Non-Antioxidant Activities of Vitamin E

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Abstract: Molecules in biological systems often can perform more than one function. In particular, many molecules have the ability to chemically scavenge free radicals and thus act in the test tube as antioxidant, but their main biological function is by acting as hormones, ligands for transcription factors, modulators of enzymatic activities or as structural components. In fact, oxidation of these molecules may impair their biological function, and cellular defense systems exist which protect these molecules from oxidation. Vitamin E is present in plants in 8 different forms with more or less equal antioxidant potential (α-, β–, γ–, δ-tocopherol/tocotrienols); nevertheless, in higher organisms only α-tocopherol is preferentially retained suggesting a specific mechanism for the uptake for this analogue. In the last 20 years, the route of tocopherol from the diet into the body has been clarified and the proteins involved in the uptake and selective retention of α-tocopherol discovered. Precise cellular functions of α-tocopherol that are independent of its antioxidant/radical scavenging ability have been characterized in recent years. At the posttranslational level, α-tocopherol inhibits protein kinase C, 5-lipoxygenase and phospholipase A2 and activates protein phosphatase 2A and diacylglycerol kinase. Some genes (e. g. scavenger receptors, α-TTP, α-tropomyosin, matrix metalloproteinase-19 and collagenase) are modulated by α-tocopherol at the transcriptional level. α-Tocopherol also inhibits cell proliferation, platelet aggregation and monocyte adhesion. These effects are unrelated to the antioxidant activity of vitamin E, and possibly reflect specific interactions of α-tocopherol with enzymes, structural proteins, lipids and transcription factors. Recently, several novel tocopherol binding proteins have been cloned, that may mediate the non-antioxidant signaling and cellular functions of vitamin E and its correct intracellular distribution. In the present review, it is suggested that the non-antioxidant activities of tocopherols represent the main biological reason for the selective retention of α-tocopherol in the body, or *vice versa*, for the metabolic conversion and consequent elimination of the other tocopherols.

Keywords: Vitamin E (tocopherols, tocotrienols), Tocopherol binding proteins, α-Tocopherol salvage pathway, Chylomicrons, Gene regulation, Non-antioxidant effects, Transport, Analogues, Metabolism.

1. INTRODUCTION

Vitamin E was first described by Evans and Bishop as an essential nutrient for reproduction in rats [1]. The action of vitamin E has been ascribed to its ability to chemically act as a lipid based (lipoprotein and membranes) free radical chain breaking molecule and to exert its action by protecting the organism against the attack of those radical [2-4]. During the last 20 years alternative roles of vitamin E have been proposed that are independent of its radical chain breaking function. Vitamin E has been shown to influence cellular signaling, enzymatic activity and gene expression. Effects of vitamin E have been observed at the level of mRNA or protein and could be consequent to regulation of gene transcription, mRNA stability, protein translation, protein stability and other post-translational events [5-7].

The proposal that vitamin E has, similarly to vitamin A and vitamin D derivatives, cell regulatory properties unrelated to its radical chain braking potential, can be supported by a number of experimental facts. In particular, there is no obvious correlation between the radical chain breaking potency of tocopherols and tocotrienols and their *in vivo* effectiveness [8,9]. In fact, other radical chain breaking molecules are in most cases not effective [10]. Furthermore, the most potent form of natural vitamin E, α-tocopherol, is taken up, transported and retained by the body much more efficiently than the other natural or synthetic derivatives [11,12]. Since they all have equal radical chain breaking properties, it is to date still unexplained why nature selected specifically the α form of tocopherol, and it is an open question whether vitamin E deficiency syndromes could be completely prevented by supplying β- γ- δ-tocopherols or tocotrienols. As discussed below, it can be speculated that α-tocopherol has some specific characteristics; e. g the fully methylated chromanol-head group may be required for optimal interactions with enzymes and/or "α-tocopherol receptors". On the other hand, the β-, γ-, and δ- tocopherols and the tocotrienols may have biological effects that interfere with normal cellular processes, so that they need to be specifically recognized, metabolized by the liver and later eliminated. A unique feature of α-tocopherol is the location of the reactive –OH group between two methyl groups; after reacting with a lipid peroxide the unpaired electron can delocalize over the fully substituted chromanol ring what is known to increase its stability and chemical reactivity [13,14]. As a consequence, α-tocopherol and α-tocotrienol, but not the other forms of tocopherol, can reduce *in vitro*

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0929-8673/04 $45.00+.00 © 2004 Bentham Science Publishers Ltd.

Cu(II) to give Cu(I) together with α-tocopheryl and α-tocotrienyl quinones, respectively, and they can exert pro-oxidant effects in the oxidation of methyl linoleate in SDS micelles [15]. Competition by β-, γ- and δ- tocopherols in reactions that require specifically α-tocopherol may be another reason that only α-tocopherol is retained. α-Tocopherol also forms specific interactions with phospholipids in membranes, but the cellular consequences of such structural alterations are not clear [13,16]. Based on this, it can be assumed that α-tocopherol is selectively recognized, retained in the liver and incorporated in VLDL, brought to peripheral cells in which it is then carried by α-tocopherol transport proteins to organelles, enzymes and receptors, where it acts either as cofactor and/or modulator of specific biochemical reactions.

In the studies discussed here, we focus on the different biological characteristics of the natural and synthetic tocopherol analogues, and attempt to summarize the current knowledge why different tocopherols have unique functions despite equally antioxidant potency. To analyze the non-antioxidant function of vitamin E, it is of paramount importance to discriminate at what level the effects of vitamin E is exerted, whether they are primary effects of a specific modulation of an enzyme by vitamin E, or secondary effects common to many molecules. A good criterion for non-antioxidant effects of vitamin E is their isoform specificity. In the next future, the analysis of specific interactions of vitamin E with proteins (enzymes, transcription factors) and the analysis of cellular effects promises to reveal mechanistic details of non-antioxidant functions of vitamin E.

In the last decade, several proteins have been cloned that recognize vitamin E and may be involved in non-antioxidant effects of vitamin E. A specific evolutionary conserved protein, with the role of selecting α-tocopherol out of other tocopherols, the α-tocopherol transfer protein (α-TTP) [17], regulates the concentration of α-tocopherol in the body. The crystal structure of phospholipase A2 with the inhibitory vitamin E is a strong example of vitamin E/enzyme interaction with regulatory function [18]. Recently, a family of novel tocopherol binding proteins, with possible receptor function, has been discovered [16,19,20]. However, despite the recent developments, the molecular mechanism of non-antioxidant function of vitamin E has not yet been clarified.

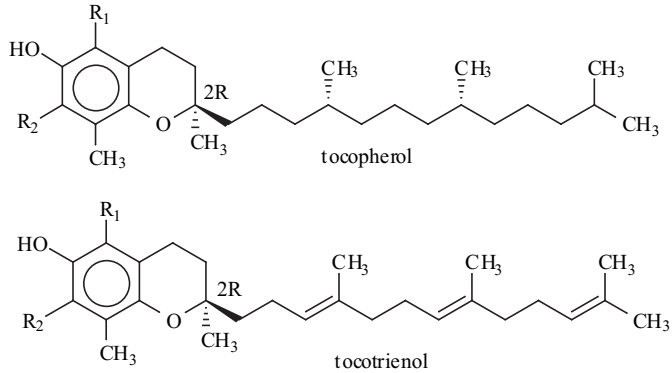
2. VITAMIN E

2.1. Natural Vitamin E

Natural vitamin E comprises 8 different forms, α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol, produced by plants alone. Tocotrienols have an unsaturated side chain, whereas tocopherols contain a phytyl tail with three chiral centres which naturally occur in the RRR configuration (Fig. **1**). Although the overall antioxidant activity of these molecules are more or less similar, clear individual chemical, physical and biological effects can be distinguished at a molecular level.

The free radical scavenging reactivity has been measured as being in the order of $\alpha > \gamma > \beta > \delta$ [21]. The chemical reactivity of the four tocopherols with singlet molecular oxygen ($^1O_2$) has been found to be very low, with $\alpha > \gamma > \delta > \beta$. The physical quenching ability of $^1O_2$ has been measured as being in the order of $\alpha \geq \beta > \gamma > \delta$ [22]. The rather complex physical and chemical properties of tocopherols have been extensively reviewed [14]. The biological potency can be summarized with the order of $\alpha >> \gamma > \delta > \beta$, which is most likely due to the selective retention of α-tocopherol in the liver.

Commercially available vitamin E consists of either a mixture of naturally occurring tocopherols and tocotrienols (from natural sources), RRR-α-tocopherol (formerly called d-α-tocopherol), synthetic α-tocopherol, consisting of the 8 possible side-chain stereoisomers at equal amounts (*all rac*-α-tocopherol, formerly called dl-α-tocopherol), or their esters (α-tocopherylsuccinate, α-tocopherylacetate). Bioavailability and bioequivalence of the different forms of vitamin E differ, what is also taken into account for the determination of total vitamin E activity in food. RRR-α-Tocopherol is the most abundant form in plasma, whereas the plasma γ-tocopherol level is only about 10% of that of α-tocopherol despite that a higher amount of γ-tocopherol is often present in the diet. This specificity is the consequence of a selective retention of RRR-α-tocopherol in the body, or



R_1	R_2	
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Fig. (1). Structure of the eight natural tocopherols. In plant tissues, four tocopherols and four tocotrienols are synthesized, all with a side-chain in the natural RRR configuration (here referred to as α-, β-, γ-, δ- tocopherol/tocotrienol). The relative concentration of the tocopherols and tocotrienols depends on the plant species and on the plant tissue. Some marine organisms contain also α-tocomonoenol, with a single unsaturated bond at the end of the phytyl side chain, which is assumed to be the result of cold-water adaptation [272].

vice versa, to the metabolic degradation of the other tocopherols and their elimination.

2.2. Synthesis of Vitamin E in Plants

Tocopherols, exclusively synthesized in photosynthetic organisms including higher plants, are found in all green tissues but significant amounts are present in seeds. They are supposed to be involved in the response to oxidative stress in plant chloroplasts. Tocopherols in plants are generated from the condensation of phytyldiphosphate and homogentisic acid (HGA), followed by cyclization and methylation reactions. Homogentisate phytyltransferase (HPT) performs the first committed step in this pathway, the phytylation of HGA. Tocopherol methylase converts δ- and γ-tocopherol into β- and α-tocopherol, respectively, but β-tocopherol is not accepted as a substrate, and thus not

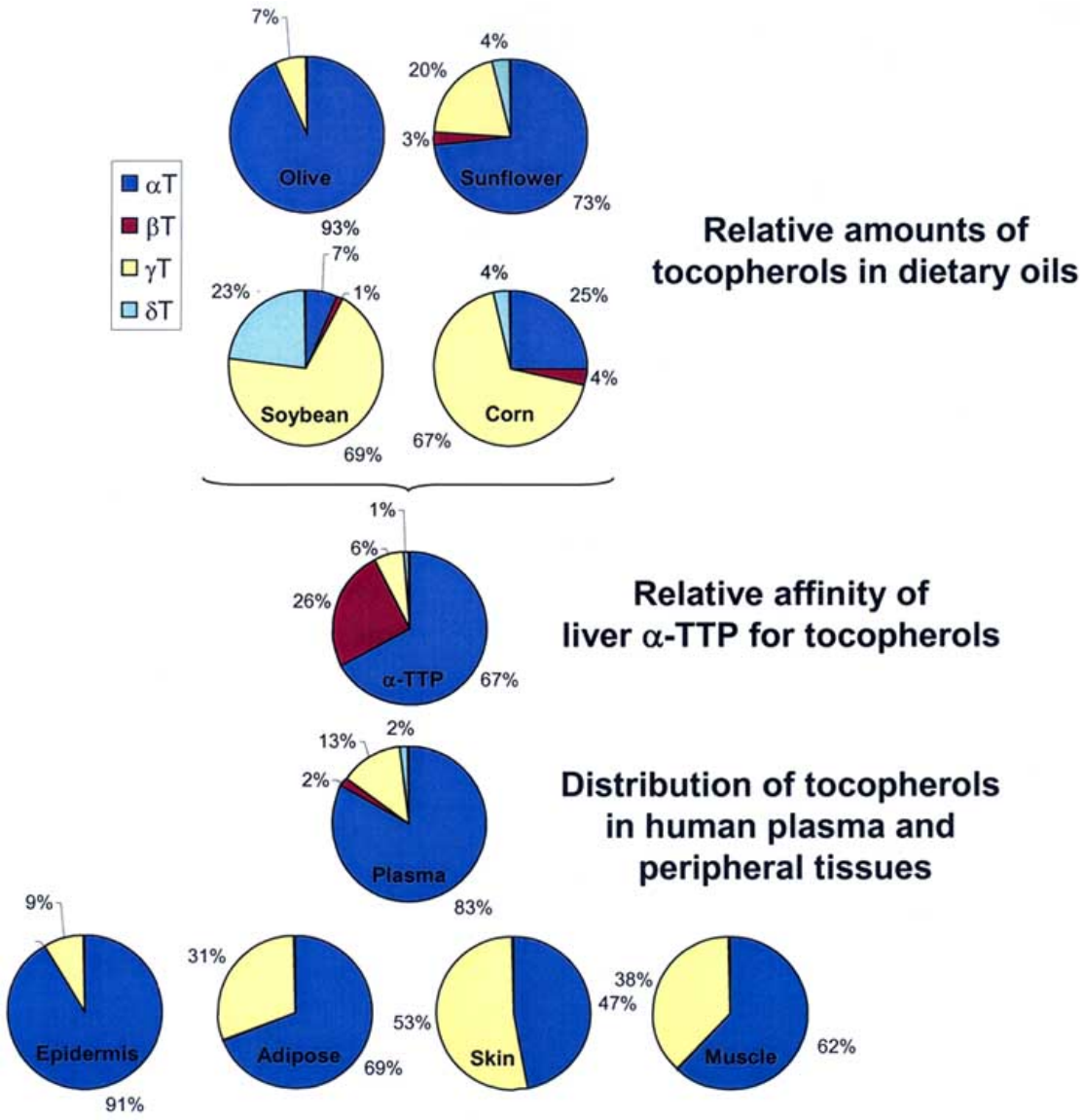


Fig. (2). Tocopherols distribution in commonly used dietary oils. More than 50% of total vitamin E uptake is derived from dietary oils. Olive and sunflower oils (common to European diet) contain mainly α-tocopherol, corn oils (common to US diet) contain mainly γ-tocopherol, and soy oils (common to Asian diet) contain mainly γ- and δ-tocopherol (data from [27,273-275]). With the exception of palm oil having a high level of tocotrienols (13% α-tocopherol, 75% tocotrienols), most oils contain only low amounts of tocotrienols (not shown). However, the uptake and retention of tocotrienols from the human diet is generally low. With the exception of skin with high levels of γ-tocopherol, α-tocopherol is the most abundant tocopherol in plasma and peripheral tissues. The relative amounts of the tocopherols in human plasma and tissues is determined by the amounts in the diet, the transport by chylomicrons (→ chylomicron tocopherol cycle), the relative affinity of liver α-TTP for tocopherols (→ α-tocopherol salvage pathway), the metabolism and the uptake efficiency in different tissues (Fig. **3**).

converted into α-tocopherol [23]. A tocopherol-deficient mutant (vte1-mutant), that lacked all tocopherols due to a deficient tocopherol cyclase activity, was isolated from Arabidopsis thaliana [24]. Growth of the vte1 mutant, chlorophyll content, and photosynthetic quantum yield were similar to wild type under optimal growth conditions. Therefore, absence of tocopherol has no large impact on photosynthesis or plant viability, suggesting that other compounds can compensate for the loss of tocopherol. However, during photo-oxidative stress, chlorophyll content and photosynthetic quantum yield were slightly reduced in vte1 as compared with wild type indicating a potential role for tocopherol in maintaining an optimal photosynthesis rate under high-light stress. Zea mays SXD1 (Sucrose Export Deficient 1) bears a mutation that causes tocopherol deficiency. In the SXD1 phenotype, the additional defective export of sucrose and impaired plasmodesmata formation suggests that, beyond presumed antioxidant activities, tocopherols or tocopherol breakdown products also function as signal transduction molecules [25,26].

2.3. Occurrence of Vitamin E in Food

The eight analogous compounds are widely distributed in nature and the richest sources are latex lipids (~80 mg/g of latex), followed by edible plant oils (Fig. **2**). Sunflower seeds contain almost exclusively α-tocopherol (59.5 mg/g of oil), oil from soybeans contains the γ-, δ-, and α-tocopherol (62.4, 20.4, and 11.0 mg/g oil), while palm oil contains high concentrations of tocotrienols (17.2 mg/g oil) and α-tocopherol (18.3 mg/g oil) [27]. As a result of different amounts of the various tocopherols in the oils and the dietary oil preferences in different countries, the plasma and tissue levels of certain tocopherols can be different. In the US the intake of γ-tocopherol is higher than that of α-tocopherol because of the high intake of corn oil, in Europe the intake of α-tocopherol is higher than that of γ-tocopherol because of high intake of sunflower and olive oil, whereas the higher intake of soybeans in Asia leads to a higher intake of δ-tocopherol. Thus, the interpretation of epidemiological studies from different countries may require taking into account the different situations at the start of the supplementation.

2.4. Vitamin E Analogues

Vitamin E acetate is the most common vitamin E analogue in supplements and creams. The esterification protects vitamin E acetate from oxidation, but in the human body the ester is rapidly cleaved by cellular esterases so that the natural vitamin E is made available. The synthetic racemic tocopherol mixture contains eight different side-chain isomers, the RRR form (natural) and all the others containing S isomers. Some of these natural and the non-natural tocopherol isomers are excluded from the plasma and secreted with the bile [11,12]. Several further analogues of vitamin E, like α-tocopherylsuccinate, α-tocopherylphosphate, trolox and others, have been synthesized and their cellular effects tested [28]. These analogues often act as completely novel molecules, are transported differently and have unique effects on e. g. apoptosis and cellular signaling. It is often unknown to what degree these vitamin E analogues enhance or disrupt pathways that are usually used by the natural tocopherols. Vitamin E has also been modified by coupling it with a lipophilic triphenylphosphonium cation through an alkyl linker to be targeted to mitochondria [29].

3. VITAMIN E UPTAKE AND DISTRIBUTION

3.1 Vitamin E Transport from the Intestine

In man, vitamin E is taken up together with dietary lipids and bile in the proximal part of the intestine with an average efficiency of about 30%. All four tocopherols are taken up equally, suggesting that at this level there is no selectivity. Consequently, a diet rich in γ-tocopherol or δ-tocopherol increases the level of γ-tocopherol in tissues, albeit in most tissues α-tocopherol is the predominant form. Since competition between the tocopherols occurs, relative tissue levels of tocopherols are depending on the relative amount of each tocopherol in the diet [30]. The tocopherols are re-assembled together with triglycerides, cholesterol, phospholipids and apolipoproteins into chylomicrons (Fig. **3**). In the course of chylomicron lipolysis, a part of the vitamin E is distributed to peripheral tissues, and the liver with the chylomicron remnants captures the other part. Lipoprotein lipase increases the transfer of tocopherol from chylomicrons into skeletal muscle cells [31]. Only 35% of α-tocopherol is still present in chylomicrons 2 hours after lymph injection of radioactive α-tocopherol [32]. Apparently, the plasma and tissue levels of the four tocopherols reached by chylomicron transport is not sufficient, since a specific salvage pathway recycles α-tocopherol from the liver into the body and mutation of this pathway leads to vitamin E deficiency syndromes (Fig. **3**). It can be speculated that the evolutionary benefit of selecting α-tocopherol for recycling by the salvage pathway did not originate only from their limited dietary availability or their antioxidant function; for a mere antioxidant function all four tocopherols could have been selected, suggesting other non-antioxidant reasons for the selection of α-tocopherol.

3.2 Vitamin E Salvage Pathway from the Liver

In the liver, α-tocopherol is specifically recognized and retained by the cytosolic 32 kDa α-tocopherol transfer protein (α-TTP), which thus plays an important role in determining the plasma vitamin E level (Fig. **3**). One of the critical determinants of vitamin E biological activity appears to be the affinity of its analogue for α-TTP [33]. The relative affinities for α-TTP calculated from the degree of competition are as follows: RRR-α-tocopherol = 100%, β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; α-tocopherol acetate, 2%; α-tocopherylquinone, 2%; SRR-α-tocopherol, 11%; α-tocotrienol, 12%; trolox, 9%. Presumably, α-TTP functions in the intracellular transport and retention of α-tocopherol in hepatocytes by sorting and incorporating it into VLDL. The secretion of cellular α-tocopherol into extracellular compartments occurs by a reaction that utilizes a novel non-Golgi-mediated pathway [34]. Vitamin E is then transported in the blood by VLDL and delivered to peripheral tissues together with triglycerides and cholesterylesters. Lipolysis of the triglycerides in VLDL

by lipoprotein lipase converts VLDL into LDL and HDL, and a part of α-tocopherol is taken up by endothelial cells together with free fatty acids and monoglycerides. The LDL and HDL fractions combined, contain approximately 90% of the total serum vitamin E in man and α-tocopherol is rapidly exchanged between the lipoproteins [35]. The plasma phospholipid transfer protein facilitates the exchange of tocopherol between LDL and HDL [36]. Another pathway of α-tocopherol uptake occurs by endocytosis of LDL via the LDL receptor.

In lung, HDL is the primary source of vitamin E for type II pneumocytes, and its uptake is regulated by the expression of scavenger receptor SR-BI [37]. In brain, HDL-associated α-tocopherol is selectively transferred into cells constituting the blood brain barrier *via* scavenger receptor SR-BI [38]. The direct uptake of hydrophobic molecules from chylomicrons may be less efficient in the nervous system (central and peripheric), possibly due to an uneven distribution and lower levels of lipoprotein lipase in certain parts of the brain [39,40]. Afamin, a protein related to albumin, specifically binds α-tocopherol and γ-tocopherol, and is assumed to transport and distribute tocopherols in body fluids such as the cerebrospinal or follicular fluids [41]. Mice deficient of SR-BI show a decrease of α-tocopherol levels in the bile and in several tissues including ovary, testis, lung and brain, but not in liver, spleen kidney and white fat, suggesting other uptake mechanisms in these tissues [42]. Similarly, scavenger receptor SR-BI transports HDL-associated α-tocopherol coming from the periphery into the liver, where it is again specifically recognized by α-TTP, recycled and secreted in VLDL [43].

When the α-TTP gene is mutated, α-tocopherol concentrations in serum and peripheral cells are very low, implying that the recycling of α-tocopherol by α-TTP is essential to maintain an adequate amount of tocopherol in the organism. Thus, in addition to the dietary availability of α-tocopherol, the expression level of liver α-TTP protein is a critical determinant of the α-tocopherol level in plasma and peripheral cells. Moreover, studies with patients expressing a mutant α-TTP gene show that after supplementation with tocopherols, peak plasma concentrations occur after 6 hours, after which the level of α-tocopherol rapidly drops [44]. In people with a normal α-TTP gene, peak concentrations are reached after 11 hours and a high concentration of α-tocopherol is maintained over several days [44,45]. These results suggest that the main physiological purpose of the α-tocopherol salvage pathway is to maintain a high and continuous plasma concentration of α-tocopherol. The maintenance of high concentrations of α-tocopherol in plasma protects the lipoproteins (VLDL, LDL, and HDL) from oxidative damage [46]; however, this may not be the only reason of this pathway. Given the neurodegenerative symptoms caused by a deficient α-TTP gene, it can be assumed that the evolutionary benefit of this pathway is to improve the delivery of α-tocopherol to the brain and the peripheral nervous system by maintaining adequate plasma levels of α-tocopherol. Other tissues, such as muscle tissues, may be less affected by vitamin E deficiency since they may receive sufficient tocopherols via the chylomicron tocopherol cycle, whereas the brain may rely more on VLDL/HDL and not chylomicrons as source for α-tocopherol. This is confirmed in α-TTP knockout mice, in which the delivery of α-tocopherol is lowest for brain and spinal cord (2.5% of normal), whereas other tissues (liver, skin, adrenal gland, muscle and others) can maintain a significant level of α-tocopherol (10-30% of normal) despite of the absence of a functional α-tocopherol salvage pathway [47].

In normal tissues, the highest contents of α-tocopherol are found in adipose tissue (150 μg/g tissue) and the adrenal gland (132 μg/g tissue), other organs like kidney, heart or liver contain between 7 and 40 μg/g tissues, and erythrocytes have a relatively low content (2 μg/g tissue) [48,49]. These differences in the amount of α-tocopherol suggest tissue specific mechanisms for enrichment and/or storage of vitamin E.

Mutations in α-TTP result in a familial disease, ataxia with vitamin E deficiency (AVED), associated with low levels of α-tocopherol in plasma and symptoms closely resembling those of Friedreich's ataxia [50]. A point mutation in four independent ataxia patients at position 101 of the α-TTP gene resulted, in three of them, in the development of retinitis pigmentosa subsequent to the onset of ataxia. Administration of vitamin E appeared to halt the progression of visual and neurological symptoms [50]. Gene analysis in another patient identified two point mutations in exon 1 of α-TTP gene, one missense mutation and a mutation upstream of the initiation codon in the 5'-untranslated region (Kozak sequence). The latter mutation is the first one identified in the translation regulatory region and is able to decreases the level of α-TTP protein expression. The clinical features include uncommon urinary disturbance, deafness and retinitis pigmentosa. Supplemental therapy increases serum vitamin E concentration to the normal range with mild improvement of the neurological symptoms [51].

The homozygote knockout male mice for α-TTP [52] are fertile, but have increased brain lipid peroxidation and develop neurological symptoms similar to AVED after 1 year [53]. Placentas of pregnant α-TTP$^{(-/-)}$ females are severely impaired, with marked reduction of labyrinthine trophoblasts, and the embryos die at mid-gestation even when fertilized eggs of α-TTP$^{(+/+)}$ mice are transferred into α-TTP$^{(-/-)}$ recipients. The use of excess α-tocopherol dietary supplement by α-TTP$^{(-/-)}$ females prevents placental failure and allows full-term pregnancies. It is unknown whether supplementation with excess amounts of β- γ- or δ-tocopherol could also prevent vitamin E deficiency in these animals. In α-TTP$^{(+/+)}$ animals, α-TTP gene expression is observed in the secretory columnar epithelial cells of the uterus and in placental trophoblasts, and its level transiently increases after implantation (4.5 days post-coitum) [54,55]. These results suggest that vitamin E is needed in the labyrinth region of the placenta during development and that in addition to the hepatic α-TTP, which governs plasma α-tocopherol level, the uterine α-TTP may be of evolutionary importance by supplying this vitamin to the embryo [52].

Two reports indicate that the protein levels of α-TTP in the liver are lowered by vitamin E deficiency [56] and that α- and δ-tocopherol induce expression of hepatic α-TTP mRNA [57]. An increased expression of α-TTP during rat neonatal liver development has also been reported [58,59]. A correlation between α-TTP expression and tocopherol blood

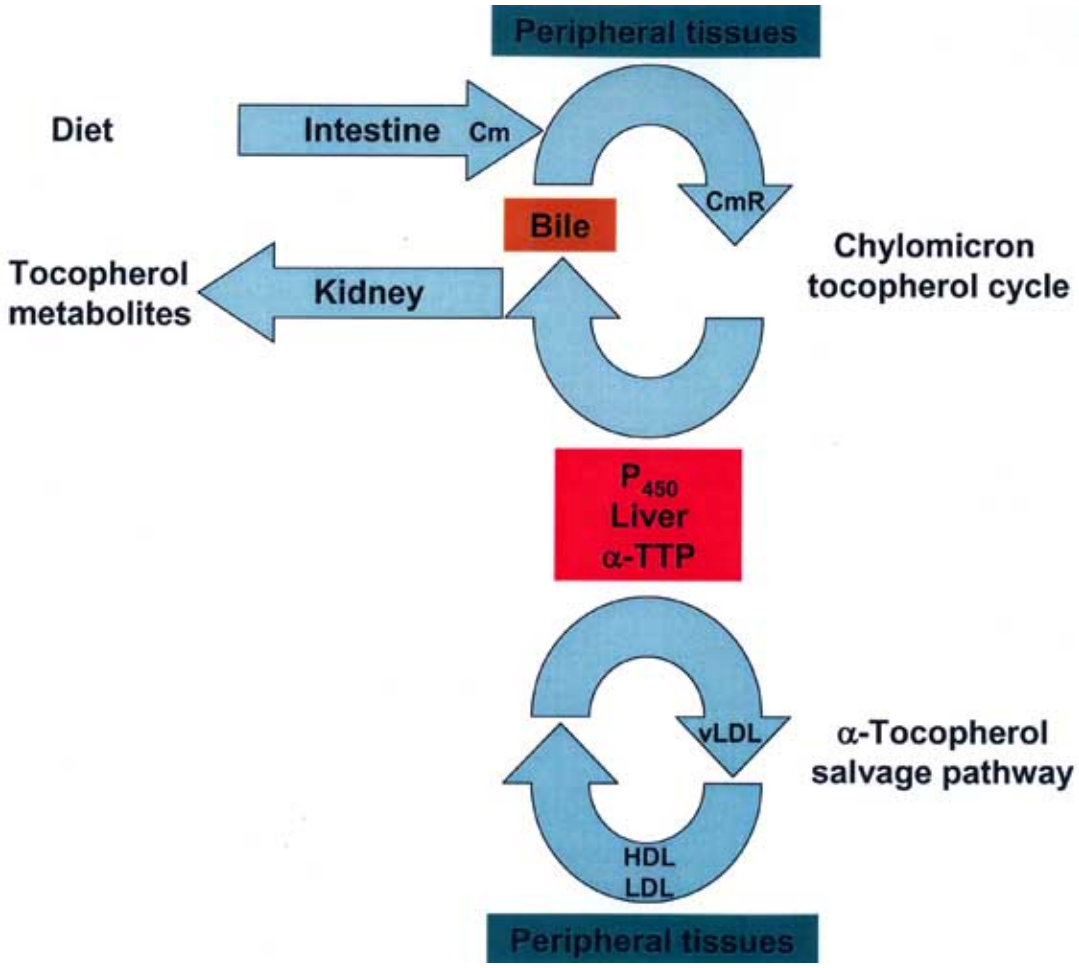


Fig. (3). Vitamin E uptake and metabolism. **Chylomicron tocopherol cycle and tocopherol metabolism**: The four tocopherols are taken up with equal efficiency (average 30%) via the intestine and distributed to peripheral tissues by chylomicrons (Cm). In the course of chylomicron lipolysis, a part of the vitamin E is distributed to peripheral tissues, and the liver with the chylomicron remnants captures the other part. Chylomicron remnants (CmR) are taken up by the liver; the α-tocopherol is recognized, sorted and secreted with VLDL. The remaining tocopherols and excess α-tocopherol is metabolized by the cytochrome P_{450} (CYP3A) enzyme, a phase I enzyme recognizing foreign compounds [72]. Thus, higher amounts of β- γ- δ-tocopherols may be recognized as "foreign", possibly since they have cellular effects that interfere with the normal cellular behavior. Alternatively, as explained in the text, the metabolites of tocopherol may have specific cellular roles. Since they are water soluble they are cleared by the kidney. Part of the liver tocopherols (up to 14%) is also secreted with bile, up to 60% of biliary α-tocopherol is reabsorbed, thus possibly undergoing a second chylomicron cycle [32,276]. Since the chylomicron tocopherol cycle is not specific for tocopherols, the uptake via this route is dependent on the dietary tocopherol composition. As described in figure 2, dietary oils are the main source of vitamin E, thus the oil source determines which tocopherol is transported by the chylomicron tocopherol cycle.

α-Tocopherol salvage pathway: In the liver α-TTP selectively recognizes α-tocopherol and incorporates it into VLDL. During circulation in the blood, VLDL converts to LDL and HDL and delivers its content including the α-tocopherol to the peripheral tissues. Excess tocopherols are transported back in LDL and HDL to the liver, where they undergo the next round in this cycle. Since the presence of α-tocopherol in chylomicrons after dietary uptake is transient, the α-tocopherol salvage pathway may allow maintaining a continuous increased level of α-tocopherol in the plasma. In particular, given the neurodegenerative symptoms caused by a deficient α-TTP gene, the key player of the α-tocopherol salvage pathway, it can be assumed that the main evolutionary benefit of this pathway is to improve the delivery of α-tocopherol to the brain and the peripheral nervous system by maintaining adequate levels of α-tocopherol in plasma VLDL and HDL. Other tissues, such as muscle tissues, may be less affected by vitamin E deficiency since they may receive sufficient tocopherols via the chylomicron tocopherol cycle, whereas the brain may rely more on HDL/VLDL and not chylomicrons as source for α-tocopherol.

level has also been described during hepatocarcinogenesis; repressed transcription of α-TTP is associated with a decrease of serum α-tocopherol and with hepatic carcinogenesis hepatic carcinogenesis [60]. Hyperoxia decreased α-TTP expression at the mRNA but not at the protein level. These findings indicate that mRNA expression of hepatic α-TTP may be responsive to oxidative stress [61].

3.3 Intracellular Distribution of Vitamin E

As described above, vitamin E in HDL is absorbed *via* scavenger receptors or endocytosed via LDL receptors. Since vitamin E is water insoluble, the intracellular distribution must either occur in the membrane of vesicles, or bound to specific transport proteins. Higher concentrations of vitamin E have been described in particular organelles, such as Golgi, lysosomes, and mitochondria. Most α-tocopherol is located in the mitochondrial fractions and in the endoplasmic reticulum, whereas little is found in cytosol and peroxisomes [62]. In mitochondria, more α-tocopherol is found in the inner membrane (83.7 %) than in the outer membrane (14.3 %) [63]. α-TTP is mainly expressed in liver, low levels are expressed in brain [64], in the retina [50], lymphocytes and fibroblasts [65]. Moreover, α-TTP expression in the labyrinthine trophoblast region of the placenta plays an important role in supplying the vitamin to the fetus, and explains the fetal resorption occurring in rats fed a vitamin E deficient diet [52].

The low level or absence of α-TTP expression in most tissues suggests that α-TTP cannot participate in the intracellular distribution in other tissues. Since in human endothelial cells and RAW 267.7 macrophages in culture α– and γ-tocopherol are differently taken up, it seems probable that the cellular transport in peripheral cells is also specific and requires tocopherol specific transporters and receptors [66]. As to the intracellular redistribution of α-tocopherol, a 14.2 kDa tocopherol binding protein (TBP) has been described to enhance up to 10 fold the transport of α-tocopherol to the mitochondria [67]. The hTAP proteins are candidates for the intracellular distribution of vitamin E, since they are ubiquitously expressed, can bind natural tocopherol analogues with reasonable affinity, and carry a carboxy-terminal GOLD domain which in other proteins (GCP60, PAP7) is known to serve as adaptor for binding to Golgi giantin or to the mitochondrial peripheral benzodiazepine receptor [20,68]. These proteins may take up tocopherols from endocytosed LDL and distribute them to specific organelles, metabolizing enzymes or possibly to receptor proteins. Non-natural tocopherol analogues that are better water soluble are easily taken up into cells, and specific analogues have been described with improved function due to targeting to mitochondria and cancer cells [29].

3.4. Cellular Export of Vitamin E

Apart from the α-TTP-mediated VLDL incorporation and export, secretion of α-tocopherol from cultured cells is also mediated by ABCA1, an ATP-binding cassette protein that transports cellular cholesterol and phospholipids to lipid-poor high density lipoprotein (HDL) and apolipoproteins such as apoA-I [69]. Induction of ABCA1 expression with cholesterol and/or 8-bromo-cyclic AMP enhanced apoA-I-mediated α-tocopherol efflux. Moreover, apoA-I were unable to remove α-tocopherol from Tangier disease fibroblasts that have a non-functional ABCA1. Cells lacking an active ABCA1 pathway markedly increased secretion of α-tocopherol to apoA-I after overexpression of ABCA1. ABCA1 only mediated a fraction of the α-tocopherol efflux promoted by lipid-containing HDL particles, indicating that HDL promotes efflux by both ABCA1-dependent and -independent processes. In analogy to the α-tocopherol enrichment by α-TTP during assembly of VLDL, the hTAP proteins may enrich lipoproteins with the four tocopherols, leading to export of the tocopherols in chylomicrons and HDL for distribution to tissues or in HDL for transport from peripheral tissues to the liver. Secretion of α-tocopherol may also facilitate vitamin transport between different cells in a tissue, however, it is unknown whether other tocopherol analogues are also exported [69].

3.5 Cellular Metabolism of Vitamin E

The selectivity of higher organisms for α-tocopherol has been impressively demonstrated in recent years by analysing the metabolism of vitamin E. Excess α-tocopherol and the other tocopherols are extensively metabolized before excretion, meaning that the organism maintains the correct vitamin E level by selective retention of α-tocopherol, and by specific metabolism of all the other tocopherols and of the excess α-tocopherol. Initially, two major metabolites of α-tocopherol, the so-called Simon metabolites (tocopheronic acid and tocopheronolactone) were described [70,71], which are excreted in the urine as glucuronides or sulfates. These metabolites have a shortened side chain and an opened chroman structure and are often quoted to demonstrate the antioxidant function of α-tocopherol *in vivo*. The level of these metabolites increases markedly in the urine of healthy volunteers after a daily intake of 2-3 g *all rac*-α-tocopherol.

However, vitamin E metabolism in humans was recently re-analyzed and a novel pathway of tocopherol metabolism was found (reviewed in [72]). Instead of Simon-metabolites, a compound with a shortened side chain but an intact chroman structure, α-carboxyethyl hydroxychroman (α-CEHC), was identified after supplementation with RRR-α-tocopherol [73]. The intact chroman structure of CEHCs suggests that they are derived from tocopherols which have not reacted as an antioxidant. This metabolite is analogous to that of δ-tocopherol found previously in rats [74] and that of γ-tocopherol identified in human urine and proposed as a natriuretic factor [75]. The proposed pathway of side-chain degradation proceeds first *via* ω- and then β-oxidation [74]. The initial step, the ω-hydroxylation of the side chain is catalyzed by the action of cytochrome P_{450} (CYP)-dependent hydroxylases. Inhibitors of the CYP3A family, like sesamin and ketoconazole, inhibit the formation of γ-CEHC, and dietary intervention with sesam oils in humans leads to increased serum γ-tocopherol levels [76,77]. Induction of CYP3A by rifampicin results in an increase of the α-tocopherol metabolites in HepG2 cells [78]. α-CEHC excretion was increased with increasing vitamin E intake after a threshold of plasma α-tocopherol had been exceeded [73].

CEHC accumulation may mediate anti-inflammatory and antioxidative effects or have other regulatory properties [79]. The metabolite of γ-tocopherol, γ-CEHC has natriuretic activity, and thus act as a "natriuretic hormone". γ-CEHC acts by inhibition of the 70 pS potassium channel of the thick ascending limb of the loop of Henle and not by inhibiting the Na^+/K^+-ATPase. The analogous α-tocopherol metabolite showed no inhibition [80]. Both γ-tocopherol

and γ-CEHC inhibit cyclooxygenase-2 (COX-2) activity and thus inhibit the synthesis of prostaglandin E2 (PGE2) in activated macrophages and epithelial cells [81,82]. In carrageenan-induced inflammation in male Wistar rats, administration of γ-tocopherol or γ-CEHC, but not α-tocopherol, reduces PGE2 synthesis at the site of inflammation, and inhibits leukotriene B4 formation, a potent chemotactic agent synthesized by the 5-lipoxygenase of neutrophils [83].

Interestingly, it was also found that vitamin E activates the human Pregnane X Receptor (PXR) in a tocopherol specific manner: α-tocopherol activates weakly, whereas β-, γ-, and δ-tocopherol and the tocotrienols lead to stronger induction, whereas the tocopherol metabolic products do not activate. PXR is involved in the drug hydroxylation and elimination pathways, it activates genes such as cytochromes P_{450} (CYP), e.g. CYP3A and some ABC Transporters [84].

A physiological reason for the selective retention of α-tocopherol and the elimination of all the others tocopherol analogues could thus be the absence of strong PXR activation by α-tocopherol and the consequent absence of induction of enzymes involved in its metabolism. In addition to that, α-tocopherol may be specifically sorted by α-TTP into vesicles destined for incorporation into VLDL. Only when the level of α-tocopherol exceeds the capacity of α-TTP, transport to the metabolic enzymes may occur. The other tocopherols are not retained by α-TTP, activate PXR and then become metabolized and eliminated by CYP3A. In this context it is interesting to compare the metabolism of tocopherol with that of retinoic acid. Retinoids occur bound to several retinoic-acid binding proteins (CRABP and CRBP), which are expressed at concentrations that exceed those of their ligands and which are believed to solubilize and stabilize their hydrophobic ligands in the aqueous phase. These proteins are assumed to restrict retinoid access to specific enzymes and thus generate specificity to the biosynthesis, metabolism and action of retinoids [85,86]. Tocopherol binding proteins, such as α-TTP and hTAPs, could perform a similar function, by generating specificity to the metabolism and action of tocopherols.

It is furthermore possible that the eliminated tocopherols or their derivatives have undesired effects at the concentration reached normally by α-tocopherol, or have homology to compounds that need to be eliminated. δ-Tocopherylquinone and γ-tocopherylquinone, but not α-tocopherylquinone, are cytotoxic in smooth muscle cell culture, acute lymphoblastic leukaemia (ALL) cells and AS52 cells [87,88]. Apoptosis is induced via caspase-9 activation and cytochrome c release by γ-tocopherylquinone in HL-60 cells and colon adenocarcinoma WiDr cells [89]. Furthermore, γ-tocopherylquinone is highly mutagenic in AS52 cells whereas α-tocopherylquinone is not, possibly giving an evolutionary advantage to organisms limiting γ-tocopherol, the precursor of γ-tocopherylquinone [88]. The reduction of α-tocopherylquinone to α-tocopherylhydroquinone occurs either via NADPH-cytochrome P-450 reductase [90], NAD(P)H:quinone oxidoreductase 1 [91,92] or ascorbate [92], for the other tocopherols these pathways have not been tested. The tocopherylhydroquinones can regenerate the tocopheroxylradical and thus preserve α-tocopherol with different efficiencies (α > β > γ-tocopherylhydroquinone) [93].

Alternatively, the eliminated tocopherols could bind and modulate a similar class of receptors like PXR and affect the expression of genes in a non-physiological manner (see below). A pharmacophore that represents key features of ligands to the PXR receptor suggests that some receptors can be activated by a number of molecules with similar structure [94].

4. MOLECULAR ACTION OF VITAMIN E

4.1. Antioxidant Effects

The antioxidant effects of α-tocopherol have been the subject of a number of reviews [6,95-101]. However, more recently interest is being developed around the novel concept that α-tocopherol is provided with more and more specific non-antioxidant functions. The following paragraphs give detailed illustrations of the non-antioxidant functions of vitamin E.

4.2. Pro-Oxidant Properties of α-Tocopherol

Although vitamin E has been described in numerous publications to act as an antioxidant, it has also been shown that lipid peroxidation of LDL is faster in the presence α-tocopherol, and is substantially accelerated by enrichment of LDL in vitamin E, either *in vitro* or *in vivo* [102,103]. It was thus proposed that peroxidation is propagated within lipoprotein particles by the vitamin E radical (*i.e.* α-tocopheroxyl radical) unless it became reduced by vitamin C or ubiquinol-10 [104]. However, it is not clear whether the pro-oxidation reactions of α-tocopherol are relevant *in vivo*, under physiological conditions.

4.3. Antialkylating Properties of α-Tocopherol

Nitric oxide released by macrophages during inflammation reacts with active oxygen to form peroxynitrite. Peroxynitrite nitrates proteins and peroxidizes lipids. γ-Tocopherol (the principal form of vitamin E in the United States diet) and α-tocopherol (the major form present in the European diet and in supplements), both protect against peroxynitrite-induced lipid oxidation [105]. γ-Tocopherol inhibits lipid hydroperoxide formation in liposomes more effectively than α-tocopherol by a non-antioxidant mechanism [106]. However, γ-tocopherol scavenging becomes significant only after α-tocopherol depletion, since α-tocopherol is able to attenuate nitration of γ-tocopherol and tyrosine, which are both susceptible to peroxynitrite attack. This would imply that, given their relative *in vivo* concentrations, γ-tocopherol alone is insufficient to remove any peroxynitrite-derived reactive nitrogen species [107].

4.4. Non-Antioxidant Effects of Vitamin E

The mechanism by which vitamin E produces cellular events could be in principle related to the known radical

chain breaking properties of the molecule. This would imply that regulation of certain cellular functions is controlled by the production and elimination of lipid soluble free radicals and that vitamin E serves as a radical scavenger. The biological difficulty of controlling the propagation of radical chain reactions makes this mechanism improbable. Furthermore, if this were the mechanism of action of tocopherols, other similar radical chain braking molecules, and in particular the eight natural tocopherol analogues, would act analogously: this is however often not the case. Thus, it can be assumed that α-tocopherol modulates cellular behavior by specific interactions with enzymes, structural proteins, lipids and transcription factors. Similarly, troglitazone, an antidiabetic drug of the thiazolidinedione class, acts as an insulin sensitizer and improves hyperglycemia. Structurally, it contains a α-tocopherol moiety similar to vitamin E and has been shown to have antioxidant properties *in vitro*; nevertheless, the main therapeutic effect occurs *via* binding to the peroxisome proliferator activated receptor gamma (PPARγ) [108]. A second example is the estrogen 17-β-estradiol, which has antioxidant capacity which has been shown to protect women from coronary artery disease [109]; however its action as a hormone is not mediated by its antioxidant activity but rather by binding to the estrogen receptor [110]. All-trans-retinol is again a potent antioxidant [111], but the main function of retinol in rhodopsin and vision is not related with this property.

Given the non-antioxidant regulatory function of vitamin E, it would be inefficient to consume it as a radical scavenger. Rather, it would be important to protect vitamin E through a network of cellular antioxidant defenses, such as catalases, superoxide dismutases, ascorbate, glutathione, α-lipoic acid etc., similarly to what occurs with proteins, nucleic acids, hormones and lipids. In the following we will focus on non-antioxidant cellular properties of vitamin E, the antioxidant actions of vitamin E have been extensively reviewed [6,95-101].

4.4.1 Modulation of Enzymatic Activity by Vitamin E

Over the last decade, vitamin E has been shown to have specific effects on cellular signalling and gene regulation [5,6]. The main effects on signalling at the enzymatic level are inhibition of PKC activity, modulation of phospholipase A2 activity [112] and inhibition of cyclooxygenase-2 activity [113]. In many situations, only α-tocopherol has been checked, and it is unclear whether other tocopherols or tocotrienols work equally well. In other experiments, the effects of vitamin E have been only tested in the test tube, and needs to be confirmed *in vivo*.

4.4.2 Inhibition of Protein Kinase C

In a first study, Vitamin E (dl-α-tocopherol) was found to inhibit *in vitro* brain protein kinase C (PKC) at a concentration that can be found in cells. It thus appeared that vitamin E, in addition to its antioxidant function, plays a role in regulating the activity of PKC [114]. In 1991 inhibition of PKC activity was found to be at the basis of the vascular smooth muscle cell growth arrest induced by α-tocopherol [115,116]. A number of reports have subsequently confirmed the involvement of PKC in the effect of α-tocopherol on different cell types, including monocytes, macrophages, neutrophils, fibroblasts and mesangial cells [117-120]. α-Tocopherol, but not β-tocopherol, was found to inhibit thrombin-induced PKC activation and endothelin secretion in endothelial cells [121]. α-Tocopherol, and not β-tocopherol or trolox, inhibits the activity of PKC from monocytes, followed by inhibition of phosphorylation and translocation of the cytosolic factor $p47^{(phox)}$ what leads to an impaired assembly of the NADPH-oxidase and lowers superoxide production [122].

Inhibition of PKC by α-tocopherol in vascular smooth muscle cells is observed to occur at concentrations of α-tocopherol close to those measured in healthy adults [123]. β-Tocopherol *per se* is ineffective but prevents the inhibitory effect of α-tocopherol. The mechanism involved is not related to the radical scavenging properties of these two molecules, which are essentially equal [124]. *In vitro* studies with recombinant PKC have shown that inhibition by α-tocopherol is not caused by a tocopherol-protein interaction and also not by affecting PKC expression. Inhibition of PKC activity by α-tocopherol occurs at a cellular level by producing dephosphorylation of the enzyme, whereby β-tocopherol is much less potent [125,126]. Dephosphorylation of PKC occurs *via* the protein phosphatase PP_2A, that has been found to be activated by the treatment with α-tocopherol [125-128]. In normal mammary epithelial cells, tocopherols and tocotrienols inhibit activation of PKCα by epidermal growth factor (EGF) *via* reduction of PKCα translocation to the membrane [129]. PMA induced phosphorylation of extracellular signal-regulated kinase (ERK) is inhibited by α-tocopherol in bovine pulmonary artery smooth muscle cells but not in HL-1 human cardiac muscles cells [130]

The group of King [131] has reported that prevention of glomerular dysfunction in diabetic rats can be achieved by treatment with α-tocopherol. Such a protection occurs through inhibition of PKC, which is activated by high glucose levels. In this case, however, α-tocopherol would act on the diacylglycerol pathway, by activating the enzyme diacylglycerol kinase with consequent diminution of diacylglycerol and PKC activation. In these studies, high glucose was responsible for increased diacylglycerol synthesis, which was counteracted, in the presence of α-tocopherol, by the activation of diacylglycerol kinase. The stimulation of diacylglycerol (DAG) kinase activity by vitamin E, and the consequent suppression of DAG by conversion to phosphatitic acid, may also contribute to the inhibition of PKC in some experimental systems [132]. It remains to be clarified why in the first experiment [114] dl-α-tocopherol was found to inhibit *in vitro* brain PKC while in later experiments the effect on PKC was shown to be mediated by the activation of the phosphatase PP2A. The answer to this question comes from the nature of the "PKC" used by Mahoney and Azzi [114], not a pure protein but a crude PKC enriched fraction. It is probable that this fraction contained also PP2A and that this was the target of the action of α-tocopherol (Azzi, unpublished).

4.4.3. Inhibition of Phospholipase A2

One of the most important functions of phospholipase A2 is the release of arachidonic acid from membrane phospholipids for the synthesis of biologically active eicosanoids. Tocol inhibits the enzyme to a greater extent

than either d- or dl-α-tocopherol, while there is little or no effect from dl-α-tocopherol acetate. These results emphasize the importance of the hydroxyl moiety of the chromanol of the vitamin E molecule for its inhibitory action, compared to that of the methyl groups which are absent in tocol. This inhibitory action of vitamin E on platelet phospholipase A2 suggests a crucial function for vitamin E in regulating arachidonate release from the membrane phospholipids and its subsequent metabolism [112,133]. Co-crystallization of α-tocopherol and phospholipase A2 showed direct binding of α-tocopherol to the enzyme [18].

α-Tocopherol also enhanced the release of prostacyclin from human endothelial cells *via* stimulation of phospholipase A2 [134]. This observation is in contrast to the role of tocopherol, which has been shown to inhibit platelet and cardiac phospholipase A2 activity in rats, and to reduce thrombin-stimulated thromboxane release in rat platelets.

4.4.4. Inhibition of Cyclooxygenase and 5-Lipoxygenase

Cyclooxygenase-2 (COX-2)-catalyzed synthesis of prostaglandin E(2) (PGE(2)) plays a key role in inflammation and its associated diseases, such as cancer and vascular heart disease. Both γ-tocopherol and γ-CEHC, but not α-tocopherol, inhibit cyclooxygenase activity and, thus, possess anti-inflammatory properties. COX-2 activity is directly inhibited by γ-tocopherol and not the result of changes of protein expression or substrate availability, and appears to be independent of antioxidant activity [81-83]. α-Tocopherol activates mouse BV-2 microglial PP2A activity and thereby silences a LPS-activated PKC/ERK/NF−κB signaling cascade resulting in significant attenuation of COX-2 synthesis. These *in vitro* results suggest that α-tocopherol could slow down pathways that are associated with acute or chronic inflammatory conditions in the central nervous system [135]. Vitamin E also plays a role in the posttranslational events related to the age associated enhancement of COX-2 activity [136,137]. Thus vitamin E reverses the age-associated increase in macrophage PGE2 production and COX activity. Vitamin E exerts its effect post-translationally, by inhibiting COX activity.

In activated human monocytes, α-tocopherol inhibits the release of the proinflammatory cytokine, IL-1β, *via* inhibition of the 5-lipoxygenase pathway [138]. A similar antioxidant, β-tocopherol, has no effect on IL-1β release. The protein kinase C inhibitor, bisindolylmaleimide, does not inhibit IL-1β release from activated monocytes, in spite of a-tocopherol decreasing protein kinase C activity, suggesting additional pathways affected by vitamin E. α-Tocopherol has no effect on IL-1β mRNA levels or stability, suggesting a posttranscriptional effect [138].

Purified α-tocopherol, but not β-, γ-, or δ-tocopherol, most remarkably induces macrophage fusion. This is not observed with similar antioxidants such as probucol or Trolox, suggesting that the α-tocopherol effects are independent of its antioxidant activity. This study indicates a novel role for α-tocopherol, as a highly potent macrophage fusion factor, with possible beneficial effects during chronic inflammation [139].

4.4.5. Inhibition of Glutathione S-Transferase Isoforms

The glutathione S-transferases GSTs perform important cellular detoxification function since they conjugate various electrophiles with glutathione. The GSTs are a diverse superfamily, and in mammals the cytosolic GSTs have been grouped into the A, Mu, Pi, Sigma, Theta and Zeta classes [140]. The Pi class is the most abundant isozyme in many tissues and is mostly expressed in tumor tissues, where it may contribute to resistance against cytostatic drugs. Therefore, compounds that inhibit GST activity in cancer cells could be used as adjuvant in cancer therapy. α-Tocopherol inhibits the GST P1-1 most efficiently, probably by binding to a lipophilic pi-like structure in the enzyme; other isoforms (A1-1, M1a-1a, A2-2) are less efficiently inhibited [141]. α- and γ-Tocotrienols, which accumulate specifically in skin (up to 13%), can also inhibit GST P1-1 [142].

4.4.6. Inhibition of NADPH-Oxidase

The NADPH-oxidase system in phagocytic cells is an electron transport system that catalyzes the reduction of O_2 to $O_2^{-\cdot}$, a process not only thought to be part of the antibacterial defenses but to contribute also to chronic inflammatory processes, including scleroderma, liver fibrosis and neurodegeneration. Activation of NADPH-oxidase requires the assembly of a multiprotein complex at the plasma-membrane. The assembly of NADPH-oxidase is inhibited by tocopherol *via* inhibition of PKC, suggesting that tocopherol may reduce scleroderma and liver fibrosis by reducing the production of superoxide by NADPH-oxidase [122]. Monocyte superoxide, in high glucose media, is released by the NADPH-oxidase but not by the mitochondrial respiratory chain, and α-tocopherol inhibits superoxide release *via* inhibition of PKC-α. PKC-α inhibition attenuates $p47^{(phox)}$ membrane translocation and phosphorylation [122,143], two events necessary for NADPH assembly and activation. In microglia cells, α-tocopherol inactivates PKC *via* phosphatase-mediated pathway (PP1 or PP2A) and, as a consequence, blocks the phosphorylation-dependent translocation of $p67^{(phox)}$ to the plasma membrane. As a result, the production of $O_2^{-\cdot}$ by the microglial NADPH-oxidase system is substantially inhibited, offering a partial explanation for the beneficial effect of α-tocopherol on a variety of neurodegenerative diseases [135]. Similarly, in alcoholic liver disease, NADPH-oxidase-derived free radicals are key oxidants [144], and inhibition of NADPH-oxidase by diphenyleneiodonium sulfate prevents early alcohol-induced liver injury in the rat [145]. Therefore, α-tocopherol could have beneficial effects in liver fibrosis by reducing NADPH-oxidase activity. Interestingly, the assembly of NADPH-oxidase is mediated by activation of phosphatidylinositol–3-kinase (PI3-kinase), which recruits the subunits to the plasma membrane [146]. At least *in vitro*, α-tocopherol modulates via hTAP proteins PI3-kinase activity [147], suggesting that α-tocopherols may also be involved in the early activation steps of the PI3-kinase/NADPH-oxidase pathway.

4.4.7. Vitamin E as a Coenzyme for Mitochondrial Fatty Acid Desaturase?

Three papers speculate that the semiquinone radical of α-tocopherylquinone functions as an essential cofactor for the fatty acid desaturases of the carnitine-dependent, channeled, mitochondrial desaturation-elongation pathway [148-150]. An efficient transport system for tocopherol or tocopherylquinone into mitochondria may be required for

Table 1. Modulation of Gene Expression by Natural Vitamin E

GENE	PATHWAY	CELL LINE/TISSUE	EFFECT	REFERENCE
CD36		smooth muscle cells, monocytes/macrophages	↓ αT	[165]
SR-BI		monocytes/macrophages	↓ αT	[37]
SR-AI/II		monocytes/macrophages	↓ αT	[277]
Tropomyosin		smooth muscle cells	↑ αT	[158]
Collagen α1(1)	ARE	liver stellate cells	↓ αT	[161,162]
MMP-1	PKC	fibroblasts	↓ αT	[159]
MMP-19	PKC	PBMC, HL-60	↓ αT	[173]
E-selectin	NF-κB	human endothelial cells	↓ αT	[278]
VCAM-1		THP-1 monocytes	↓ αT	[231]
ICAM-1		keratinocytes, neutrophils, endothelial cells, monocytes	↓ αT	[279-282]
Integrins		human erythroleukemia cells (HEL)	↓ αT	[170]
Glycoprotein IIb	PKC	platelets	↓ αT	[160]
CTGF	TGF-β-RE	smooth muscle cells, fibroblasts	↑ αT	[164]
Il-2		mouse T cells	↑ αT	[283]
IL-4	NF-κB, AP-1	human T cells	↓ αT	[284]
IL-1β		THP-1 monocytes	↓ αT	[171]
TGF-β		rat liver	↓ αT	[163]
α-TTP		liver	↑αT, δT	[57]
Cytochrome P_{450} (CYP3A)	PXR/RXR	HepG2	_↑ βT,γT, δT, δTT	[84]
Bcl2-L1		rat liver	↑ αT	[198]
p27		LNCaP, PC-3	↑ αT	[186]
γ-Glutamyl-cysteine sythetase heavy subunit		HaCat keratinocytes, rat liver	↑ αT	[198,285]
Cyclin D1		DU-145, rat liver	↓ αT, γT	[175,198]
Cyclin E		DU-145	↓ αT, γT	[175]
LDL receptor		HepG2	↑ αT ↓ γT, δT	[286]
HMG-CoA reductase		HepG2	↑ αT	[286]
PPARγ		SW480, LoVo	↑ αT, γT	[287]
Leptin		human	↑ αT	[172]
CD95L (CD95 APO-1/Fas ligand)	NF-κB, AP-1	T-cells	↓ αT	[288]
BACE		NT(2) neurons	↓ αT	[270]

such a function, possibly mediated by proteins binding tocopherols, such as α-TTP or hTAPs.

4.4.8. Modulation of Gene Expression by Vitamin E

Regulation of gene expression requires transcription factors and the specific effects of the tocopherols analogues in cells require recognition proteins capable of distinguishing the different tocopherols from each other, from tocotrienols and from synthetic analogues. The search for transcription factors modulated by tocopherol and proteins specifically binding it starts to give some results. Several genes have been described as being modulated by tocopherol (Table **1**) (reviewed in [151]). However, how tocopherol can modulate gene expression is not yet clearly resolved, and indeed it may involve several different molecular mechanisms. To explain all the effects seen at the

level of gene expression several regulatory pathways have to be considered:

1) α-Tocopherol can change the activity of transcription factors and signal transduction pathways by modulating enzymes, such as protein kinase C α (PKC-α), the phospholipase A_2, 5-lipoxygenase and cyclooxygenase 2, which therefore could indirectly influence gene expression.

2) α-Tocopherol may also influence gene expression by direct modulation of the activity of specific transcription factors in a non-antioxidant fashion, for example *via* the pregnane X receptor [84], possibly other nuclear receptors such as the peroxisome proliferators activated receptors (PPARs), orphan nuclear receptors, or *via* one of three human tocopherol associated proteins, hTAPs, recently reported to modulate gene expression [20,152].

3) α-Tocopherol may also influence gene expression by binding to proteins like hTAP, that may act as "molecular chaperones", which generate specificity to the action of the tocopherols. These proteins may regulate tocopherol access to specific enzymes and transcription factors or control the level of "free" tocopherol. The hTAPs modulate *in vitro* the activity of recombinant phosphatidylinositol-3-kinase and α-tocopherol modulates kinase activity in an hTAP-dependent manner, possibly by competition with phosphatidylinositol. Thus, by modulating the intracellular targeting of the ligands to enzymes and organelles, the hTAPs may influence the activity of lipid dependent enzymes [20].

4) Tocopherols may be metabolized to bioactive compounds, which can bind to transcription factors and enzymes and modulate their activity. The metabolite of γ-tocopherol, γ-CEHC has natriuretic activity, inhibits cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) synthesis in activated macrophages and epithelial cells, two events that could change the cellular behavior and affect gene expression [81,82]. Recently a metabolite of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol (PMCol), has been found to inhibit growth of androgen-sensitive prostate carcinoma cells, which is due to the potent anti-androgenic activity of this compound [153].

4.4.9. Modulation of Gene Expression by Natural Tocopherols

An early study suggested that vitamin E affects the expression of the aryl hydrocarbon hydroxylase gene, a P_{450} oxygenase (P_1-450) involved in the detoxification of polynuclear aromatic hydrocarbons and in the disposition of certain drugs [154]. Since α-tocopherol inhibits PKC activity, and PKC ultimately regulates the phosphorylation of several transcription factors, it was postulated that several genes may be regulated by tocopherols. Early studies have shown that α-tocopherol, but not β-tocopherol, can regulate AP-1-mediated gene expression [155,156], in particular after activation of PKC by 12-O-Tetradecanoylphorbol 13-acetate (TPA). α-Tocopherol also increases *de novo* synthesis of protein kinase C molecules [157]. The regulation of α-tropomyosin gene transcription and protein expression was found by using a differential display technique. Northern and Western blot analyses revealed a time-dependent transient up-regulation of the amount of mRNA (with a peak between 2 and 3 h) and protein (with a peak at 5 h) in α-tocopherol-treated cells. No effect is observed in cells treated with β-tocopherol [157,158]. In human skin fibroblasts, PKC-α protein expression increases during *in vivo* aging as a function of the donor's age. Concomitant with the increase in PKC-α, also collagenase (MMP-1) gene transcription and protein expression increases with age. α-Tocopherol is able to diminish collagenase gene transcription without altering the level of its natural inhibitor, tissue inhibitor of metalloproteinase, TIMP-1 [159]. Glycoprotein IIb is the α-subunit of the platelet membrane protein glycoprotein IIb /IIIa, which functions as a specific receptor for platelet aggregation. Transient transfection of the glycoprotein IIb promoter-reporter plasmid into cells in which PKC was stimulated with TPA shows that α-tocopherol inhibits glycoprotein IIb promoter activity. This event may result in a reduction of glycoprotein IIb protein expression by α-tocopherol and thus contribute to anti-platelet aggregation [160].

Later on, genes were found to be modulated by tocopherols independent of PKC, and specific regulatory elements were found in their promoters, but a specific transcription factor responsive only to tocopherol or a "tocopherol nuclear receptor" has so far not been identified. In primary cultures of quiescent stellate cells, inhibition of collagen α1(I) transactivation by α-tocopherol requires only -0.44 kb of the 5' regulatory region. Transfection of stellate cells with a collagen-luciferase chimeric reporter construct allowed localization of an "antioxidant"-responsive element (ARE) [161,162]. Long- and short-term supplementation with α-tocopherol to mice selectively decreases liver collagen mRNA by approximately 70% [162]. Similarly, chronic treatment of rats with carbon tetrachloride increases TGF-β1 gene expression and α-tocopherol inhibits both TGF-β and α2(I) procollagen mRNA expression [163]. α-Tocopherol induces a 2-3 fold increase of connective tissue growth factor expression in human vascular smooth muscle cells by a non-radical chain braking mechanism, and a TGF-β-response element which mediates the effect of α-tocopherol has been identified [164].

A series of studies have pointed to the effect of α-tocopherol on macrophages and smooth muscle cells with possible relationships with atherosclerosis and inflammatory events. CD36 scavenger receptor (a specific receptor for oxidized LDL, oxLDL) is expressed in macrophages and cultured human aortic smooth muscle cells. Studies indicate that CD36 transports oxLDL into the cytosol of these cells and that α-tocopherol inhibits oxLDL uptake by a mechanism involving down-regulation of CD36 mRNA and protein expression. Therefore, the beneficial effect of α-tocopherol against atherosclerosis can be explained, at least in part, by its effect of lowering the uptake of oxidized lipoproteins, with consequent reduction of foam cell formation [165,166]. A reduction of the scavenger receptor SR-A expression and activity in presence of α-tocopherol was also observed [167]. The α-tocopherol role of diminishing scavenger receptor activity (CD36 and SR-B1) has been confirmed *in vivo* [168,169]. Correspondingly, rats depleted of vitamin E show an increased expression of the

scavenger receptor SR-B1 [37]. Additionally, reduction of integrins expression by α- but not β-tocopherol has been observed, possibly reducing monocyte cell adhesion, an important event both in inflammation and atherosclerosis [170].

Several events that are associated with inflammation appear to be down-regulated by tocopherol. The cytokine interleukin-1 (IL-1α) is decreased by α-tocopherol by a mechanism involving down regulation of IL-1α mRNA expression [171]. Combined α-tocopherol and selenium deficiency is characterized by alterations in the expression level of genes encoding for proteins involved in inflammation (multispecific organic anion exporter, SPI-3 serine protease inhibitor) and acute phase response (α-1 acid glycoprotein, metallothionein 1). Isermann *et al* have shown that α-tocopherol induces leptin expression in healthy individuals and also *in vitro* [172]. The matrix metalloproteinase-19 (MMP-19) in myeloid cells is down regulated by α-tocopherol and not by β-tocopherol [173]. The adhesion-dependent expression of MMP-19 is down-regulated or even abrogated by blockade of adhesion or interfering with adhesion-controlling signaling using α-tocopherol.

A protective role of tocopherols against a number of tumors has been described. Tumors development in animals exposed to 7,12 dimethylbenz(a)anthracene (DMBA) is significantly reduced after vitamin E supplementation which is possibly the result of a notable increased expression of the p53 tumor suppressor gene [174]. γ-Tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulating cyclins D1 and E [175]. Because γ-tocopherol has a weaker antioxidant capacity than α-tocopherol and γ-tocopherol more significantly inhibits cell proliferation and DNA synthesis than α-tocopherol, a non-antioxidant mechanism appears to be at the basis of this effect.

4.4.10. Modulation of Gene Expression by non-Natural Tocopherol Analogues

The most studied tocopherol analogue is tocopherylsuccinate but many other analogues have been described [28]. Regarding the cellular effect of tocopherol derivatives it can be asked if they simply serve as vehicle of tocopherol into cells, enhance the transport of tocopherol into specific organelles or act as completely different molecules. It is also often not investigated whether the tocopherol analogues are metabolized to release tocopherol e. g. by esterases, or whether they undergo other metabolic conversions. At least the selective antiproliferative effects of α-tocopheryl hemisuccinate on murine leukemia cells result from the action of the intact compounds possibly *via* modulation of gene expression [176]. c-Jun involvement has been established in α-tocopherylsuccinate induced apoptosis in reticuloendotheliosis virus transformed avian lymphoid cells [177].

α-Tocopherylsuccinate is a potent, novel anti-neoplastic agent with high tumor selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) *in vivo* [178]. Interestingly, α-tocopherylsuccinate affects specifically tumorigenic cells, since it induces apoptosis in malignant cell lines but not, in general, in normal cells, and inhibits tumorigenesis *in vivo* [179]. Mitochondria play a central role in apoptosis induced by α-tocopherylsuccinate, since mitochondrial DNA-deficient cells or cells treated with mitochondrially targeted radical scavengers are resistant to apoptosis [180]. α-Tocopherylsuccinate induces Fas-mediated apoptosis in estrogen receptor-negative human breast cancer cells. This may be of clinical use in the treatment of aggressive human breast cancers, particularly those that are refractory to anti-estrogen therapy [181].

α-Tocopherylsuccinate was found to reduce the surface expression of CD178 (CD95/Fas-ligand), which is expressed on several tumor cells and probably influences the interaction of the tumor with the host immune system. Accordingly, α-tocopherylsuccinate treatment diminishes the ability of tumor cells to kill CD95/Fas-sensitive lymphocytes. *In vivo*, such treatments may play an important role in the outcome of tumor sensitivity or resistance to host immune mechanisms [182]. α-Tocopherylsuccinate can suppress the expression of prostate-specific antigen, a marker for the progression of prostate cancer [183]. A role of extracellular signal-regulated kinase pathway in α-tocopherylsuccinate induced differentiation of human MDA-MB-435 breast cancer cells has been indicated [184]. α-Tocopherylsuccinate also suppresses androgen receptor expression by means of transcriptional and posttranscriptional modulation, but not by ligand binding, nuclear translocation, or androgen receptor dimerization. The α-tocopherylsuccinate mediated inhibition of androgen receptors is selective because it does not repress the expression of other nuclear receptors [185]. In LNCaP and PC3 prostate carcinoma cell lines, α-tocopherylsuccinate reduces cell cycle progression, which is mediated by a 3-fold up-regulation of p27 and cyclin E [186]. In another study α-tocopherylsuccinate decreases expression of the cell cycle regulatory proteins cyclin D1, D3, E, cdk2 and 4, and reduces cdk4 kinase activity, retinoblastoma protein (Rb) phosphorylation, and cyclin E mRNA expression [187]. α-Tocopherylsuccinate inhibits growth and induces cell differentiation in culture by decreasing the levels of c-myc and H-ras specific mRNAs in B-16 melanoma cells [188], and the expression of c-myc and N-myc and H-ras in murine neuroblastoma cells [189].

Other tocopherol analogues, such as the α-tocopheryl ester with 9-*cis*-retinoic acid, inhibits the proliferation of acute promyelocytic leukemia cell-derived NB4 and HT93 cells and induces differentiation markers in these cells, such as granulocytic maturation, nitroblue tetrazolium reduction, and CD11b expression. The α-tocopheryl 9-*cis*-retinoic acid ester similarly induces the trans-activating action of RARs, but is not effective on RXRs [190]. In hippocampal slices, α-tocopherylphosphate induces a slowly developing long-term potentiation of excitatory postsynaptic potentials, which was impaired in slices from vitamin E-deficiency animals [191,192].

From what has been described above α-tocopherylsuccinate and some other tocopheryl esters act by diverse mechanisms in different tumor cell lines, leading to inhibition of growth, activation of apoptosis or cellular differentiation. More studies are required to establish in detail the molecular aspects involved in the tumor protecting effects of α-tocopherylsuccinate.

4.4.11. Modulation of Gene Expression by Tocotrienols

Potential antiproliferative effects of tocotrienols, the major vitamin E component in palm oil, were investigated with both estrogen-responsive and estrogen-unresponsive human breast cancer cells. Complete suppression of growth is achieved at 8 μg/ml (in the estrogen-responsive) and at 20 μg/ml tocotrienol (in the estrogen unresponsive cells), in both the presence and absence of estradiol. The γ- and α-tocotrienols are the most potent inhibitory forms [193]. α–Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and consecutive adhesion of monocytes [194]. Supplementation with α-tocotrienol improves bone calcium content in vitamin E deficient rats, but supplementation with α-tocopherol does not, suggesting that tocotrienols play an important role in bone calcification [195].

When compared to tocopherols, tocotrienols show additional effects in mammalian cells by influencing the mevalonate pathway. Tocotrienols inhibit the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) at the posttranscriptional level by specifically modulating the intracellular mechanism for its controlled degradation. α-Tocotrienol inhibits the rate of [^{14}C] acetate but not [^{3}H] mevalonate incorporation into cholesterol in a concentration- and time-dependent manner, with 50% inhibition observed at approximately 2 μM. Maximum inhibition (80%) was observed in HepG2 cells within 6 h. HMG-CoA-reductase total activity and protein levels are reduced concomitantly with the decrease in cholesterol synthesis [196]. Tocotrienols were recently reported to increase transcription of IKAP mRNA in patients with familial dysautonomia, a neurodegenerative genetic disorder that is caused by mutations in the IKBKAP gene which encodes the IkappaB kinase complex-associated protein (IKAP). These findings suggest that *in vivo* supplementation with tocotrienols may elevate IKBKAP gene expression [197].

4.4.12. Modulation of Gene Expression as Analyzed by Gene Array Experiments

In recent years gene expression arrays have allowed to screen for genes modulated by vitamin E. Cell culture experiments allow to isolate responsive genes that are immediately regulated by vitamin E and these studies may give insight into the regulatory mechanisms and possible transcription factors modulating gene expression by vitamin E. In smooth muscle cells several genes were found to be consistently regulated by vitamin E, some of these genes were confirmed by other methods (like the CTGF gene and the prostacyclin stimulating factor) [164]. However the evidence of a regulatory function by α-tocopherol for most of these genes still needs confirmation by other methods.

Animal studies allow screening for genes that ultimately may be the causes of diseases associated with vitamin E deficiency. However, since the vitamin E deficient state needs long time to be reached, these studies may also show genes that are secondary or tertiary modulated by vitamin E. A myriad of genes were found to be regulated in rats by combined selenium and vitamin E deficiency [198]. Vitamin E increases the expression level of genes important in the inhibition of apoptosis (defender against cell death 1 protein, Bcl2-L1), in cell cycle progression (G1/S-specific cyclin D1) and in antioxidant defense (α-glutamylcysteine synthetase catalytic subunit) [198].

Vitamin E and selenium supplementation was also studied in rats fed a high fat diet and several genes in rat skeletal muscle were found to be modulated by supplementation [199]. In rats fed vitamin E deficient diet many genes show an altered expression level, and vitamin E (α-tocopherol and α-tocotrienol) supplementation regulates some of them [200]. Some of these genes were confirmed by RT-PCR; heme oxygenase 3 (HO-3), cyclin D1, high mobility group protein 1 (HMG1), and nuclear phosphoprotein p140 (NOPP140) are up-regulated and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is down-regulated.

Although it is mechanistically interesting that vitamin E can modulate gene expression *in vivo*, these changes in order to be relevant for the prevention of disease should be detectable at the protein level. Many of the above described vitamin E regulated genes show also alterations at the protein level, others have not been tested. The proteomics experimental approach was taken in one study using human cytokine antibody arrays to show that vitamin E can affect many genes at the protein level in healthy human individuals [201]. Several cytokines, like the monocyte chemoattractant protein 1 (MCP-1), ENA-78, IL-1α, RANTES, MIG and TNF-β were found to be significantly down-regulated by supplementation with vitamin E [201]. Some of these chemokines, like RANTES, MCP-1 and MIP-1α, were also found to be regulated by vitamin E in other studies [202].

5. NOVEL TOCOPHEROL BINDING PROTEINS

Several tocopherol binding proteins have been studied [17,67,203-205], but so far only α-TTP and hTAPs have been cloned and shown *in vitro* to bind tocopherol with reasonable affinity [206]. Initially, the novel α-Tocopherol-Associated Protein (TAP) has been isolated from bovine and human liver [19], later it was found that human and rat TAP1 are identical to the previously described microsomal supernatant protein factor (SPF), which stimulates squalene epoxidation required for cholesterol synthesis, either by directly stimulating squalene transport or possibly by increasing the transport of vesicles carrying squalene [207]. However, this protein is also known to bind phospholipids, such as phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and phosphatidic acid [208]. Sequence analysis has established that TAP structural motifs have similarity with a family of hydrophobic ligand-binding proteins (RALBP, CRALBP, α-TTP, SEC 14, PTN 9, RSEC45). TAP may be involved in the regulation of cellular α-tocopherol concentration, tocopherol transport and α-tocopherol-mediated signaling [209]. Several human TAPs (hTAP1, hTAP2 and hTAP3) have been cloned recently and their localization, ligand binding ability and functions are presently under investigation. hTAPs can bind phosphatidylinositol, phosphatidylcholine, and tocopherols can compete with binding, suggesting that tocopherols may modulated *via* hTAP phospholipid-dependent signaling pathways [20]. The hTAP proteins could be involved in tocopherol transport to the Golgi apparatus or to the

mitochondria, since they carry a carboxy-terminal GOLD domain which in other proteins (GCP60, PAP7) is known to serve as adaptor for binding to Golgi giantin or to the mitochondrial peripheral benzodiazepine receptor [20,68]. By construction of a fusion between hTAP1 and the green fluorescent protein (GFP), it was observed that TAP1 translocates from cytosol to nuclei in an α-tocopherol-dependent manner [152]. As described above, vitamin E activates the human pregnane X receptor (PXR) in HepG2 cells [84]. The role of TAPs and similar proteins may be that of conferring specificity to the action of the different tocopherols, through recognition and selective transport to enzymes, transcription factors, nuclear receptors such as PXR, or organelles. Indeed, the hTAP1 protein recognizes the different natural tocopherols with different specificity [206].

To date no diseases linked with the hTAPs proteins have been described. However, mutations in related proteins with similar function can lead to hereditary disease, clearly showing the importance of a correct protein-mediated distribution of hydrophobic ligands. The *Drosophila melanogaster* RdgB protein prevents retinal degeneration [210]. The vibrator knockout mouse (vb^-/vb^-), which is deficient in the phosphatidylinositol-transfer-protein alpha (PI-TPα), shows degeneration of neurons of the spinal chord, brain stem and dorsal root ganglions, leading to symptoms similar to progressive neurodegenerative disorders [211]. CRALBP deficient humans show autosomal recessive retinitis pigmentosa, caused by a deficient transport of 11-cis-retinol and 11-cis-retinaldehyde [212]. Patients with ataxia with vitamin E deficiency (AVED), caused by α-TTP gene mutations, are affected by ataxia, loss of neurons, retinal atrophy, massive accumulation of lipofuscin in neurons and retinitis pigmentosa [213]. These symptoms are similar to Friedreich ataxia, caused by defective expression of frataxin and the consequent increased mitochondrial oxidative damage and cell death [50]. In Friedreich ataxia fibroblasts cell damage can be more efficiently prevented by using a mitochondria-targeted vitamin E derivative (MitoVit E) than by using α-tocopherol [214]. Thus, the confirmation that the tocopherol binding proteins, such as hTAPs, can enrich tocopherol in cells and specific organelles like mitochondria could explain the beneficial function of vitamin E in several degenerative diseases, ranging from cardiomyopathy, to Alzheimer's, Parkinson's and several ataxias with unknown etiologies. These diseases have in common that they often show mitochondrial impairment, and reduced levels of in mitochondrial vitamin E may accelerate this process [215-219].

6. PREVENTIVE EFFECTS OF VITAMIN E

6.1 Vitamin E Deficiency Syndromes

It should also be noted that vitamin E deficiency in humans with full neurological symptoms is rare and usually is the consequence of mutations of the α-tocopherol transfer protein leading to AVED. What is more likely to occur is vitamin E insufficiency, with suboptimal vitamin E supply in the diet or inefficient uptake and distribution of vitamin E. The normal plasma concentration of vitamin E is 23.2 μM [220], a plasma level below 11.6 μM is regarded as deficient. Certain diseases, like abetalipoproteinemia, chronic cholestatic liver disease, cystic fibrosis, chronic pancreatitis, progressive systemic sclerosis, short-bowel syndrome or several other lipid malabsorption syndromes are associated with a low efficiency of vitamin E uptake leading to similar symptoms as in AVED. These symptoms clearly can be prevented and in some situation reversed by supplemental vitamin E (reviewed in [220]).

6.2 Vitamin E Supplementation for Prevention of Diseases

Up to now, most if not all epidemiological studies with vitamin E have been carried out without detailed knowledge of the cellular functions of vitamin E. It has been constantly assumed that vitamin E is only an antioxidant, thus preventing diseases in which oxidative stress may occur. Several studies have presumed that vitamin E could even be beneficial in situations where the disease is already fully developed. However, the disease process is usually developing over years, hence supplementation of vitamin E may be most effective when given early on and over years, thus slowing down inherent disease progression or even preventing it completely.

The role of vitamin E (and its related molecular effects) may acquire importance mainly in a selected patient population with low plasma levels of vitamin E. In fact, it was reported that patients respond differently to vitamin E, and achieve different levels of plasma vitamin E after supplementation (discussed in [6]). Plasma vitamin E concentrations could vary in different individuals as a consequence of excessive consumption by oxidative stress or of a deficient uptake and transport into plasma and tissues. Polymorphisms and cellular expression levels of tocopherol binding proteins, like hTTP or hTAPs, could be the reason for the individual response and uptake of vitamin E and explain the differential susceptibility to disorders such as atherosclerosis, certain cancers and neurodegenerative diseases. Patients undergoing therapy with orlistat, a gastrointestinal lipase inhibitor that reduces dietary fat absorption, show also lower vitamin E absorption [221].

6.3 Prevention of Atherosclerosis

Several epidemiological studies have shown a preventive effect of α-tocopherol on atherosclerosis (reviewed in [6,222]), however, a more recent clinical trial (the HOPE study) has failed to demonstrate the clinical utility for α-tocopherol in the advanced cardiovascular patient. Vitamin E may act more as long term preventive agent, and in patients with advanced disease (as in the HOPE study) its effect may not be evident. Although lipid peroxidation in the subendothelial space has been hypothesized to play a central role in atherogenesis, the role of vitamin E in preventing lipid peroxidation and lesion development remains uncertain [101].

The best support for a preventive role of vitamin E supplementation comes from studies with animals that are either fed a high atherogenic diet or in animals susceptible to atherosclerosis because of mutations in specific genes. In New Zealand white rabbits fed a high cholesterol diet (0.1% (w/w)), α-tocopherol protected against atherosclerosis and

lowered plasma cholesterol concentrations [223]. In apoE deficient mice fed an atherogenic diet, α-tocopherol did not affect cholesterol levels, but reduced the incidence of atherosclerotic lesions by 60% [224]. The reduction in the lesion size was correlated with the reduced expression of MCP-1 mRNA and protein [225]. In primates (*Macaca fascicularis*) receiving an atherogenic diet, mean percent ultrasound stenosis at 36 months posttreatment was lower in the tocopherol-supplemented groups (61 and 18%) than in the unsupplemented group (87%). Percent stenosis in the regression group decreased from 33 to 8%, 8 months after tocopherol supplementation, suggesting that d-α-tocopherol may be prophylactically and therapeutically effective in atherosclerosis prevention [226].

α-TTP$^{(-/-)}$ knockout mice are a genetic model of vitamin E deficiency and should be valuable for studying other diseases in which oxidative stress is thought to play a role. In atherosclerosis-susceptible apolipoprotein E (apo E) knockout mice, vitamin E deficiency caused by disruption of the α-tocopherol transfer protein gene (α-TTP) increased the severity of atherosclerotic lesions in the proximal aorta [227]. The increase was associated with increased levels of isoprostanes, a marker of lipid peroxidation, in aortic tissue. Whether the effect of vitamin E in keeping the levels of isoprostanes low can be referred to its "antioxidant" properties or rather to the tocopherol dependent inhibition of radical production by pathways such as the NADPH oxidase is an open question. These results show however that vitamin E deficiency promotes atherosclerosis in a susceptible setting (apo E-deficient) and support the hypothesis that lipid peroxidation contributes to lesion development. Similarly, an up 76.5% decrease in aortic tree lesion was observed in CD36-apo E-double knockout mice when compared with controls. These results support a major role for CD36 in atherosclerotic lesion development *in vivo* and suggest that blockade of CD36 or reduction of its expression by α-tocopherol could be protective even with proatherogenic circumstances [228].

The α-tocopherol effect was also studied on the expression of monocyte chemoattractant protein (MCP)-1 in aortic lesions of apolipoprotein E knockout mice. α-Tocopherol did not reduce serum cholesterol nor change lipoprotein profile, but it reduced the area of the aortic lesion by 55%. The reduction in the lesion size was correlated with the reduced expression of MCP-1 mRNA and protein [225]. Apoptosis induced by oxLDL or oxysterols, caspase-3 activity and DNA fragmentation in vascular endothelial cells were inhibited by α-tocopherol, but not by β-tocopherol [229]. Similarly, despite the increased uptake of γ-tocopherol, only α-tocopherol, and not γ-tocopherol or α-tocopherylacetate was effective at inhibiting 7 beta-hydroxycholesterol-induced apoptosis in U937 cells [230]. Reduction of apoptosis may contribute to the preventive effects of α-tocopherol in atherosclerosis by decreasing he formation endothelial lesion [229]. Vitamin E also inhibits the adhesion of THP-1 monocytes to endothelial cells, an initiating event for plaque formation, which is most likely the result of inhibition of integrin and vascular cell adhesion molecule-1 (VCAM-1) expression [231].

In cardiology the inverse epidemiological correlations between plasma vitamin E levels and mortality due to ischemic heart disease, as well as beneficial effects of vitamin E on experimentally induced oxidative damage to the heart support the hypothesis, that vitamin E might have a protective role against myocardial ischemia-/reperfusion injury [232,233].

6.2 Prevention of Cancer

Since the discovery that the vitamin E group had fewer incidences of cancers of the prostate and colorectum compared with the group not receiving vitamin E in the Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention Study [234], a number of other epidemiological observations have pointed to a protective effect of tocopherol on different cancers. [235-237]. High serum α-tocopherol levels are associated with lower lung cancer risk [238]. Studies *in vitro* have shown a number of possible reactions involved in the decrease of tumour cell proliferation produced by tocopherols. Vitamin E inhibits cell proliferation due to PKC inhibition in a number of tumor lines [6,170,175,239]. γ-Tocopherol treated prostate carcinoma DU-145 cells show decreased progression into the S-phase. This effect is associated with reduced DNA synthesis and decreased levels of cyclin D1 and cyclin E [175]. Vitamin E inhibits cell proliferation and activation of the Erk cascade during urethane-induced lung tumorigenesis in mice independent of its antioxidant effect [240]. In animal models α-tocopherol has been shown to be useful for the chemoprevention for liver cancer [241] and other tumors [241-243]. Recently it has been found that growth modulation by vitamin E in androgen-sensitive prostate carcinoma cells is due, at least in part, to the potent anti-androgenic activity of its metabolite, 2,2,5,7,8-pentamethyl-6-chromanol (PMCol) [153]. Chronic alcohol consumption results in colorectal mucosal hyperregeneration, a condition associated with an increased risk for colorectal cancer. In this respect it is interesting to note that alcohol-associated colonic hyper-regeneration is inhibited by α-tocopherol [244] and may also play a preventive role against upper gastrointestinal cancers [245].

6.3 Prevention of Fibrotic Diseases

In alcoholic liver disease, NADPH-oxidase-derived free radicals are key oxidants [144], and inhibition of NADPH-oxidase by diphenyleneiodonium sulfate prevents early alcohol-induced liver injury in the rat [145]. Interestingly, the assembly of NADPH-oxidase is inhibited by tocopherol, suggesting that tocopherol may reduce the symptoms of scleroderma and alcoholic liver fibrosis by reducing the production of superoxide by NADPH-oxidase [122]. Similarly, maintenance of the scleroderma fibroblast phenotype is mediated in part by the constitutive upregulation of reactive oxygen species generated through the NADPH-oxidase complex pathway [246].

Connective tissue growth factor (CTGF) has recently received much attention as a possible key determinant of progressive fibrosis and excessive scarring. CTGF is highly up-regulated in numerous fibrotic diseases, including lung-, skin-, pancreas-, liver- and kidney-fibrosis. During alcoholic liver fibrogenesis, hepatic stellate cells seem to be the major cellular sources of CTGF in the liver [247]. In liver cirrhosis secondary to alcoholic abuse, CTGF is also highly

overexpressed [248]. α-Tocopherol treatment of patients affected by chronic hepatitis and hepatic fibrogenesis prevents the fibrogenesis cascade [249]. Although in the normal setting CTGF is weakly upregulated by α-tocopherol, it can be speculated that in the pathologic setting the α-tocopherol responsive transcription factors may interfere with the fibrosis-inducing transcription factors because they may bind to the same element [164].

6.4 Prevention of Neurodegenerative Diseases

Mutations of the α-TTP gene lead to drastically reduced α-tocopherol concentrations in plasma and tissues, that ultimately lead to a rare, severe syndrome named ataxia with vitamin E deficiency (AVED) with similar symptoms as in Friedreich Ataxia [50,250,251]. These patients show loss of neurons, symptoms of retinal atrophy, massive accumulation of lipofuscin in neurons including dorsal root ganglions, and retinitis pigmentosa [53]. The role played by α-tocopherol is consistent with that of a specific survival factor for neuronal cells, e. g. for cerebellar Purkinje cells [64,213]. Very recently it has been found that vitamin E is an exogenous factor playing a direct role in regulation of adult hippocampal neurogenesis at different stages [252].

Specific mutations of the α-TTP gene are linked to the severity of symptoms in patients with AVED [253]. Reduced α-TTP gene expression, as in hepatocellular carcinoma, could also lead to reduced plasma level of α-tocopherol [60]. Moreover, the uptake of dietary hydrophobic antioxidants (tocopherols, carotenoids, and flavonoids) and transport by chylomicrons from intestine to the liver is impaired in abetalipoproteinemia and a number of lipid malabsorption syndromes, such as cholestatic liver disease, short bowel syndrome and cystic fibrosis, which often show symptoms very similar to AVED [254]. In these diseases the transport of α-tocopherol is impaired either in the liver or in the intestine by the complete absence of a transport pathway, leading to extremely low plasma α-tocopherol levels [255].

Furthermore, it can be assumed that conditions may exist with partially impaired vitamin E uptake and transport, such as a heterozygotic mutation of vitamin E binding proteins [256], less penetrating mutations [253] or other reasons, with consequent less severe symptoms or delayed outcome. In fact, the age of symptoms onset in AVED patients is dependent on the type of mutation in the α-TTP gene [253]. The neurological symptoms of AVED are stabilized in patients following vitamin E treatment [253,257].

A model mouse of AVED with deleted α-TTP gene showed ataxia and retinal degeneration after 1 year of age [53]. Because the brain α-TTP may function in maintaining α-tocopherol levels in the central nervous system, α-tocopherol was completely depleted in the α-TTP$^{(-/-)}$ mouse brain, and the neurological phenotype of α-TTP$^{(-/-)}$ mice is much more severe than that of wild-type mice when maintained on an α-tocopherol-deficient diet. Lipid peroxidation in α-TTP$^{(-/-)}$ mice brains showed a significant increase, especially in the degenerating neurons. α-Tocopherol supplementation suppressed lipid peroxidation and almost completely prevented the development of neurological symptoms. Thus, this therapy almost completely corrects the abnormalities in a mouse model of a human neurodegenerative disease. α-TTP$^{(-/-)}$ knockout mice may prove to be excellent animal models for diseases caused by chronic oxidative stress, such as delayed onset, slowly progressive neuronal degeneration. It is still unknown whether the degenerative neurological symptoms in patients with vitamin E deficiency syndromes are the result of insufficient protection by antioxidants or to a lack of specific and non-antioxidant effects mediated by α-tocopherol.

In patients with moderately severe impairment from Alzheimer's disease, treatment with α-tocopherol slowed the progression of disease [258,259]. *In vitro*, α-tocopherol protects HT4 hippocampal neuronal cells against cell death caused by amyloid β protein, hydrogen peroxide and the excitatory amino acid glutamate [260]. In alzheimer brains, the nitration product 5-nitro-γ-tocopherol is increased, and peroxynitrite produced by SIN-1 *in vitro* could be attenuated by γ-tocopherol, but not by α-tocopherol [261]. Nanomolar amounts of α-tocotrienol, but not α-tocopherol, block glutamate-induced death by suppressing glutamate-induced early activation of c-src kinase and inhibition of 12-lipoxygenase, and thus appears to be independent of antioxidant properties. *In silico* docking studies identified that α-tocotrienol may hinder the access of arachidonic acid to the catalytic site of 12-lipoxygenase by binding to the opening of a solvent cavity close to the active site [262,263].

Oxidation has been proposed to be an important factor in the pathogenesis of Alzheimer's disease (AD) and amyloid beta is considered to induce oxidation [264]. Thus, an obvious explanation of the beneficial effects of α-tocopherol could be that related to its antioxidant function. Alternatively, the implication of inflammatory events and the possible role of scavenger receptors in the onset of the disease [265-269] suggest that the effect of α-tocopherol may be linked more to its non-antioxidant anti-inflammatory role, e. g. via down-regulating scavenger receptor gene expression (see above). α-Tocopherol also reduces 4-hydroxynonenal (HNE)-induced expression of the aspartyl protease with β-secretase activity (beta-site amyloid precursor protein (APP)-cleaving enzyme, BACE) which cleaves the amyloid β protein precursor at the amino-terminus, and thus may play a central role in alzheimer's disease prevention [270].

Environmental and endogenous factors have been suggested to cause Parkinson's disease by producing mitochondrial oxidative stress and damage in the substantia nigra, leading to cell death. It has been suggested that a high dose of dietary vitamin E supplementation or parenteral vitamin E administration may serve as a successful therapeutic strategy for the prevention or treatment of Parkinson's disease by enriching substantia nigra mitochondria with protective levels of α-tocopherol [217]. However, to what extent antioxidants may be beneficial in Parkinson's disease prevention and treatment is an open question [271].

7. CONCLUSION

The results summarized in this review strongly indicate three rather new concepts regarding tocopherols and related

compounds. The first is that the data discussed in this study are altogether suggestive for an *in vitro* gene regulatory function of α-tocopherol, γ-tocopherol, tocotrienols and tocopheryl esters. It is not always clear whether the observed effects are the result of changes of gene expression or may be due to other events such as changes in mRNA or protein stability. The second is that vitamin E interacts with specific proteins, and affects the activity of these proteins in a manner specific for the different tocopherol analogues. The third clear point of this survey is that no obvious correlation exists between the described regulatory functions of the tocopherols and their free radical chain interrupting properties established to take place only within a lipid phase. As a consequence it is reasonable to assume that the basis of the selective retention of α-tocopherol is found in some of the non-antioxidant properties of this analogue, or that the other tocopherol analogues at higher concentrations perform biological activities that interfere with the normal cellular performance.

ACKNOWLEDGEMENTS

This study was made possible thanks to the support of the Swiss National Science Foundation and the Foundation for Nutrition Research in Switzerland. We thank A. Munteanu for critically reading the manuscript. The terms vitamin E, tocopherols and tocotrienols currently give 22999 hits in PubMed data bank, indicating the interest in this field. We apologize if some published non-antioxidant effects have not been cited. Suggestions for overlooked non-antioxidant effects are welcome for inclusion in future reviews.

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