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Vitamin E: non-antioxidant roles

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Abstract

Vitamin E was originally considered a dietary factor of animal nutrition especially important for normal reproduction. The significance of vitamin E has been subsequently proven as a radical chain breaking antioxidant that can protect the integrity of tissues and play an important role in life processes. More recently α-tocopherol has been found to possess functions that are independent of its antioxidant/radical scavenging ability. Absorption in the body is α-tocopherol selective and other tocopherols are not absorbed or are absorbed to a lesser extent. Furthermore, pro-oxidant effects have been attributed to tocopherols as well as an anti-nitrating action. Non-antioxidant and non-pro-oxidant molecular mechanisms of tocopherols have been also described that are produced by α-tocopherol and not by β-tocopherol. α-Tocopherol specific inhibitory effects have been seen on protein kinase C, on the growth of certain cells and on the transcription of some genes (CD36, and collagenase). Activation events have been seen on the protein phosphatase PP2A and on the expression of other genes (α-tropomyosin and Connective Tissue Growth Factor). Non-antioxidant molecular mechanisms have been also described for γ-tocopherol, δ-tocopherol and tocotrienols.

Contents

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1. Introduction

1.1. Vitamin E discovery and history

The term "Vitamin E", introduced in 1922 by Evans and Bishop [1] described a dietary factor in animal nutrition considered at the time to be especially important for normal reproduction. After feeding female rats accidentally with rancid fat they observed a deficiency syndrome, in which foetal resorption was the most characteristic symptom. From the fact that adding fresh salad to the diet reversed the symptoms they concluded that plants contained a

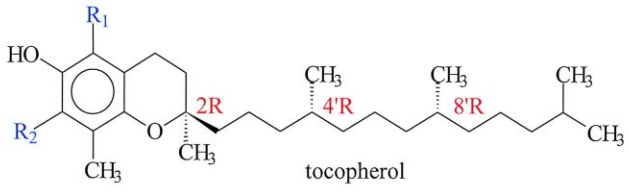
specific factor being responsible for the observations. The multiple nature of the vitamin began to appear in 1936, when two compounds with vitamin E activity were isolated and characterized from wheat germ oil [2]. These compounds were designated as α- and β-tocopherol, deduced from the Greek expressions "tokos" (childbirth) and "phorein" (to bring forth). In the following years two additional tocopherols, γ- and δ-tocopherol [3,4] as well as the tocotrienols [5] were isolated from edible plant oils, so that today a total of four tocopherols and four tocotrienols are known to occur in nature. The American Food and Nutrition Board in 1968 officially recognized the essential nature of vitamin E. Researchers have since confirmed the significance of vitamin E as a radical chain breaking antioxidant that can protect the integrity of tissues and play an important role in life processes.

1.2. Chemistry of tocopherols and tocotrienols

Unlike other vitamins that represent one well-defined chemical structure, natural vitamin E includes two groups of closely related fat-soluble compounds, tocopherols and tocotrienols. The compounds of both groups are all derivatives of 6-chromanol. The first group derives from tocol, which carries a saturated isoprenoid C-16 side chain and three chiral centres with configuration R at position 2, 4′ and 8′ (Fig. 1).

The members of the second group have a triply unsaturated side chain at the positions 3′, 7′, and 11′. Within one group the members are designated α, β, γ and δ depending on the number and the position of the methyl groups attached to the aromatic ring [6].

The tocopherols are naturally occurring phenolic benzopyrans, which display antioxidant activities in vivo and in vitro. Since their initial discovery, they have been investigated to elucidate their mechanism of action and to identify potential metabolites. Much interest has been focused on their reactivity towards peroxy radicals as well as on their remarkable regiospecificity towards oxidation and electrophilic substitution [7,8]. Burton and Ingold [9] have studied extensively the effects of chemical structure of phenolic compounds on the reactivity towards peroxyl radicals. By measuring the rate constant for hydrogen abstraction from the tocopherols and related phenols they found that α-tocopherol had the highest value



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

Fig. 1. Naturally occurring components of vitamin E.

($k_1 = 2.35 \times 10^6$ M^{-1} s^{-1} at 30°C) of all the compounds examined. It was concluded that the rate constant k_1 is determined primarily by the bond dissociation energy of the phenolic O–H bond, which is influenced by stereoelectronic effects as well as by constituent effects. They could demonstrate that the heterocyclic chromanol ring has an optimised structure for resonance stabilization of the unpaired electron of the α-tocopheroxyl radical and that electron-donating substituents e.g. methyl groups, increase this effect [9] (Fig. 2).

Besides its radical trapping properties, α-tocopherol can act as a strong reductant and as electrophilic agent in chemical reactions, depending on its environment. It has recently been shown that the chemical oxidation of α-tocopherol proceeds in a two-electron process, which does not involve the formation of any radical intermediate [10]. This mechanistic approach has led to a general understanding of the oxidation mechanism of α-tocopherol. The results indicate that the oxidation of α-tocopherol by non-radical processes is always accompanied by the formation of a transition state, or perhaps intermediate, which is characterized by a *para*-quinoid structure and its mesomeric *ortho*-quinone methide (Fig. 3). Investigations on the chemistry of the methide indicate that this important intermediate is reactive towards nucleophilic agents and also towards protic solvents. Reactions of the *ortho*-quinone methide that is formed by the phenolic OH-group and a ring methyl group always occur in the 5-position, never by the 7-methyl group. The preference of the 5-position, the so-called "Mills–Nixon-Effect", has been calculated and ascribed to simple changes in conjugative effects, as already discussed by Behan et al. [11]. Oxidative coupling in the absence of methyl groups, (e.g. in γ-tocopherol) still occurs in the 5-position for the same reasons. In the light of these results, previously reported investigations on the reactivity of α- and γ-tocopherol towards reactive oxygen and nitrogen species have to be re-evaluated [12,13].

1.3. Relationship between structure and biological activity of vitamin E

Natural-source and synthetic vitamin E are not identical. Unlike naturally occurring α-tocopherol, the synthetic form of vitamin E, *all-rac*-α-tocopherol, contains eight different stereoisomers arising from the three stereocenters of the molecule. The bioavailability and the biopotency of the various tocopherol derivatives and stereoisomers have been extensively studied in mammals by several authors [14–18]. Most studies assessing the biological activities of tocopherols are based on the classical rat foetal gestation–resorption assay [1,19] (Table 1).

The data of the rat gestation–resorption assay show a preference for the most abundant natural derivative of vitamin E, RRR-α-tocopherol. Despite the fact that the synthetic stereoisomers of α-tocopherol must possess, for theoretical reasons, equal antioxidant properties, they have impaired relative biological activities. This indicates that structural

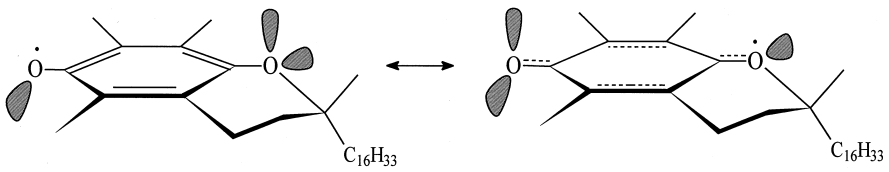


Fig. 2. The resonance forms of the α-tocopheroxyl radical.

features of α-tocopherol are of vital importance and that the antioxidant properties of tocopherols are not necessarily reflecting their biological activities. Consequently, alterations in biological activity of tocopherols are structure specific, including the presence or absence of ring methyl groups, stereochemistry of the chiral carbon centres, branching or desaturation of the side chain, respectively.

Numerous supplementation studies have been carried out in animals in order to assess the relative bioavailability of RRR-α-tocopherol compared to its stereoisomers in plasma and tissues [14,16,17,20]. In these studies a ratio of 1.36 for natural RRR-α-tocopherol relative to *all-rac*-α-tocopherol was established. In a previous study, the biodiscrimination of the eight α-tocopherol stereoisomers was followed over a period of 90 days. The stereoisomer profiles showed a remarkable preference in the accumulation of 2R forms in brain (74%), adipose tissue (74%), liver (70%) and plasma (86%) [21]. The somewhat lesser accumulation effect in liver was explained by its involvement in primary biodiscrimination. Time dependent changes in serum concentrations of all eight α-tocopherol stereoisomers were also assessed in humans under comparable conditions [22]. The authors of the human studies consistently conclude that the bioavailability of RRR-α-tocopherol is up to three times higher than that of *all-rac*-α-tocopherol. In phase studies it was also clearly shown that all 2R epimers compared to the 2S epimers are preferentially retained in man, which implies the existence of tocopherol binding factors with stereospecificity towards the natural RRR-stereoisomer of α-tocopherol [15].

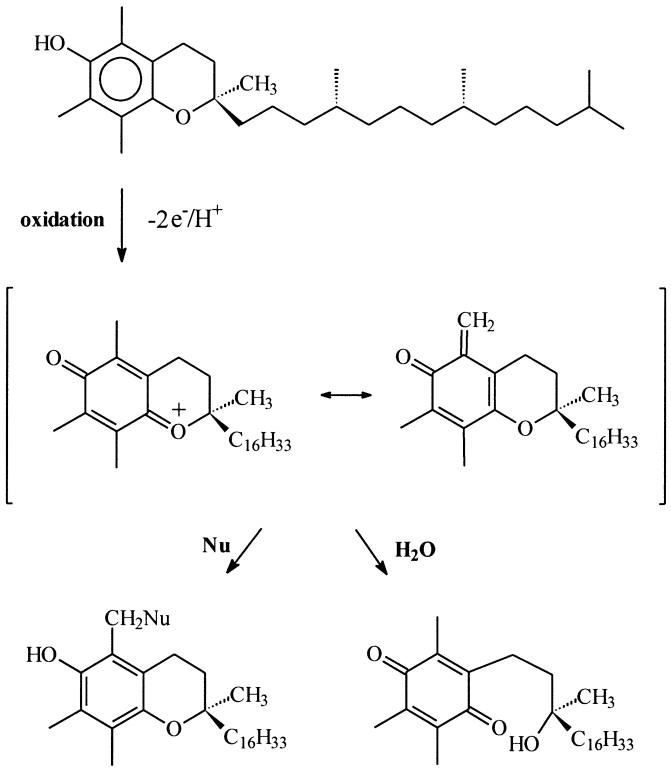


Fig. 3. Non-radical oxidation pathway of α-tocopherol.

The space filling models of the R- and S-epimers of α-tocopherol indicate that the configuration at C-2 has a major impact on the three dimensional structure of the molecule (Fig. 4). Changing the configuration from R to S at C-2 leads to inversion of the angle between the phytyl tail and the chromanol ring. The influence of the chiral centres at C-4′ and C-8′ on structural changes in the phytyl tail are less pronounced but have an impact on the biological activity of the corresponding isomers (see Table 1).

1.4. Availability in food

Vitamin E is an essential nutrient in the human body and thus it must be provided by foods and supplements [23]. The eight isomers of vitamin E are widely distributed in nature. Vitamin E has been detected in varying compositions (4–160 μg/g fresh weight) in all plants having been examined so far [24]. The richest sources of natural vitamin E are latex lipids with an exceptional high tocopherol content (8% w/v) followed by edible oils originating from plants (cf. Table 2) [25]. Sunflower seeds contain almost exclusively α-tocopherol as single-isomer product [26]. The strong correlation between the content of tocopherols and the amount of unsaturated fatty acids in plant oils suggests that vitamin E represents the most important antioxidant in plant tissues [27]. In contrast to plants mammalian tissues contain almost exclusively α-tocopherol. The highest content of α-tocopherol, (150 μg/g) is found in adipose tissue whereas erythrocytes have a relatively low content (2 μg/g) [28]. Investigations on the vitamin E content of microorganisms have revealed a non-uniform picture. The groups of Skinner, Powls and Woggon have investigated the α-tocopherol content of phototrophic algae [29–31]. Significant amounts of α-tocopherol were detected in the green algae *Chlorella* (7.6

Table 1
Relative biological activity of natural tocopherols, tocotrienols and synthetic stereoisomers of α-tocopheryl acetate (determined by the foetal resorption–gestation test in rats [19]

	Activity %
Natural derivatives	
RRR-α-tocopherol	100
RRR-β-tocopherol	57
RRR-γ-tocopherol	37
RRR-δ-tocopherol	1.4
R-α-tocotrienol	30
R-β-tocotrienol	5
Synthetic derivatives	
RRR-α-tocopheryl acetate	100
RRS-α-tocopheryl acetate	90
RSS-α-tocopheryl acetate	73
SSS-α-tocopheryl acetate	60
RSR-α-tocopheryl acetate	57
SRS-α-tocopheryl acetate	37
SRR-α-tocopheryl acetate	31
SSR-α-tocopheryl acetate	21

μg/g dry weight), *Stichococcus bacillaris* (134.2 μg/g dry weight), *Dunaliella salina* (63.8 μg/g dry weight) and *Cladophora stichotoma* (0.7 μg/g dry weight) as well as in the blue green alga *Anabaena variabilis* (213.5 μg/g dry weight) and also in the brown algae *Macrocystis integrifolia* (12.2 μg/g dry weight) and *Fucus distichus* (11.1 μg/g dry weight). No tocopherol has been found in the red algae *Gigartina corymbifera*, *Drionitis lanceolata* and in five yeast strains including *Torula 1N*.

1.5. Absorption and distribution in the body

Vitamin E requires, because of its hydrophobicity, special transport mechanisms in the aqueous environment of plasma, body fluids and cells. In humans, vitamin E is taken up in the proximal part of the intestine depending on the amount of food lipids, bile and pancreatic esterases. It is emulsified together with the fat-soluble components of the food. Lipolysis and emulsification of the formed lipid droplets then lead to the spontaneous formation of mixed micelles, which are absorbed at the brush border membrane of the mucosa by passive diffusion. Together with triglycerides, phospholipids, cholesterol and apolipoproteins, the tocopherols are re-assembled to chylomicrons by the *Golgi* of the mucosa cells [32]. The chylomicrons are stored as secretory granula and eventually excreted by exocytosis to the lymphatic compartment from where they reach the blood stream via the *ductus thoracicus*. The rather high clearance rate (24–48 h) of a bolus of vitamin E from the plasma and the concomitant rapid uptake by the liver parenchyma indicates that the intravascular degradation

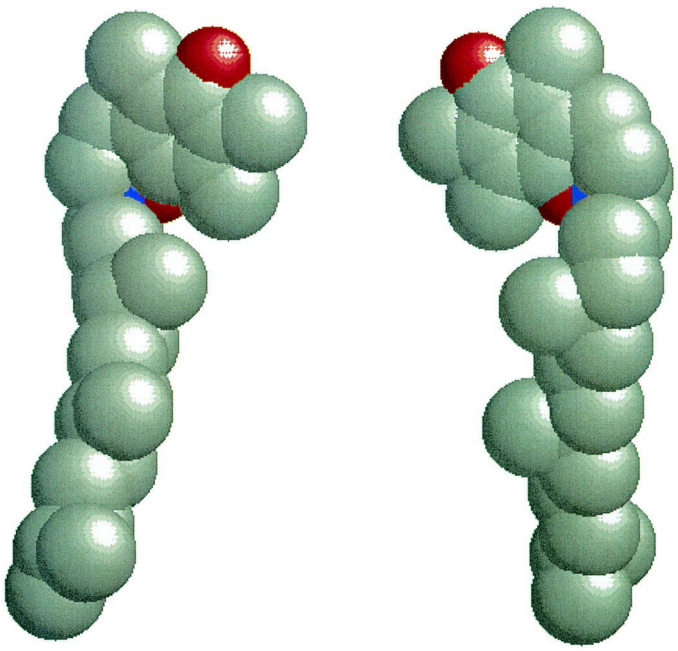


Fig. 4. Space filling models of natural RRR-α-tocopherol and its synthetic 2S-epimer SRR-α-tocopherol. Carbon atoms are shown in gray; C-2 is shown in blue, oxygen atoms in red, hydrogen atoms not shown.

Table 2
Typical vitamin E content of selected foods (based on α-tocopherol content) [25]

Food (100 g portion)	Vitamin E[a]	
	mg	IU
Wheat germ oil	119	178
Sunflower oil	49	73
Safflower oil	40	59
Peanut oil	19	28
Margarine, soft	14	21
Mayonnaise	13	19
Margarine, hard	11	16
Soybean oil	8.1	12
Butter	2.2	3.2
Wheat germ, stabilized	11	17
Oatmeal, rolled, cereal	1.3	2.0
Brown rice, boiled	1.3	2.0
Bread, whole wheat	0.5	0.8
Bread, white	0.1	0.2
Corn flakes, cereal	0.1	0.2
White rice, boiled	trace	0.1
Sunflower seeds, raw	50	74
Almonds	27	41
Peanuts, dry roasted	7.4	11
Peanut butter	6.2	9.2
Cashews	0.2	0.3
Liver, grilled	0.6	0.9
Shrimp, frozen, baked	0.6	0.9
Chicken, fried	0.6	0.9
Eggs	0.5	0.7
Bacon	0.5	0.7
Haddock	0.4	0.6
Chicken breast, grilled	0.4	0.6
Steak, grilled	0.3	0.5
Whole milk	trace	trace
Apples, fresh	0.3	0.5
Bananas, fresh	0.2	0.3
Cantaloupe, fresh	0.1	0.2
Strawberries, fresh	0.1	0.2
Asparagus, fresh	1.8	2.7
Spinach, fresh	1.8	2.7
Peas, fresh	0.6	0.8
Broccoli, fresh	0.5	0.7
Beans, baked	0.1	0.2
Potato, baked	trace	0.1

[a] 1 mg or 1 mg α-tocopherol equivalent to 1.49 IU.

of the chylomicrons to remnants by the endothelial lipoprotein lipase (LPL) is a prerequisite for the hepatic uptake of tocopherols [33]. Most probably the exchange between apolipoproteins of the chylomicrons (type AI, AII and B_{48}) and HDL (type C and E) triggers the formation of the remnants and in this way favours the rapid uptake of the tocopherols via the hepatic receptors for apo-E and apo-B [34–36]. This hypothesis is supported by the fact that, comparing subcellular compartments the highest concentrations of α-tocopherol are found in lysosomes (14.6 mmol/mol lipid) [37] (Fig. 5).

In contrast to the unspecific uptake of vitamin E from food by the liver cells, the specific α-tocopherol transfer protein (α-TTP) mediates the transfer of α-tocopherol from the hepatic lysosomes into lipoproteins [38]. This protein specifically separates α-tocopherol from all incoming tocopherols and promotes its net mass transfer into VLDL [39]. α-TTP has been shown to possess both stereospecificity as well as regiospecificity towards the most abundant isomer of vitamin E, RRR-α-tocopherol. As a consequence of the selective transfer mechanism, major parts of the natural homologues and non-natural isomers of α-tocopherol are excluded from the plasma and secreted with the bile [40]. Several studies have shown that α-TTP is expressed only in liver in significant amounts. Thus, the incorporation of extracellular α-tocopherol into extrahepatic tissues would relate to a series of unknown processes. Because of its hydrophobicity, α-tocopherol is mainly transported in association with lipoproteins in the plasma compartment. All plasma lipoproteins can constitute α-tocopherol vehicles, and the

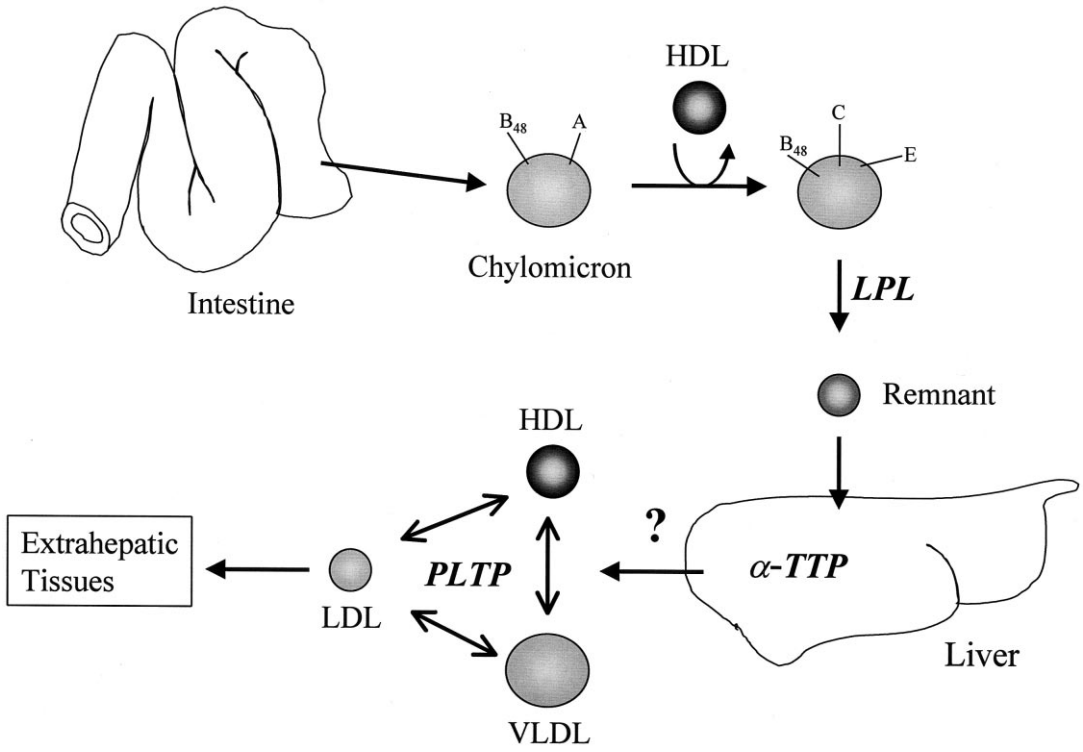


Fig. 5. Absorption, transport and distribution of the tocopherols.

contribution of distinct lipoprotein fractions to α-tocopherol transport actually depends on their relative proportions in one given plasma sample [41]. The plasma phospholipid transfer protein (PLTP), which is known to catalyse the exchange of phospholipids and other amphipatic compounds between lipid structures has been shown to facilitate the exchange of α-tocopherol between HDL and LDL [42].

Recently a cytosolic tocopherol binding protein with broad tissue distribution has been discovered in our group [43]. The function of this protein is still unknown and therefore it was given the name of tocopherol-associated protein (TAP). So far TAP has been shown to be ubiquitous, but more highly expressed in adult liver, prostate and brain tissue. Sequence homology of TAP ascribes it to a family of hydrophobic ligand binding proteins including α-TTP [44]. Another member of this family is the phosphatidylinositol-transfer protein (SEC14). This protein catalyses the transfer of phospholipids between membrane bilayers and plays an essential role in yeast Golgi function [45]. X-ray data of SEC14 provide structural insights in its function and new information concerning the architecture of the entire family of evolutionary conserved proteins [46] (Fig. 6).

The structural homology of TAP with phosphatidylinositol-transfer protein (SEC14) and its broad tissue distribution make TAP a possible candidate responsible for the regulation of tissue α-tocopherol levels. Nevertheless, the mechanisms by which vitamin E is transported,

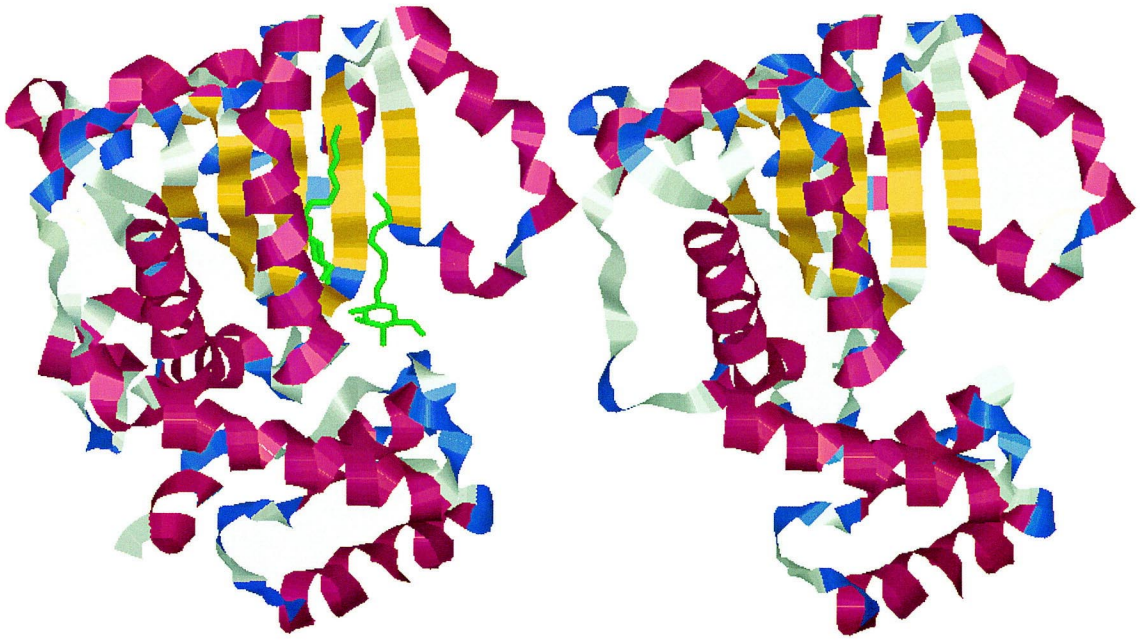


Fig. 6. Left: crystal structure of yeast phosphatidylinositol-transfer protein (SEC14), residues 1–246. Right: computational model (Swissmodel) of putative three-dimensional structure of human tocopherol associated protein (TAP), residues 1–246; helices are shown in red, β sheets in yellow, β turns in blue, backbone in grey and ligands in green.

regulated within cells and how it is involved in cellular signalling still remains obscure. Furthermore, the possibility that α-tocopherol acts similar to the retinol derivatives, is being considered. The major urinary metabolite (α-CEHC) of α-tocopherol has been discovered recently [47]. It appears in human urine after vitamin E supplementation and is formed directly from α-tocopherol without previous oxidative splitting of the chromane ring. The correlation of tocopherol intake and urinary excretion of α-CEHC was examined in human volunteers supplemented with RRR-α-tocopherol in the range from 0 to 800 mg/d. The analysis revealed that α-CEHC was only excreted above a daily intake of 150 mg α-tocopherol. This amount was interpreted as an indicator of plasma saturation by vitamin E (ca. 80 μM) and may be considered as marker of maximum vitamin E intake [48]. If prevention of oxidative damage and promotion of an optimal health status are the objectives, current estimates implicate that roughly an amount ten times higher than that recommended to prevent symptoms of deficiency is needed [49]. In the light of our current knowledge it might be reasonable to conclude that human supplementation with RRR-α-tocopherol is preferable in disease prevention.

2. Molecules may be provided with antioxidant capacity as well as with additional functions

The fact that α-tocopherol has properties that are independent of its antioxidant/radical scavenging ability does not represent an unusual situation. A number of bio-molecules are provided with an antioxidant function as well as additional properties, the latter being sometimes of bigger importance than the former. Among the multiple examples that could be cited here, only few will be discussed, which more clearly illustrate this notion.

2.1. Estrogens

Estriol and 17-β-estradiol belong to the family of estrogens and are naturally occurring antioxidants [50]. 2-Hydroxyestrone was found to posses an antioxidant activity 2.9-times higher than α-tocopherol as measured by the inhibition of lipid peroxidation. It was also found that the estrogens having an OH group at the aromatic ring have an ability to regenerate the tocopheroxyl to tocopherol with up to three orders of magnitude higher reactivity than ascorbic acid [51]. Exposure of LDL to physiological levels of 17-β-estradiol in a plasma milieu is associated with enhanced resistance to Cu^{2+}-mediated oxidation and incorporation of 17-β-estradiol derivatives into LDL. This antioxidant capacity could be indeed very important, being another means by which 17-β-estradiol limits coronary artery disease in women [52]. However, the most evident function of estrogens, responsible for the determination of secondary sexual characters, is unrelated with their antioxidant activity.

2.2. Retinol

At low oxygen partial pressure and low concentrations, *all-trans*-retinol behaves as an effective antioxidant [53–55]. Vitamin A as well increases the antioxidant potential of the tissues [56]. Age-related macular degeneration correlates with low plasma concentrations of

carotenoids [57]. However, the principal function of retinol in rhodopsin and vision is not related with its antioxidant properties.

2.3. Melatonin

Melatonin has been involved in ageing and age-related diseases as a free radical scavenger [58,59] and it may act as an antioxidant in the brain. Hydroxyl radicals and peroxyl radical in vitro are scavenged more effectively by melatonin than by glutathione and vitamin E respectively [59]. Due to its antioxidant effects, it protects against lesions induced by ischemia-reperfusion [60]. All these events, however, are not associated with the function of melatonin related to the sleep-wake regulation in humans that occurs through a receptor-mediated signalling function [61–65]. Further, with a non-antioxidant mechanism, melatonin regulates the glucocorticoid receptor [66] and blocks the activation of estrogen receptor for DNA binding [67].

2.4. Polyphenols and flavonoids

Conclusive evidence for its absorption by human subjects in biologically significant amounts of polyphenols is lacking [68]. However, significant effects of polyphenols have been observed in vitro that have been attributed to both antioxidant and non-antioxidant properties of this molecule.

A modulation of the ultraviolet A radiation activation of haem oxygenase, collagenase and cyclooxygenase gene expression by epigallocatechin has been found in human skin cells. These effects may involve direct action on signal transduction as well as changes that may be associated with its antioxidant activity [69]. Tea polyphenols induce in vitro inhibition of cell proliferation, cell apoptosis and cell cycle arrest. These events may be related to their binding properties to the epidermal growth factor receptor [70].

Flavonoids as well have properties that may be due to their radical scavenging function but also effects that are produced through inhibition of various enzymes, recently shown to play an important role in signal transduction and cell transformation [71].

The bioavailability in human subjects of non-nutrient dietary flavonoids and phyto-oestrogens is of great importance relative to their reported health protective effects. Human subjects [72] absorb the isoflavone phyto-oestrogen genistein. However, whether in vivo it acts as an antioxidant is doubtful due to its potent effect at low concentrations in inhibiting tyrosine kinases.

2.5. Carotenoids, vitamin A and retinoids

All-trans retinoic acid is the major biologically active form of vitamin A, and nuclear retinoid receptors are the major mediators of *all-trans* retinoic acid actions [73,74]. Retinoic acid metabolites of vitamin A are key regulators of gene expression involved in embryonic development and maintenance of epithelial tissues [75]. The absence of retinoic acid receptor gamma is associated with a loss of the retinoic acid-inducible expression of the Hoxa-1, Hoxa-3, laminin B1, collagen IVα1, GATA-4, and BMP-2 genes [76]. Furthermore, the loss of

retinoic acid receptor gamma is associated with a reduction in the metabolism of *all-trans*-retinoic acid to more polar derivatives, while the loss of retinoic acid receptor α is associated with an increase in metabolism of retinoic acid [76]. Retinoic acid also induces osteopontin gene expression, in concert with vitamin D [77], *all-trans* retinoic acid 4-hydrolase [78] and suppresses that of collagenase [79]. Carotenoids, both with and without provitamin A function, upregulate gap junctional communication and connexin43 gene expression in human dermal fibroblasts and inhibit carcinogen-induced neoplastic transformation [80–82]. All the above effects are caused by antioxidant independent mechanisms.

2.6. Vitamin C

Calcineurin (protein phosphatase 2B) is inhibited by superoxide radicals that are capable of oxidizing the Fe^{2+} of the enzyme active center to Fe^{3+}. The enzyme can be activated by ascorbate but not by other strong reducing agents, such as dithiothreitol or mercaptoethanol indicating that ascorbate plays a more specific role than that of a generic antioxidant [83]. Since inactivation of calcineurin (by cyclosporin A or FK506), results in the inhibition of interleukin-2 gene expression, its specific (not obtained by other reducing agents) activation by ascorbate may be on the basis of an immune response enhancement by vitamin C [84]. Although it has been suggested [85] that vitamin C acts as a pro-oxidant, it appears that such events do not take place under physiological conditions [85–89].

2.7. α-Tocopheryl quinone

α-Tocopheryl quinone is an oxidized product of α-tocopherol and an efficient antioxidant [90]. α-tocopherol shows very modest anticoagulant activity. In contrast, tocopherylquinone is a potent anticoagulant as an inhibitor of the vitamin K-dependent carboxylase that controls blood clotting. This action is unrelated to the antioxidant properties of the α-tocopheryl hydroquinone and requires attachment of the active site thiol groups of the carboxylase to one or more methyl groups on tocopherylquinone [91].

2.8. Molecular mechanism of tocopherol protection against free radicals

Lipid peroxyl radicals present in the plasma membrane interact with α-tocopherol whereby a lipid peroxide and the α-tocopheroxyl radical result. Ascorbic acid, by donating electrons to the α-tocopheroxyl radical reduces it back to α-tocopherol. This property makes ascorbic acid, the primary plasma and cell antioxidant. Recycling of α-tocopherol by ascorbate helps to protect membrane lipids from peroxidation [92].

The antioxidant capacity of α-tocopherol is also evident in low-density lipoproteins. The tocopherol mediated radical propagation is observed at a low rate of radical flux, and it is suppressed by reductants, such as ascorbic acid and ubiquinols [93–96].

2.9. Pro-oxidant effect of tocopherols

It has been found [97,98] that lipid peroxidation in LDL induced by a steady flux of aqueous

peroxyl radicals declines as vitamin E is consumed, (ii) is faster in the presence of vitamin E than following its complete consumption, (iii) is substantially accelerated by enrichment of the vitamin in LDL, either in vitro or by diet, and (iv) is virtually independent of the applied radical flux. It was thus proposed that the vitamin E radical (i.e. α-tocopheroxyl radical) propagates peroxidation within lipoprotein particles unless it becomes reduced by vitamin C (or ubiquinol-10). The importance of pro-oxidation reactions of α-tocopherol in vivo, under physiological conditions however appears to be questionable.

2.10. Anti-nitrating action of tocopherols

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, Lipid hydroperoxide formation in liposomes was reported to be inhibited more effectively by γ-tocopherol than α-tocopherol. From these results it was concluded that α-tocopherol action is that of an antioxidant and that γ-tocopherol is required to effectively remove the peroxynitrite-derived nitrating species [99]. However, Goss et al. [100] concluded that α-tocopherol alone is sufficient to remove any peroxynitrite-derived reactive nitrogen species, as the presence of γ-tocopherol attenuates nitration of both γ-tocopherol and tyrosine [100].

3. Non-antioxidant molecular mechanisms of tocopherols

As discussed in the introduction, some properties of tocopherol cannot be assigned to their known antioxidant or pro-oxidant function. A number of those properties are discussed below.

3.1. Effects of α-tocopherol on protein kinase C

Protein kinase C has been originally suggested to be regulated, at a cellular level, by α-tocopherol [101–104]. A number of reports have subsequently confirmed this finding in different cell types, including monocytes, macrophages, neutrophils, fibroblasts and mesangial cells [105–118]. Animal work has also confirmed the importance of protein kinase C inhibition by α-tocopherol in vivo [119,120].

The activity of PKC from monocytes is inhibited by α-tocopherol in a specific manner compared with that of β-tocopherol (see Tables 3 and 4) or Trolox(R). This event leads to inhibition of phosphorylation and translocation of the cytosolic factor p47(phox) and to an impaired assembly of the NADPH-oxidase and of superoxide production [121]. α-Tocopherol also produces a significant decrease in monocyte superoxide anion release, lipid oxidation, interleukin-1 β (IL-1 β) release and adhesion to endothelium. A similar antioxidant, β-tocopherol, had no effect on IL-1 release. Thus, α-tocopherol has the novel biological effect of inhibiting the release of the proinflammatory cytokine, IL-1 β, via the inhibition of the 5-lipoxygenase pathway [122].

The inhibition by α-tocopherol of protein kinase C activity and of proliferation are parallel events in vascular smooth muscle cells. Inhibition is observed to occur at concentrations of α-

tocopherol close to those measured in healthy adults [123–125]. While α-tocopherol inhibits protein kinase C activity, β-tocopherol is ineffective. When both are present, β-tocopherol prevents the inhibitory effect of α-tocopherol. The inhibition by α-tocopherol and the lack of inhibition by β-tocopherol of cell proliferation and protein kinase C activity shows that the mechanism involved is not related to the radical scavenging properties of these two molecules, which are essentially equal [126]. The measurements at both protein kinase C and proliferation level of the effect of the natural form of α-tocopherol relative to the racemic mixture of the phytyl chain isomers shows that the former is twice as potent as the latter (Özer et al., unpublished).

3.1.1. Inhibition by α-tocopherol is not caused by a direct interaction with protein kinase C

Addition of α-tocopherol to recombinant protein kinase C in the test tube does not result in inhibition of protein kinase C. Inhibition of protein kinase C is obtained only at a cellular level. Protein kinase C activity increases during the cell cycle progression, reaching a maximum in the late G_1-phase. The time of addition of α-tocopherol during the cell cycle determines the extent of protein kinase C inhibition. If α-tocopherol is added in the Go-phase of the cycle and incubated for 7 h, in the absence of foetal calf serum, no inhibition is observed. If, together with α-tocopherol, foetal calf serum is added and cells are stimulated to enter in the G_1-phase, after 7 h of incubation inhibition of protein kinase C by α-tocopherol is observed. If α-tocopherol is added in the G_1-phase (and protein kinase C activity measured after 7 h) α-tocopherol shows no inhibition.

3.1.2. α-Tocopherol does not inhibit protein kinase C expression

When smooth muscle cells are supplemented in the G_0 phase with foetal calf serum, a time-dependent α-tocopherol sensitive increase in protein kinase C activity is observed. A mRNA analysis carried out for the different isoforms (protein kinase C-α, protein kinase C-δ, protein kinase C-ε, and protein kinase C-ζ) does not show any significant change. Similarly, the protein levels expressed during the transition are essentially the same. α-Tocopherol does not affect the mRNA of the protein kinase C isoforms. The protein level of protein kinase C after 7 h incubation with α-tocopherol is slightly increased (14%) rather than decreased.

3.1.3. α-Tocopherol inhibits protein kinase Cα phosphorylation-state and its activity

Nanomolar concentrations of the indolocarbazole Gö 6976 are known to inhibit protein kinase C-α and β1, whereas even micromolar concentration of Go 6976 have no effect on the activity of protein kinase C-δ, protein kinase C-ε and protein kinase C-ζ [127]. After inhibition of protein kinase C-α by Gö 6976, the residual protein kinase C activity is not sensitive to α-

Table 3
Comparison of α- and β-tocopherol antioxidant properties (from ref. [126]

Compound	Relative antioxidant potency	Stoichiometry factor
α-Tocopherol	[1]	[2]
β-Tocopherol	0.89	2.04

Table 4
Effect of α-tocopherol and β-tocopherol on protein kinase C-α phosphorylation state, autophosphorylating activity and activity towards histone III-S [104]

	^{32}P-Protein kinase C-α (%)	Autophosphorylating activity of protein kinase C-α (%)	Histone activity (%)	Cell proliferation
PMA	100	100	100	100
α-Tocopherol	18.5	36.4	56.0	30
β-Tocopherol	74.1	84.9	79.0	90

tocopherol. From this experiment it can be concluded that the isoforms of protein kinase C-δ, ε, ζ and μ are not involved in the α-tocopherol induced protein kinase C inhibition. The experiments suggest that protein kinase C-α is the specific target of α-tocopherol.

If protein kinase C is immunoprecipitated from cells incubated in the presence of ^{32}P and α-tocopherol with antibodies specific for protein kinase C-α, inhibition of protein kinase C phosphorylation is observed. β-Tocopherol is much less potent in inhibiting protein kinase C phosphorylation. Pre-treatment of smooth muscle cells with α-tocopherol (but not with β-tocopherol) results in inhibition of the immunoprecipitated protein kinase C-α activity [128].

3.1.4. Effects on PP2A

The experiments presented above suggest that protein kinase C activation occurring in the G_0 to G_1 transition is related with a change of phosphorylation of the enzyme. Moreover, the inhibitory effect of α-tocopherol has been correlated with a dephosphorylation of protein kinase C-α. PP_2A has been found in vitro to be activated by the treatment with α-tocopherol [128]. This event may be crucial in the dephosphorylation of protein kinase C with its consequent diminution of activity.

3.2. Effect on gene expression

Using a differential display technique several candidate cDNAs of differentially expressed mRNAs have been detected. Sequence analysis of one of the cDNA fragments and comparison with DNA sequence databases has revealed 100% homology with the 3′ region (exon 9b) of the α-tropomyosin isoform TMBr-2. The mRNA level increased transiently in the α-tocopherol treated, synchronously growing cells, reaches a maximum after 2 h of restimulation. Maximum protein expression was observed after 4 h. After 7 h, mRNA and protein levels had returned to baseline levels. In the light of these observations, it might be possible that the induction of this tropomyosin isoform is an early event caused by α-tocopherol and leading to cell proliferation inhibition. Another gene, that of smooth muscle cells scavenger receptor (CD36), is down-regulated by α-tocopherol (but not by β-tocopherol) at a transcriptional level [129].

3.3. Effects of tocopherols on cell proliferation

α-Tocopherol at concentrations of 50 μM inhibits rat A7r5 smooth muscle cell proliferation, while β-tocopherol is ineffective. When α-tocopherol and β-tocopherol are added together, no

inhibition of cell growth is seen. Both compounds are transported equally in cells and do not compete with each other for the uptake [130]. The prevention by β-tocopherol of the proliferation inhibition by α-tocopherol suggests a site-directed event as the basis of α-tocopherol inhibition rather than a general radical scavenging reaction. The oxidized product of α-tocopherol, α-tocopherylquinone, is not inhibitory, indicating that the effects of α-tocopherol are not related to its antioxidant properties [130]. α-Tocopherol is not only responsible for the proliferation control of smooth muscle cells but it exhibits similar functions in a number of different cell lines (Table 5).

δ-Tocopherol, α-tocopherol and γ-tocopherol are (within experimental error) equally inhibitory [102]. On the other hand it appears that the inhibition by β-tocopherol is ten-fold less potent relative to the others compounds. Tocotrienols, although possessing a greater antioxidant activity than tocopherols [131], inhibit cell proliferation to the same extent [102]. Janero et al. have shown in a series of 6-hydroxy-chroman-2-carbonitrile tocopherol derivatives whose antioxidant properties strongly depend upon the nature and length of their side chains [132]. These compounds were tested in smooth muscle cells (A7r5) and their relative potency in inhibiting cell proliferation established. A Student *t*-test analysis between the two data sets (antioxidant and antiproliferative activity) has given a $p = 0.006$, indicating a lack of significant correlation between them [102]. Probucol, a potent hydrophobic antioxidant, similar in its general properties to RRR-α-tocopherol has been shown not to inhibit smooth muscle cell proliferation, but to prevent the inhibition by α-tocopherol, as is the case for β-tocopherol.

3.4. Other situations in which tocopherol effects result from a non-antioxidant action

One of the best evidence of a non-antioxidant function of α-tocopherol is related to its recognition and transfer. α-Tocopherol transfer protein in the liver specifically sorts out RRR-α-tocopherol from all incoming tocopherols in order to incorporate it into plasma lipoproteins. Relative affinities (RRR-α-tocopherol = 100%) calculated from the degree of competition are as follows: β-tocopherol, 38%; γ-tocopherol, 9%; δ-tocopherol, 2%; α-tocopherol acetate, 2%; α-tocopherol quinone, 2%; SRR-α-tocopherol, 11%; α-tocotrienol, 12%; trolox, 9% [39].

These observations indicate that such a stereospecific binding cannot be due to the antioxidant properties of the molecule. Absorption of δ-tocopherol is also selective relative to the different methyl derivatives of the chromane ring [133–135].

α- or δ-Tocopherol or both equally induce the expression of the hepatic mRNA for the α-tocopherol transfer protein. This indicates that, due to the different radical scavenging properties of these molecules, the phenomenon cannot be considered to be antioxidant in nature [135].

A dissimilar protection of tocopherol isomers against membrane hydrolysis by phospholipase A2 has been shown, suggesting biological actions of compounds with vitamin E activity other than their classical roles as antioxidants [136].

Interestingly, γ-tocopherol, which is a homologue of α-tocopherol with a comparable antioxidative capacity, showed only a weak suppression of SR (scavenger receptor: as found in monocytes) activity, SR-A expression and AP-1 (activator protein-1: a transcription factor) activity. These observations point to the conclusion that the reduction of SR-A expression and

activity in presence of α-tocopherol is possibly related to its direct action on cell signalling [137].

A unique tissue distribution of tocopherols is also indicative of selective mechanisms for maintaining in each tissue some but not all of those molecules needed to specifically regulate cellular functions. By simultaneous determination of individual tocopherols, tocotrienols, ubiquinols and ubiquinones a variety of hairless mouse tissues were analysed. Brain contained virtually only α-tocopherol (5.4 ± 0.1 nmol/g; 99.8%) and no detectable tocotrienols were found. By contrast, skin contained nearly 15% tocotrienols and 1% γ-tocopherol. In other tissues, the α-tocopherol content was higher (20 nmol/g), while each of the other forms represented about 1% of the total (γ-tocopherol 0.2–0.4 nmol/g, α-tocotrienol 0.1, γ-tocotrienol 0.2). Ubiquinol-9 concentrations were highest in kidney (81 nmol/g) and in liver (42 nmol/g), while the highest ubiquinone-9 concentrations were found in kidney (301 ± 123 nmol/g) and heart (244 ± 22 nmol/g). Liver contained nearly identical concentrations of each of the redox couple ubiqu inol-9 (41 ± 16 nmol/g) and ubiquinone-9 (46 ± 18 nmol/g) [138].

Plasma α- and γ-tocopherol were found in pregnant women to change their ratio during gestation and to be different from non-pregnant women, suggesting a specific role for these tocopherols [139].

Table 5
The growth inhibitory effect of α-tocopherol on different cell lines

Sensitive lines	Insensitive lines	Tissue and origin
A10		Rat aorta smooth muscle
A7r5		Rat aorta smooth muscle
T/G		Human aorta smooth muscle
NB2A		Mouse neuroblastoma
Balb/3T3		Mouse fibroblast
Human fibroblast		Primary cell lines
DU-145, PC-3		Human Prostate Cancer
Human Pigmented Retinal Epithelial Cells		hPRE
LNCaP		Human Prostate Cancer (androgen sensitive)
Human leukaemia		U937
Mouse fibroblast		Balb/c-3T3
Glioma		C6
	P388 Dl	Mouse monocyte macrophage
	LR73	Chinese hamster ovary
	Saos-2	Human osteosarcoma
	Human hepatocarcinoma	HepG2
	Human colon adenocarcinoma	CaCo2

3.5. Non-antioxidant molecular mechanisms of γ-tocopherol and δ-tocopherol action

Peroxynitrite, a powerful oxidant and nitrating species, is formed by the near diffusion-limited reaction of NO and O^{2-}. Chronic inflammation induced by phagocytes is a possible source of peroxynitrite and a major contributor to cancer and other degenerative diseases. γ-Tocopherol at the nucleophilic 5-position is capable of scavenging peroxynitrite. These results suggest that γ-tocopherol effectively removes, by a non-antioxidant mechanism, the peroxynitrite-derived nitrating species [99]. Nitric oxide released by macrophages during inflammation reacts with active oxygen to form peroxynitrite. Peroxynitrite nitrates protein and peroxidizes lipids. γ-Tocopherol traps peroxynitrite and is more effective than α-tocopherol in protecting lipids against such peroxidation [140]. However, more recently it was found that nitration of γ-tocopherol becomes significant only after levels of α-tocopherol have been depleted [100].

Vitamin E inhibition of O^{2-} production in the promonocyte cell line THP-1 is essentially due to RRR-δ-tocopherol [141].

3.6. Non-antioxidant molecular mechanisms of tocotrienol effects

Tocotrienols and δ-tocopherol (but not other tocopherols) have been shown to inhibit proliferation of breast cancer cells in vitro [142]. δ-Tocopherol, α-tocopherol and γ-tocopherol are, within experimental error, equally inhibitory. [102]. On the other hand it appears that the inhibition by β-tocopherol is ten-fold less potent relative to the other compounds. Tocotrienols, although possessing a greater antioxidant activity than tocopherols [131], inhibit cell proliferation to the same extent [102].

4. Disease and α-tocopherol

The disease that has best been directly linked with an α-tocopherol deficiency is ataxia with vitamin E deficiency (AVED). Other diseases have a less clear relationship. Epidemiological (as well as, in some cases, direct intervention studies) have indicated a probable involvement of vitamin E deficiency in the pathogenesis of atherosclerosis, diabetes and of some types of cancer, as well as a modulation of the inflammatory and immune responses.

However, to what extent the beneficial effects of vitamin E can be referred to an antioxidant or a non-antioxidant mechanism cannot be assessed at the present time.

4.1. Ataxia with vitamin E deficiency

AVED, or familial isolated vitamin E deficiency is an uncommon autosomal recessive neurodegenerative disease, whose clinical presentation is remarkably similar to that of Friedreich ataxia. AVED is caused by mutations in the gene for α-tocopherol transfer protein (α-TTP). Therapeutic and prophylactic vitamin E supplementation prevents the onset of the disease before irreversible damage develops [143–153].

4.2. Atherosclerosis

Low levels of α tocopherol have been associated with increased risk of coronary artery disease and increased intake has been shown to be protective [154–156]. Data support a pathogenetic role for oxidized LDL in atherosclerosis and a number of studies show that α tocopherol reduces the susceptibility of LDL to oxidation [96]. In addition, α-tocopherol can cause partition in the artery smooth muscle cells, monocyte-macrophages, endothelial cells, and platelets, exerting advantageous effects. The anti-atherogenic effects of α-tocopherol on crucial cells in atherogenesis such as the inhibition of smooth muscle cell proliferation, preservation of endothelial function, inhibition of monocyte-endothelial adhesion, inhibition of monocyte reactive oxygen species and cytokine release, and inhibition of platelet adhesion and aggregation [107] may be at the basis of α-tocopherol effect.

Furthermore, plasma phospholipid-transfer protein prevents vascular endothelium dysfunction by delivering α-tocopherol to endothelial cells [157].

In several animal studies it has been shown that α-tocopherol cannot be substituted by other antioxidants [119,158–160].

4.3. Cancer

In intervention studies, vitamin E treated probands had fewer incidents of prostate and colorectal cancers compared to the group not receiving vitamin E. Protection by α-tocopherol seems to occur also for certain types of breast cancers [154,161–163]. Induction of apoptosis in human breast cancer cells by tocopherols and tocotrienols has been also described [142] and the effect is specific for α-, γ- and δ- tocotrienols and δ-tocopherol.

4.4. Diabetes

In vivo as well as in vitro evidence that PKC activation could be responsible for the hyperglycaemia-induced vascular dysfunctions in diabetes has been provided. Animal and clinical studies have shown that high doses of vitamin E treatment can apparently reverse some of the changes in the retinal and renal vessels. [164–166].

4.5. Inflammation

α-Tocopherol addition to polymorphonuclear cells results in inhibition of O^{2-} generation [110,111,121]. Vitamin E has been shown to protect well against polymorphonuclear leukocyte-dependent adhesion to endothelial cells [167]. Monocytes, after α-tocopherol supplementation, show a significant decreases in release of reactive oxygen species, lipid oxidation, IL-1 beta secretion and monocyte-endothelial cell adhesion, both in resting and activated cells. Inhibition of the release of reactive oxygen species and of lipid oxidation is due to an inhibition of protein kinase C activity by α-tocopherol [105]. All the above described effects are α-tocopherol specific. During hypoxia NF-kappa B activation takes place, with associated transcriptional activation of the IL-6 gene. α-Tocopherol inhibits NF-kappa B activation induced by hypoxia. It is improbable that this event is related to reactive oxygen intermediates, given the fact that

the entire phenomenon takes place in the absence of oxygen [168]. During inflammation γ-tocopherol traps peroxynitrite and is more effective than α-tocopherol in protecting lipids against such peroxidation [140].

4.6. Immune response

Vitamin E, in amounts greater than currently recommended, enhances certain clinically relevant in vivo indexes of T-cell-mediated function in healthy elderly persons [169–171].

5. Final considerations

In the above discussion emphasis has been given to three notions. In general molecules can be provided with different properties, and the importance of one does not exclude the existence of a second and may be of a third property. Research can certainly profit from the investigation of hidden properties of compounds provided with already well-known functions. The second and more precise paradigm has been that of the identifications of some antioxidants provided with different and more specific functions. One of the most important consequences of this concept is that through specific recognition interactions more precise and site directed events could take place in a cell. This idea points to the uniqueness of some natural compounds, whose combination of antioxidant and non-antioxidant properties cannot be imitated or substituted for by simple synthetic antioxidants. Thirdly, oxidant sensitive molecules change their concentration as a function of their more or less oxidant environment. This concentration change acts as a probe of the surroundings and produces signals apt to regulate cellular events. Future research should be directed towards understanding and dissecting in more detail the cellular effects of these bifunctional molecules and to create mimics that may posses more specific and more precise properties.

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