studies on the influence of α-tocopherol on vesicular trafficking.

Discussion

The array analysis of this study was originally undertaken to identify enzymes involved in the metabolism of xenobiotics, the expression of which is under the control of the PXR, because vitamin E had been demonstrated to activate PXR [34]. Further, an induction of the PXR-driven murine homolog to human CYP3A4, Cyp3a11 by high levels of α-tocopherol had been verified *in vivo* by us [14] and others [15,16]. In addition, α-tocopherol has been suggested to generally regulate the expression of enzymes involved in its own metabolism (reviewed in Azzi, 2004), including CYPs, ABC-transporters ([16]), α-TTP [35,36] and SR-BI ([37]. In this respect, however, the present investigation did not yield any new insight. Apart from Cyp3a11 [14], we did not observe an up-regulation of MDR-1, MRP-2, SR-BI, or α-TTP expression, neither in the arrays nor in real time PCR experiments (not shown) by α-tocopherol. Even Cyp3a11 by the stringent criteria the evaluation of the microarray data were based on did not show up in the list of α-tocopherol up-regulated genes (Supplemental Tables I and II). Lack of α-TTP regulation confirms the results of Bella et al. [38] who also did not find an effect of α-tocopherol on α-TTP expression in mice.

A total of 389 genes were differently expressed with the 132 mg α-tocopherol/kg diet compared to the 5 mg/kg group. The response to the 15 mg/kg dosage, which was anticipated to be adequate, proved to be indistinguishable from that of the 5 mg group. The marginal change in gene expression is, however, in accordance with the similar hepatic content in both groups (Table II). Our observation is supported by the response of gene expression in lungs of mice fed diets containing different amounts of α-tocopherol. Whereas a cluster of 13 genes was up-regulated in lungs of female mice fed an α-tocopherol-high diet (1000 IU/kg α-tocopherol in the form of *all rac*-α-tocopheryl acetate, equivalent to 671 mg *RRR*-α-tocopherol/kg diet) compared to lungs of mice in the α-tocopherol-poor diet (10 IU/kg diet), in lungs of mice fed an α-tocopherol normal diet (35 IU/kg diet) an up-regulation of only two genes of the cluster was observed [23].

Cluster analyses revealed a surprisingly high number of genes involved in the vesicular transport which were induced in livers of the supplemented

mice. In this respect, our data proved to be largely complementary to the transcriptional analysis performed by Gohil et al. [24] with brain of α-TTP-deficient mice that is extremely low in α-tocopherol: A large number of the transport-related genes that were found up-regulated in our study were described to be down-regulated in the brain cortex of the α-TTP-deficient mice (indicated in bold in Table IV). In rats fed diets containing 0, 1.5 and 5 mg *all rac* α-tocopherol/kg diet for 14 months, vitamin E-deficiency obtained by the 0 diet affected the expression level of mRNAs encoding ion channels and proteins involved in synaptic vesicle trafficking and the regulation of G-protein signaling [39]. The observed changes correlated with the impaired neurological functions in the vitamin E-deficient rats observed by the same group [40]. Taken together, these studies strongly argue in favour of a pivotal function in processes involving membrane fusion, be it the release of preformed compounds from vesicles in general, transmitter release in the nervous system, cell adhesion, endocytosis, recycling of vesicles, phagocytosis, or fusion of cells and organelles.

Release from vesicles requires the fusion of a vesicle with a membrane, which is achieved by the concerted action of membrane associated proteins. Minimum requirements of the general fusion machinery include soluble Nsf attachment protein receptors, so-called SNAREs, NSF itself, syntaxin binding protein (Stxbp or Munc18), and small GTPases of the Rab family (reviewed in Ref. [41]). SNAREs at the vesicular membrane (v-SNAREs), comprise the vesicle-associated membrane proteins (VAMPs), SNAREs at the target membrane (t-SNAREs) include syntaxins and the synaptosomal associated protein-25 (SNAP25) (Figure 3). Interaction of syntaxin with VAMP and SNAP25 initiates the fusion process. The resulting SNARE complex is stabilized by complexin. Influx of Ca^{2+} triggers binding of synaptotagmin to the SNARE complex and fusion of the vesicle with the membrane. After release of vesicular content, the SNARE complex is dissociated by the SNAPs-stimulated ATPase activity of NSF, then the SNAREs can be recycled for a further round of exocytosis [41–43]. Strikingly, all genes encoding the proteins described above were found to be up-regulated by α-tocopherol (Table IV, Figure 3).

Recycling of vesicles requires the refolding of SNARE proteins. CSPα (cysteine string protein) is a molecular chaperone involved in this process [44,45]. It contains a DNAJ domain similar to that of Hsp40 chaperones. CSPα has Hsp40-like activity and forms a trimeric complex with Hsc70 and SGT (glutamine-rich tetratricopeptide repeat protein). In this way CSPα supports the correct folding and refolding of SNARE proteins. Also a DNAJ (Hsp40) homolog as well as tetratricopeptide repeat domain 3 proved to be

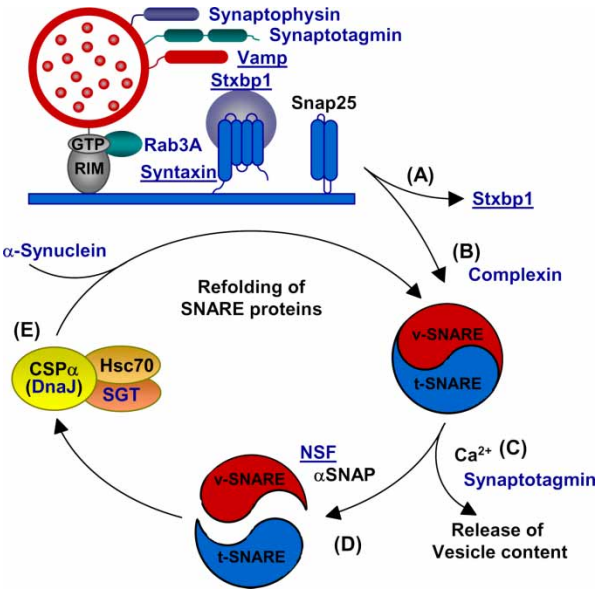

Figure 3. Proteins and processes involved in vesicular transport. (A) Vesicle and membrane SNARE proteins. Stxbp controls SNARE complex formation. Phosphorylation of Stxbp leads to its dissociation from syntaxin which now can interact with VAMP and SNAP25. (B) SNARE complex assembly and stabilization by complexin. (C) Ca^{2+}-triggered binding of synaptotagmin to the SNARE complex, fusion and release of vesicular content. (D) Disassembly of the SNARE complex by NSF. (E) Refolding of SNARE proteins, recycling of the vesicle. CSPα: Cysteine string protein. It forms a trimeric complex with Hsc70 and SGT (glutamine-rich tetratricopeptide repeat protein) and contains a DNAJ domain. Gene names indicated in blue belong to the genes up-regulated by α-tocopherol, genes in blue and underlined were confirmed by real time PCR. (colour figure published online).

up-regulated in mice of the 132 mg α-tocopherol group (Table IV).

Also absorption and distribution of α-tocopherol over the organism may require vesicular transport at several steps (for reviews see [46,47]). Intestinal absorption follows the route of dietary fatty acids. α-Tocopherol is taken up with micelles and enters the lymph incorporated into chylomicrons. Two independent pathways exist for the secretion of vitamin E from cultured intestinal cells. One is the incorporation into apoB-containing chylomicrons, the other the extracellular efflux via the ABCAI transporter [48], both dependent on intracellular vesicle trafficking. Cellular efflux of α-tocopherol via ABCA1 has been shown in fibroblasts and macrophages [3] and in mice lacking the gene for the microsomal triglyceride transfer protein required for chylomicron assembly [4].

Liver takes up α-tocopherol with chylomicron remnants by LDL-receptor- or LDL-receptor-related protein-mediated endocytosis [49]. In late endosomes/lysosomes apo-lipoproteins and transporting proteins are degraded or sorted for further distribution [50]. HDL-bound lipids can also be taken up by the scavenger receptor BI (SR-BI). The process was called selective lipid uptake since HDL was not degraded

and loss of apo-lipoproteins avoided. However, also SR-BI-mediated HDL-uptake is an endocytotic process in which HDL is "retroendocytosed" via the endosomal recycling compartment [51]. Also HDL-bound α-tocopherol is taken up via SR-BI in liver [52] and brain [53]. Insofar the uptake of α-tocopherol is similar to the uptake of cholesterol.

LDL-derived cholesteryl esters are transported to late endosomes/loysosmes. There, cholesterol is deliberated by acid lipase and transported back to the plasma membrane and/or the endoplasmic reticulum. Within lysosomes it is packed into intralysosomal multivesicle bodies (MVB) [54]. MVB are lysophosphatidic acid-rich membranes [55] which can undergo backfusion to the late endosome membrane, a process allowing the transport out of lysosomes to other intracellular destinations [50]. Removal of cholesterol from lysosomes requires transport facilitators such as the Nieman-Pick disease type I class 1 (NPC-1), a protein spanning the late endosomal membrane [56].

Certain analogies have been found for α-tocopherol: HDL-bound α-tocopherol is taken up by cultured liver cells and transported to late endosomes/lysosomes [57]. The transport of α-tocopherol from lysosomes to the cell membrane appears to be supported by the α-TTP. Neutralisation of the intra-lysosomal pH by chloroquine causes a block in the release of α-tocopherol from the cell and an accumulation of α-TTP at the cytosolic side of lysosomes thereby preventing coordinated intracellular traffic of α-tocopherol [58]. Co-localisation of α-tocopherol, α-TTP, and a lysosomal marker (LAMP-1) as well as an interaction of α-TTP with ABC-type transporters further confirms the role of α-TTP in the transport of α-tocopherol from lysosomes to the plasma membrane and release from the cell via ABC transporters [57]. Release of α-tocopherol from lysosomes has been postulated to require a protein which interacts with α-TTP which might be similar to NPC-1.

Proteins from the same families as involved in exocytosis but different isoforms are required for the pathway of regulated endocytosis, and release of endocytosed ligands from the cell [59]. Although not yet studied in detail these proteins may be equally important in the intracellular traffic of α-tocopherol, too. If this turns out to be true α-tocopherol itself may contribute to its traffic in the cell and between organs.

Remains the question whether α-tocopherol directly regulates gene activity or might rather influence gene expression by alternative pathways. The first alternative may be related to phosphorylation events. Release, recycling and synthesis of vesicles are regulated at the post-translational level, in particular by Ca^{2+}-dependent and phosphorylation events (reviewed in Ref. [60]). In view of the amply documented inhibition of PKC-dependent phosphorylation by α-tocopherol, which is presumably due to stimulation of phosphatases [61], vesicular transport may equally be modulated beyond a transcriptional activation of related genes. A few intriguing examples to support this perspective may be compiled: (i) Munc18 controls the function of syntaxin in the fusion machinery in a phosphorylation-dependent manner [62]. Kinases implicated in Munc18 phosphorylation are PKCα [63] and Ca^{2+}-stimulated Cdk5 [64], (ii) Synaptotagmin, a key player in the fast Ca^{2+}-mediated neurotransmitter release, interacts with the SNARE core proteins syntaxin and SNAP25 upon phosphorylation, e.g. by CaMKII, whereas de-phosphorylation is required for reversal of the interaction and the initiation of vesicle recycling [65], (iii) Synapsins, coat proteins on synaptic vesicles [66] of the reserve pool [67], contain a single conserved CaMKI and PKA phosphorylation site. Phosphorylation prevents synapsin binding to phospholipids, which causes dissociation of synapsins from the vesicles, a process implicated in neuronal plasticity [68], (iv) Phosphorylation of NSF, the key regulator in the vesicle recycling process, inhibits its interaction with SNARE-bound SNAPs and, thereby, prevents SNARE complex disassembly (reviewed in Ref. [69]). Dephosphorylation is catalyzed by an internal membrane protein tyrosine phosphatase, PTP-MEG2 and restores SNAP-binding capacity of NSF and, in consequence, maintains the vesicular fusion process [70], (v) α-Tocopherol enhances neuronal plasticity by increasing the expression of PSA-NCAM, the polysialylated form of neuronal CAM, of synaptophysin as well as of synaptic profile density most probably by an inhibition of PKCδ [71]. Modulation of phosphorylation cascades may result in a continuous stimulation of vesicular release and, in turn, lead to transcriptional activation of the machinery which is known to respond to persistent activity with the induction of key components such as syntaxin1B [62,72]. The long time required to observe gene regulatory effects of vitamin E rather supports an indirect effect.

A second alternative may be the stabilization of membranes by α-tocopherol. Vesicular transport is particularly dependent on intact membranes. Impaired mitochondrial membrane stability and function [73] as well as electrophysiological abnormalities of neuronal function [39] have been described in vitamin E-deficient rats. The effects were explained by the protection of unsaturated fatty acids by vitamin E [40], thus, favouring the antioxidant theory, which in fact cannot be completely excluded. NO or H_2O_2, e.g. inhibit exocytosis of platelet and endothelial granules by modulating distinct cysteine residues in Nsf which inhibits its disassembly activity [74].

In sum, the gene expression pattern observed upon supplementation of mice with α-tocopherol strongly suggests an involvement of the vitamin in vesicular transport phenomena and increased stimulated

release of β-hexosaminidase by α-tocopherol in two different cell culture models corroborates this hypothesis. The precise role of α-tocopherol in this context remains to be established. The concerted transcriptional activation of key players of the vesicular transport machinery is not necessarily and not even likely due to binding of the vitamin to specific transcription factors, as it is known for vitamin A and D. This option can, however, not be ruled out either, after tocopherols and tocotrienols have been demonstrated to activate PXR [34]. Irrespective of the mechanistic uncertainties, the data presented here as well as quoted circumstantial evidence open up a novel perspective to unravel the molecular function of α-tocopherol. The proposed role of the vitamin in vesicular transport might indeed have a better chance to explain the known deficiency syndromes than its chemical ability to scavenge radicals: Both prominent vitamin E-deficiency symptoms, disturbed female reproduction and neuronal dysfunction, are not easily explained by unspecific oxidative tissue damage, whereas a compromised vesicular transport would certainly be incompatible with optimal neurotransmission and normal placental development.

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