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Invited Review

Anti-atherosclerotic effects of vitamin E - myth or reality?

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Abstract

Atherosclerosis and its complications such as coronary heart disease, myocardial infarction and stroke are the leading causes of death in the developed world. High blood pressure, diabetes, smoking and a diet high in cholesterol and lipids clearly increase the likelihood of premature atherosclerosis, albeit other factors, such as the individual genetic makeup, may play an additional role. Several epidemiological studies and intervention trials have been performed with vitamin E, and some of them showed that it prevents atherosclerosis. For a long time, vitamin E was assumed to act by decreasing the oxidation of LDL, a key step in atherosclerosis initiation. However, at the cellular level, vitamin E acts by inhibition of smooth muscle cell proliferation, platelet aggregation, monocyte adhesion, oxLDL uptake and cytokine production, all reactions implied in the progression of atherosclerosis. Recent research revealed that these effects are not the result of the antioxidant activity of vitamin E, but rather of precise molecular actions of this compound. It is assumed that specific interactions of vitamin E with enzymes and proteins are at the basis of its non-antioxidant effects. Vitamin E influences the activity of several enzymes (*e.g.* PKC, PP2A, COX-2, 5-lipooxygenase, nitric oxide synthase, NADPH-oxidase, superoxide dismutase, phopholipase A2) and modulates the expression of genes that are involved in atherosclerosis (*e.g.* scavenger receptors, integrins, selectins, cytokines, cyclins). These interactions promise to reveal the biological properties of vitamin E and allow designing better strategies for the protection against atherosclerosis progression.

Keywords: vitamin E • atherosclerosis • non-antioxidant • gene expression • signalling • transcription factors • tocopherol binding proteins • clinical trials

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Introduction

Vitamin E has been entrusted with therapeutic properties against cardiovascular disease (CVD) for almost 60 years (Vogelsang & Shute, 1946) [1]. Not accepted in the beginning, confirmed at a later date by a number of epidemiological studies, but denied by some others, Dr. Even Shute's discovery is still a subject of controversy. The fact that most of the clinical studies have been carried out without a detailed knowledge of the cellular functions of vitamin E, made an evaluation of epidemiological data more difficult. The beneficial effects of vitamin E, considered to be one of the most effective natural hydrophobic scavengers for reactive oxygen and nitrogen species, were supposed to be due to its antioxidative properties. Although lipid peroxidation in the subendothelial space is assumed to be a central event in the development of atherosclerosis, the ability of vitamin E to prevent this process and the progression of *in vivo* lesions remains uncertain. Alternative non-antioxidant mechanisms of vitamin E action have been recently suggested, such as its involvement in cellular signalling, interference with enzymatic activity, apoptosis and gene expression modulation [2-4].

Vitamin E

Vitamin E - structure, occurrence in food, analogues

Natural vitamin E is a mixture of tocopherols and tocotrienols (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol), synthesized only by plants. All of them consist in a chromanic nucleus (6-chromanol) with an aliphatic side chain (C16), which is saturated for tocopherols, with three chiral centers occurring in the RRR stereoisomeric configuration, and unsaturated for tocotrienols, with three double bonds at 3', 7', and 11' positions.

The relative antioxidant potency of tocopherols is rather similar, with α > β > γ > δ. However, at the molecular level clearly distinctive biological effects have been observed that cannot be the consequence of the differences in antioxidant activity. RRR-α-tocopherol is the most abundant form in human plasma (about 25 μmol/l), while γ-tocopherol reaches only 10% of this value, even in the USA population, where the γ-tocopherol is predominant in foods. This specificity is most likely the consequence of selective retention of α-tocopherol at the hepatic level, or alternatively, due to the metabolic conversion and subsequent elimination of the other analogues.

The natural vitamin E sources are vegetal oils: sunflower seeds contain almost exclusively α-tocopherol (59.5 mg / 1g of oil), soy oil is rich in γ-, δ-, and α-tocopherol (62.4, 20.4, 11.0 mg / 1g of oil, respectively), and palm oil contains tocotrienols (17.2 mg / 1g of oil) in addition to α-tocopherol (18.3 mg / 1g of oil) [5].

Vitamin E is commercially available in different forms: a mixture of natural tocopherols and tocotrienols (extracted from natural sources), RRR-α-tocopherol, synthetic α-tocopherol, which is an equi-molar racemic mixture of 8 stereoisomers (*all rac*-α-tocopherol), or a mixture of the synthetic tocopheryl esters (α-tocopheryl-succinate or α-tocopheryl-acetate).

Vitamin E acetate, the most often used analogue in food supplements and cosmetic products, is more stable due to its esterification and consequent protection from oxidation. In the guts, vitamin E esters are split to their unesterified forms under the action of intestinal esterases. Other vitamin E analogues, such as α-tocopheryl-succinate, α-tocopheryl-phosphate or trolox, have been also synthesized, and their cellular effects investigated [6]. These molecules often act as completely novel compounds, are differently transported and have their own effects on cellular signalling and apoptosis [6]. The biological effectiveness *in vivo* of the four forms of vitamin E has been established as α >> γ > δ > β [7].

Vitamin E absorption and transport

In humans vitamin E is taken up together with nutritional lipids in the proximal part of the intestine and released in the lymph within chylomicrons (Fig. 1). All forms of vitamin E are equally absorbed, which suggests the absence of selectivity at this level. After passing through the lymphatic pathway the chylomicrons reach the systemic circulation, and are progressively hydrolyzed

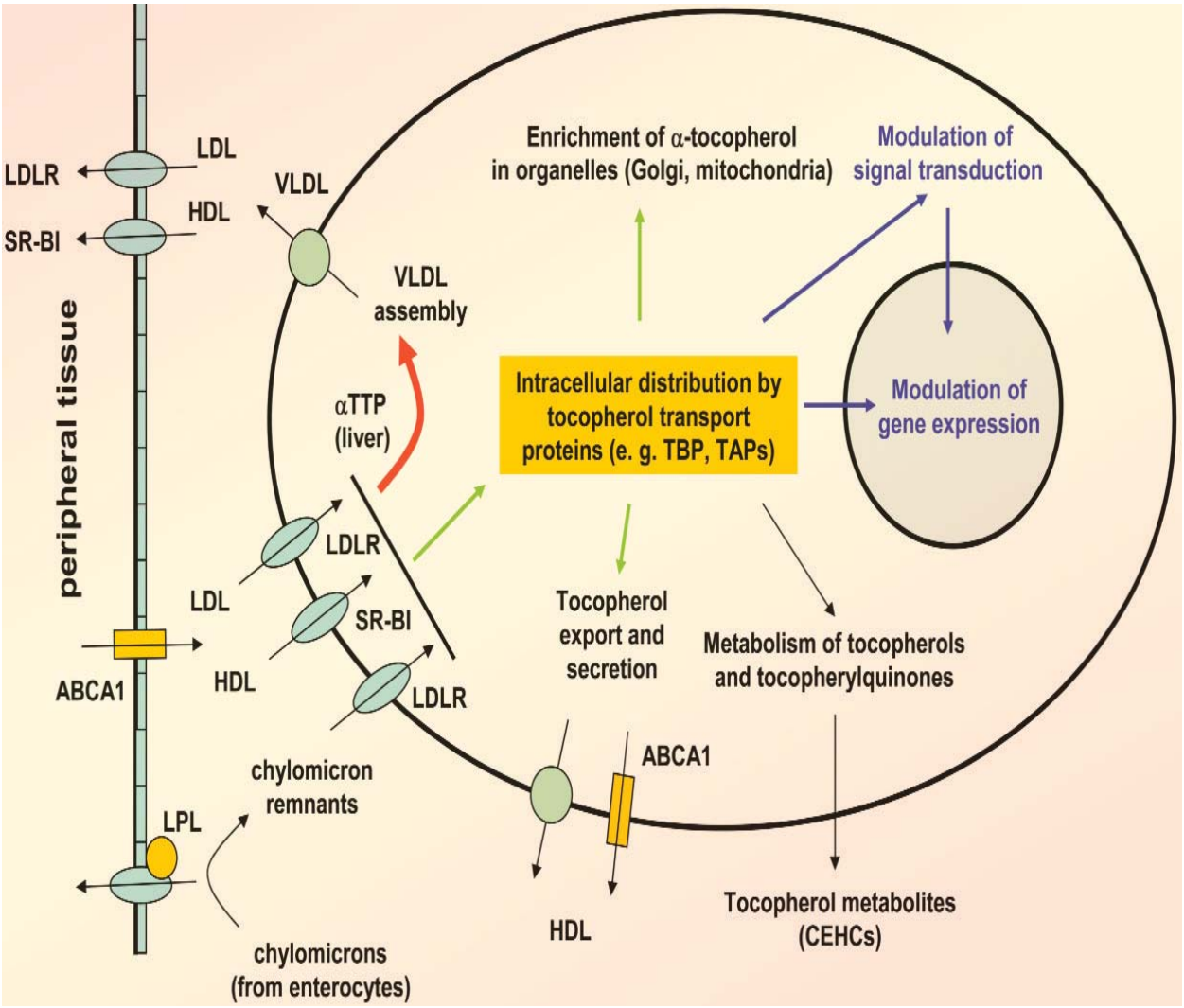

Fig. 1 The four tocopherol forms are equally taken up from the diet and transported to peripheral cells by chylomicrons. After hydrolysis by endothelial lipoprotein lipase (LPL), part of the transported tocopherols is released from chylomicrons to peripheral tissues. The chylomicron remnants deliver the remaining vitamin E to the liver mainly *via* the LDL-receptor (LDLR), and α-tocopherol is specifically recognized by α-TTP (red arrow, occurring only in the liver), incorporated into VLDL and re-distributed to peripheral tissues in LDL and HDL *via* LDL-receptor or scavenger receptor SR-BI, respectively. The remaining tocopherols (β-, γ-, δ-, and also excess α-tocopherol) and tocopheryl-quinones are metabolized and eliminated. Thus, the α-TTP mediated α-tocopherol salvage pathway is essential to maintain an adequate plasma α-tocopherol concentration. In the cells, the tocopherols modulate the enzymatic activity (*e.g.* PKC/PP2A, 5-lipooxygenase, phospholipase A2, cyclooxygenase-2, NADPH-oxidase, SOD, NOS), or either directly or indirectly influence the expression of several genes involved in atherosclerosis (CD36, CTGF, integrins, cytokines etc.). Several tocopherol transport proteins (TAPs, TBP) may be involved in the correct distribution of the four tocopherols to lipoproteins, organelles (Golgi, mitochondria, nucleus, endoplasmic reticulum), enzymes, transporters (ABCA1) and transcription factors, and in this way confer specificity to the different tocopherols in the cell.

under the action of the endothelial lipoprotein lipase present in the target tissues. During this process, a part of vitamin E is released in the plasma and taken up by the cells.

In contrast to vitamins A and D, vitamin E does not seem to have any specific plasma carrier protein. Incorporated into plasma lipoproteins it is non-specifically transported to the tissues. In the

liver, the tocopherols are taken up from chylomicron remnants mainly *via* LDL receptor, and the α-tocopherol transfer protein (α-TTP) [8,9] channels specifically α-tocopherol to organelles where very low density lipoproteins (VLDL) are synthesized (rough endoplasmic reticulum, Golgi apparatus) (Fig. 1). The α-TTP protein (32 kDa) is expressed at high levels only in hepatocytes. As compared with the non-specific intestinal absorption, α-TTP stereospecificity allows an almost exclusive incorporation of RRR-α-tocopherol in the VLDL particles. As a consequence of this selective transfer mechanism, most of the plasma tocopherol analogues and synthetic isomers, not being recognized by α-TTP, are metabolized and eliminated through the bile and urine (Fig. 1). The relative affinity of tocopherols for α-TTP when compared to the α form is: RRR-α-tocopherol 100%, β-tocopherol 38%, γ-tocopherol 9%, δ-tocopherol 2% [9].

A large amount of the total secreted VLDL are hydrolyzed by the lipoprotein lipase and converted to LDL. In the blood α-tocopherol is rapidly transferred between lipoprotein particles, such that the LDL and HDL fractions contain more than 90% of it [10]. Part of the HDL-carried tocopherol is taken up once more by hepatocytes, specifically recognized by α-TTP, recycled and released for a second time in the plasma together with the VLDL (Fig. 1) [11].

α-TTP is considered to be the major element needed to maintain adequate α-tocopherol plasma levels. In rats supplementation with α-tocopherol modulates the mRNA levels for α-TTP in the liver [12]. Mutations of the α-TTP gene induce a significant drop of plasma and tissue α-tocopherol concentrations, which can lead to a severe neurodegenerative disease called ataxia with vitamin E deficiency (AVED) [13]. These observations underline the importance of α-tocopherol recycling *via* α-TTP. Furthermore, the association between vitamin E deficiency and atherosclerosis was studied in α-TTP knockout mice ($Ttpa^{+/-}$ and $Ttpa^{-/-}$) [14]. In these animals, the plasma and tissue levels of α-tocopherol were reduced, α-TTP was absent in the hepatic homogenate of $Ttpa^{-/-}$ and lowered by 50% in the $Ttpa^{+/-}$ mice as compared to the controls. Furthermore, the α-tocopherol deficit was associated with a raise of atherosclerotic lesions in the proximal aorta and increased lipid peroxidation. These results document the importance of the α-TTP-mediated salvage pathway for the maintenance of adequate plasma tocopherol level and its possible relevance in decreasing the risk of cardiovascular disease.

The increase of α-tocopherol plasma concentration caused by supplementation is limited. In subjects with normal blood tocopherol values (25 μmol/l), the plasma levels cannot be increased more than 2-3 times, whatever is the amount or duration of dietary supplementation. Moreover, the plasma concentration was not equally raised in all healthy subjects supplemented with α-tocopherol (e.g. 12 hours after the administration of 75 mg of RRR-α-tocopherol the measured increases were between 0.3 and 12.4 μmol/l) [15]. This could be the consequence of individual differences in α-TTP activity, in transport efficiency, in the rate of metabolism, the structure and plasma levels of lipoproteins, the status of other micronutrients involved in α-tocopherol recycling, as well as of some environmental factors [3]. Thus, despite an adequate supplementation vitamin E plasma levels can significantly vary from one individual to another.

Intracellular distribution of vitamin E

The transport of vitamin E, due to its hydrophobicity, must necessarily take place either through membrane contacts, or through some specific proteins. High levels of vitamin E have been measured in the Golgi apparatus, lysosomes, endoplasmic reticulum and mitochondria, while peroxisomes and the cytosol had the lowest tocopherol concentrations [16]. α-TTP is present mainly in the liver and in small amounts inside the brain [17], retina [13], lymphocytes, fibroblasts [18] and placenta. The low levels or absence of α-TTP in the majority of tissues suggest the existence of other alternative intracellular transporters. A recently identified group of proteins, called tocopherol associated proteins (TAPs), seems to be specifically involved in the tocopherol intracellular traffic (Fig. 1) [19]. TAPs have GTPase activity and they may modulate cellular signalling in a tocopherol-dependent manner [19]. Another tocopherol transport protein (the 14.2 kDa TBP - tocopherol binding protein), which preferentially

binds the α form, is also supposed to be involved in the intracellular distribution of the vitamin [20].

Vitamin E metabolites

The main metabolite after α-tocopherol oxidation is considered to be α-tocopheryl-quinone, which can be reduced to α-tocopheryl-hydroquinone by microsomal and mitochondrial NADPH-dependent enzymes.

In urine two main α-tocopherol metabolites have been initially isolated, tocopheronic acid and tocopheronolactone, also called the Simon's metabolites [21,22]. These are often considered as markers for the *in vivo* antioxidant activity of α-tocopherol as a consequence of their shortened side chain and opened chromanic ring.

Another metabolite, the α-carboxyethyl hydroxychromane (α-CEHC), has recently been identified in the human urine [23]. Due to its intact chromanic structure, this metabolite is assumed to be derived from α-tocopherol that has not reacted as an antioxidant. This compound is analogous to the one produced by δ-tocopherol degradation, previously described in rats [24], and to the one resulted from γ-tocopherol, isolated from human urine [25]. CEHCs have been investigated for their anti-inflammatory and antioxidant functions [26]. γ-CEHC has natriuretic properties [27] and inhibits the cyclooxygenase-2 (COX-2) in macrophages, which thus leads to the diminution of prostaglandin synthesis [28, 29].

Vitamin E and atherosclerosis

Studies on animal models and clinical trials have investigated the effects of α-tocopherol in atherosclerosis prevention. The clearest *in vivo* evidence of the vitamin E cardiovascular protective effects has been provided in animal models of atherosclerosis. In humans, although epidemiological studies have given indications that α-tocopherol protects against atherosclerosis progression, clinical intervention trials have reported contradictory results. The reasons of this ambiguity may possibly lay on the enrollment in clinical studies of patients with already manifested atherosclerotic lesions or increased risk of cardiovascular disease [2,3].

Studies on animal models

The incidence of atherosclerotic lesions in New Zealand white rabbits fed a high cholesterol diet was decreased after α-tocopherol supplementation [30]. *ApoE*$^{-/-}$ knockout mice fed an atherogenic diet showed, after vitamin E supplementation, a 60% decrease of the atherosclerotic lesions [31]. This event was correlated with a reduction of mRNA and protein expression for MCP-1 (monocyte chemoattractant protein-1) [32]. In primates (*Macaca fascicularis*) fed a cholesterol enriched diet the thickness of carotid artery has been significantly lower after 36 months of vitamin E treatment, as compared to the control group [33]. However, most of the studies in vitamin E supplemented animals fed a standard chow, which already contains higher amounts of tocopherol compared to the Western type diet, showed no beneficial effect with respect to atherosclerosis development [34]. In vitamin E deficient animals tocopherol supplementation reduced atherosclerosis. Moreover, the *ApoE*$^{-/-}$ knockout mice crossed with an α-TTP deficient mouse, which had a consequent decrease of α-tocopherol tissue and plasma levels, developed severe atherosclerotic lesions in the proximal aorta [14].

The α-TTP deficient mice did not show an increased level of oxLDL but broad changes in gene expression patterns without significant changes in enzymes dealing with scavenging reactive oxygen species [35]. This suggests that α-tocopherol plays alternative roles in the development of atherosclerosis. Similarly, the finding that probucol, which strongly protected LDL against oxidation, failed to decrease atherogenesis in *LDLR*$^{-/-}$ mice suggests that the effect of α-tocopherol could not be due to its antioxidant properties.

Clinical trials

Scientific studies describing lipid oxidation as a major event in the development of atherosclerotic

Table 1 Summary of clinical trials for the effect of vitamin E supplementation in cardiovascular disease.

Trial	Subjects' description	Number of subjects	Dose and type of vitamin E	Follow-up (years)	Parameters	Relative risk
Primary prevention						
ATBC	Male smokers; 50 - 69 years	29133	50 mg *all rac*-tocopheryl-acetate	6.1	MI, stroke deaths	0.95
PPP	At least one major risk factor for CVD; mean age 64.4 years	4495	300 mg *all rac*-α-tocopherol	3.6	CVD mortality, MI Peripheral-artery disease	0.87 0.54
ASAP	Plasma cholesterol > 5 mM; age 45 - 69 years	458	136 IUx2/day RRR-α-tocopheryl- acetate	3.0	IMT progression in common carotid artery	0.56
Secondary prevention						
CHAOS	Clinical and angiographic evidence of CAD; mean age 62 years	2002	400-800 IU RRR-α-tocopherol	1.4	CVD and total mortality Non-fatal MI	1.18 0.23
SPACE	Hemodialysis and known CVD; age 40 - 75 years	196	800 IU RRR-α-tocopherol	1.4	MI, CVD mortality	0.46
GISSI	Recent MI (< 3 months); age > 50 to < 80 years	11324	300 mg *all rac*-α-tocopherol	3.5	CVD mortality, non-fatal MI	0.88
HOPE	Known CVD or diabetes; mean age 66 years	9541	400 IU RRR-α-tocopheryl-acetate	4.5	CVD mortality	1.05

CAD - coronary artery disease; CVD - cardiovascular disease; MI - myocardial infarction; IMT - intima-to-media thickness, see text for references.

lesions has prompted the investigation of antioxidant vitamins effects in preventing the initiation and progression of cardiovascular disease. The results of the major clinical trials aimed to check the cardiovascular protective properties of vitamin E are summarized in Table 1.

In primary prevention studies all subjects had risk factors but not pre-existing CVD. The Finnish Alpha-Tocopherol Beta Carotene study (ATBC) [36] reported no effect of vitamin E supplementation on the cardiovascular mortality, a modest decrease of the angina pectoris incidence and an increase of hemorrhagic strokes compared to the control subjects. In the Collaborative Primary Prevention Project (PPP) [37] the absence of effect was constant for the main combined endpoint (CVD death, myocardial infarction (MI), stroke) and for other endpoints (all deaths, all MI, angina pectoris, transient ischaemic attack, revascularisation procedure), except for the incidence of peripheral-artery disease, which was significantly lower among subjects treated with vitamin E (relative risk ratio of 46%). The Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study [38] investigated the effect of RRR-α-tocopheryl-acetate, vitamin C, a combination of both or placebo on the progression in intima-to-media thickness (IMT) of common carotid artery, used as a marker of on-going atherosclerotic disease. Only the male smokers treated with both vitamins showed a relevant decrease in the rate of IMT progression.

The subjects enrolled in the secondary prevention trials had clinical evidence of CVD (Table 1).

In the Cambridge Heart Antioxidant Study (CHAOS) [39], supplementation with vitamin E induced a major decrease in the risk of non-fatal acute MI. Moreover, the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage Renal Disease (SPACE) study [40] reported a significant reduction of the acute MI incidence in hemodialysed patients who received vitamin E. Two other large prospective trials, the Gruppo Italiano per lo Studio della Supervienza nell'Infarto miocardico (GISSI) [41] and the Heart Outcomes Prevention Evaluation (HOPE) study [42], showed no effect of vitamin E treatment on any cardiovascular event.

After a general review the results appear to be inconsistent, but as described below, several reasons can be postulated that some of the trials gave negative results. An accurate interpretation needs to take into account the modality of selection for enrollment, the geographical area of selected subjects connected to the diet differences, the stage of the disease, the chosen markers, the dosage and the type of vitamin E used in each of these trials [3].

Although within a study designed for evaluating the efficiency of vitamin E supplements the vitamin E status of the subjects should be carefully quantified, the tocopherol plasma levels of subjects were not always measured. Moreover, approximately 20% of the individuals do not respond to the vitamin E treatment by an α-tocopherol plasma concentration increase, but so far this aspect has not been considered.

Sex can also be an evaluation criterion because of the lower cardiovascular disease incidence among women. A global analysis of subjects is susceptible of diminishing the statistical relevance of the reported results.

Neither the genetic status of subjects has been considered within the presented trials. Polymorphisms of genes for Apo A IV [43], iNOS (inducible nitric-oxide synthase) [44], or CD36 [45,46] could facilitate the development of atherosclerosis. Moreover, modifications of the structure of proteins directly involved in the transportation and cellular distribution of α-tocopherol can alter its bioavailability.

The age of subjects within the analyzed studies differs (Table 1). Consequently, the stage of atherosclerotic lesions could have been also different. Antioxidant therapy is supposed to be rather effective in the early stages of atherosclerosis by preventing the oxidative lesion of endothelium and the LDL oxidation, than preventing clinical events in the advanced stages. In all animal studies with positive results the vitamin E administration started before the constitution of histological atherosclerotic changes in the structure of the arterial wall. Furthermore, the protective effect showed by vitamin E and C supplementation in the prevention of immune atherosclerotic lesions occurring after cardiac transplant also supports this assumption [47].

The significant reduction of MI incidence reported in some of the secondary prevention studies (Table 1) is unlikely to be only the result of an antioxidant action of vitamin E. In order to have an impact during any of the disease stages, it should have pharmacodynamic effects superior to the antioxidant function. In fact, none of the trials measured the markers of lipid peroxidation to check the antioxidant efficacy of supplementation [48]. Moreover, in a randomised placebo-controlled study in healthy adult subjects, Meagher *et al.* [49] reported no changes in the amount or structure of breakdown products of lipid peroxidation, despite the high vitamin E supplementation (up to 2000 mg daily). These data may allow us to presume the involvement of alternative non-antioxidant mechanisms for the anti-atherosclerotic effect of tocopherols.

The different type and dose of α-tocopherol supplements administered within the trials (Table 1) can also explain the incongruence between their results. Much smaller amounts of vitamin E were used in the GISSI trial (50 mg daily) compared to the others (300 - 800 IU daily) [37]. The conventional synthetic form, all rac-α-tocopherol, can increase the α-tocopherol only half as much as compared to the RRR-α-tocopherol [7,50,51]. Moreover, it is degraded to α-CEHC 3-4 times as much as the RRR-α-tocopherol [50]. Yet, it has not been very clearly established whether the efficiency of all-rac-α-tocopherol is only half as much as the one of RRR-α-tocopherol. The α-tocopheryl-acetate necessitates the hydrolytic action of intestinal esterases, whose activity may vary from one individual to another.

Vitamin E action mechanisms involved in the anti-atherosclerotic protection

Antioxidant properties

Two hypotheses are currently accepted to explain the initiation of the atherogenic process: the oxidative theory [52,53] and the theory of "injury response" [54]. Both theories consider the oxidative modification of LDL as key event in atherosclerosis induction and/or progression. The ability of α-tocopherol to inhibit the *in vitro* LDL oxidation has been unequivocally proved. This observation suggested the possibility that vitamin E may reduce the occurrence of atherosclerotic lesions through the prevention of the initial oxidative event.

α-Tocopherol is the main vitamin E analogue involved in this phenomenon because it is the most abundant and active scavenger of peroxyl radicals present within low density lipoproteins (LDL). Lipid peroxidation is an initial event within LDL oxidative modification induced by the reactive oxygen and nitrogen species. Vitamin E acts by inhibiting the radical chain propagation within lipid domains thus leading to stable lipid species. The resulting α-tocopheroxyl free radical can be reduced by electron donors such as ascorbate or ubiquinol-10 [55].

If LDL is confronted with large amounts of aggressive reactive oxygen species, α-tocopherol is quickly consumed, thus leading to accumulation of lipid hydroperoxides. A lowered intensity of the radicals attack over a prolonged period of time may induce a continuous consumption of α-tocopherol and a higher degree of lipid peroxidation [55].

Following α-tocopherol consumption, the secondary reactions of peroxidation become predominant, and the lipid hydroperoxides are converted into aldehydes. These can modify the apolipoprotein B-100 (the most abundant protein within LDL) thereby inducing an increase of electronegativity and the possible aggregation and conversion of lipoproteins into proatherogenic forms. Vitamin E may also reduce LDL oxidative modification by decreasing the assembly of active NADPH-oxidase responsible for reactive oxygen species production [56]. To what extent the radical scavenging properties or the radical production inhibition of α-tocopherol are important in the prevention of LDL oxidation is not clear at the present time.

Oxidized LDL (oxLDL) stimulate the expression of endothelial adhesion molecules, have a chemotactic effect and inhibit the migration of macrophages outside the subendothelial space, thus increasing the number of leukocytes and proinflammatory factors involved in atherogenesis [55]. They also stimulate the expression of scavenger receptors CD36 and SR-A within monocytes, macrophages and smooth muscle cells (SMC). These receptors internalize the oxidized lipoproteins in a specific manner, until foam cells are formed. Moreover, oxLDL can promote the proliferation of SMC, followed by the narrowing of the vascular lumen.

The bioavailability and antioxidant efficiency of tocopherols, tocotrienols, and their synthetic analogues are different for each of them. Every LDL particle contains 6-10 molecules of α-tocopherol (next to other hydrophobic antioxidants: ubiquinol-10, alpha- and beta-carotene, lycopene, and cryptoxanthine, in much smaller amounts) [57]. Chylomicrons also carry the other forms of tocopherol and tocotrienol in concentrations similar to α-tocopherol, or depending on the diet even in higher amounts, which contribute as well to antioxidant protection of lipids. *In vitro* studies suggest that tocotrienols are more effective in LDL oxidation inhibition than tocopherols [58,59] however, they are less important *in vivo* since their plasma levels are low.

Prooxidant properties of α-tocopherol

Lipid peroxidation of LDL can be enhanced by the presence of α-tocopherol, and substantially accelerated by increasing the amount of vitamin E within LDL, both *in vitro* as well as *in vivo*. It is assumed that peroxidation is propagated by the vitamin E radical, α-tocopheroxyl, if the latter is not reduced by vitamin C or ubiquinol-10 [55]. It is not yet certain whether the prooxidant reactions of α-tocopherol are relevant *in vivo*, within physiological conditions.

The anti-alkylating properties of α-tocopherol

Nitric oxide produced by macrophages during inflammation, combined with reactive oxygen species, form peroxynitrite, which can induce lipid peroxidation. Both γ-tocopherol (abundant in the United States diet) and α-tocopherol (abundant in the European diet and supplements) protect against peroxynitrite-induced lipid peroxidation [60]. The effect of γ-tocopherol becomes significant only after the depletion of the α form, which is capable of neutralizing by itself *in vivo* peroxynitrite-derived reactive species of nitrogen [61].

Anti-atherogenic non-antioxidant mechanisms of α-tocopherol

The antioxidant properties of tocopherols and tocotrienols have for long time been the interest of many researchers. However, during the last decade, specific non-antioxidant effects of vitamin E in cellular signalling and regulation of gene expression have been demonstrated (Fig. 1).

Effects on protein-kinase C and protein phosphatase 2A activity

The involvement of vitamin E in cellular signalling was described for the first time in 1991, when the inhibition of protein-kinase C (PKC) activity was identified to be at the basis of growth inhibition by α-tocopherol of smooth muscle cells within the arterial wall, an event being present at physiological plasma concentrations of the vitamin [62,63]. The effect of β-tocopherol was much weaker; this observation together with the similar antioxidant potency of the two molecules suggested a non-antioxidant mechanism for the α-tocopherol inhibitory effect. Subsequently, numerous studies have confirmed the involvement of PKC in the action of α-tocopherol on monocytes, macrophages, neutrophils, fibroblasts, and mesangial cells [64-67].

Within endothelial cells, the thrombin-induced PKC activity and endothelin secretion are also inhibited by α-, but not by β-tocopherol [68]. In monocytes the superoxide anion is produced mainly by the NADPH-oxidase. In these cells the inhibitory effect of α-tocopherol on PKC prevents phosphorylation and translocation of the cytosolic factor p47 (phox), an indispensable event for NADPH-oxidase assembly [69]. Consequently, the production of superoxide is reduced, and the inflammatory processes attenuated.

Furthermore, α-tocopherol inhibits human thrombocyte aggregation in a PKC-dependent manner, both *in vivo* and *in vitro* [66, 70-72]. In another study, α- and γ-tocopherol have been shown to diminish platelet aggregation and decrease intra-arterial thrombus formation [71]. The significantly higher effect of γ-tocopherol suggests a non-antioxidant mechanism, as the two tocopherol forms have similar antioxidant capacity.

PKC inhibition is achieved through protein phosphatase 2A (PP2A) activation by α-tocopherol, which will catalyze PKC dephosphorylation [73-75]. The many of the α-tocopherol effects on cell signaling can be explained through PKC inhibition.

Inhibition of cyclooxygenase-2 and 5-lipooxygenase

Cyclooxygenase-2 (COX-2) catalyzes the synthesis of prostaglandins, which are important elements within the inflammatory process. COX-2 activity is inhibited by γ-tocopherol and γ-CEHC (but not by α-tocopherol), which feature anti-inflammatory effects in this way [28, 29, 76]. In aged mice, vitamin E decreased COX activity, without any effect on mRNA and protein levels, indicating a post-translational regulation of COX by vitamin E [77]. Further experiments indicated that vitamin E decreases COX activity through reducing formation of peroxynitrite, a hydroperoxide shown to be involved in the activation of COX-2 [77]. Other homologues of tocopherols were also effective in inhibiting COX activity, but their degree of inhibition varied. The varied potency to inhibit COX activity was not explained totally by differences in their antioxidant capacity. Vitamin E-induced inhibition of COX activity might contribute to its effect of reducing CVD risk.

Within activated human monocytes the α-tocopherol inhibits the release of interleukin-1β (IL-1β), a proinflammatory cytokine, through 5-lipooxygenase activity inhibition [78]. Since β-tocopherol did not have any effects on the enzyme, a non-antioxidant mechanism is suggested. The involvement of PKC was excluded after bisindoylmaleimide (a PKC inhibitor) did not induce a decrease in IL-1β release from activated monocytes.

Nitric oxide synthase and superoxide dismutases

In splenocytes from old mice α-tocopherol reduced the production of reactive nitrogen species (NOS) by inhibiting INF-γ synthesis, leading to a reduction of the inducible nitric oxide synthase (iNOS) [79]. On the other hand, α- and γ-tocopherol increased endothelial nitric oxide synthase (eNOS) activity and NO production, but only γ-tocopherol induced eNOS protein expression [80].

In rats, both α- and γ- tocopherol inhibited platelet aggregation, delayed the time of occlusive thrombus formation, decreased arterial superoxide anion generation, lipid peroxidation and LDL oxidation, however, γ- was more efficient than α-tocopherol [71]. The activity of superoxide dismutases was more increased by γ-tocopherol than α–tocopherol.

Transcription factors responsive to tocopherols

The expression of a variety of genes involved in inflammatory response and cellular proliferation is controlled by the nuclear factor kappa B (NF-κB) transcription factor. Activated NF-κB has been identified *in situ* in human plaque, but not in normal vascular cells unaffected by atherosclerosis [81]. Moreover, NF-κB is activated by a cholesterol-rich diet [82] and by oxidized LDL [83]. These findings suggest a critical role for NF-κB in atherosclerosis.

A series of genes known to be involved in atherosclerosis development are regulated by NF-κB, such as the genes of tumour necrosis factor α, interleukin 1, macrophages or granulocyte colonies stimulating factor, monocyte chemotactic protein 1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule (ICAM) [84, 85].

Because it has a central role within the inflammatory response, attempts have been made to therapeutically regulate the activity of NF-κB. Experimental data suggest that the anti-inflammatory properties of vitamin E are partly a consequence of NF-κB activity inhibition. The incubation of human T cells with α-tocopheryl-acetate or α-tocopheryl-succinate has shown a concentration-dependent inhibition of NF-κB activation [59]. Also, studies carried out on human THP-1 macrophages treated with α-tocopheryl-succinate and activated afterwards have shown a 43% reduction of NF-κB activation as compared to the control cells.

It has not yet been clearly established whether vitamin E directly participates in some of the key steps in NF-κB activation, or through intracellular redox status modulation, which is known to be one of the major determinants of NF-κB activation [4].

Several other transcription factors have been shown to be modulated by tocopherols. α-Tocopherol promoted AP-1 activation in quiescent aortic smooth muscle cells but prevented its activation by phorbol myristate acetate, which activates PKC [86]. None of the described effects of α-tocopherol were shared by β-tocopherol, suggesting a non-antioxidant mechanism as the basis of its action.

The expression of PPARγ mRNA and protein are increased by α- and γ-tocopherols and these effects are more pronounced with γ-tocopherol, suggesting relevance to the management of inflammatory and cardiovascular disorders [87]. Vitamin E is able to activate gene expression via the pregnane X receptor (PXR), a nuclear receptor regulating a variety of drug metabolizing enzymes [88]. Strongest induction was seen with α- and γ-tocotrienol followed by rifampicin, δ-, α- and γ-tocopherol. PXR is a member of the family of nuclear receptors, and it can be assumed that other receptors of this family could also be modulated by tocopherols.

Table 2 Genes modulated by vitamin E with possible involvement in atherosclerosis.

Gene class	Genes	Normal function	Effect of tocopherols	Reference
Scavenger receptors	CD36, SR-BI, SR-AI/II	uptake of oxLDL	inhibition by α-tocopherol	[100, 110, 111]
Extracellular matrix	E-selectin, L-selectin, ICAM-1, Integrins, Mac-1	rolling and adhesion of monocytes/macrophages	inhibition by α-tocopherol	[93, 94, 107, 108, 112-114]
	Collagen α1(1), Glycoprotein IIb	platelet adhesion	inhibition by α-tocopherol	[115, 116]
Growth factors	CTGF	proliferation and plaque stabilization	induction by α-tocopherol	[117]
Inflammatory cytokines	TGF-β, IL-4, IL-1β	inflammation and chemotaxis of inflammatory cells	inhibition by α-tocopherol	[118-120]
Cell cycle regulation	P27	inhibition of smooth muscle cells proliferation and aortic thickening	induction by α- and γ-tocopherol	[121, 122]
	cyclin D1, cyclin E	induction of proliferation	inhibition by α- and γ-tocopherol	[122]
Apoptosis	CD95L (CD95 APO-1/Fas ligand)	induction of apoptosis	inhibition by α-tocopherol	[123]
	Bcl2-L1	inhibition of apoptosis	induction by α-tocopherol	[124]
Transcription	PPARγ	induction of transcription	induction by α-tocopherol	[125]
Metabolism	HMG-CoA reductase	cholesterol synthesis	induction by α-tocopherol	[126]
	LDL-receptor	uptake of LDL	induction by α-tocopherol, inhibition by γ- and δ-tocopherol	[126]
	α-TTP	α-tocopherol transfer, plasma level of α-tocopherol	induction by α-and δ-tocopherol	[12, 109]

Cellular adhesion proteins and chemokines

In early atherosclerosis stages the monocytes adhere to the vascular endothelium. The endothelial selectins mediate the monocytes rolling, and the leukocytes integrins along with the endothelial cellular adhesion molecules (VCAM-1, ICAM) mediate the firm adhesion of monocytes to the vascular endothelium. Later on, the monocytes enter through diapedesis in the subendothelial space, where they differentiate into macrophages. These cells can probably oxidize LDL through reactive oxygen and nitrogen species generation, *via* NADPH-oxidase, lipooxygenase, myeloperoxidase or nitric oxide synthase. As a consequence of excessive oxLDL accumulation, the macrophages become foam cells, and the atherosclerotic lesion develops. The activated endothelial cells secrete cytokines, *e.g.* IL-1β and TNF-α, which will maintain their activation, and the production of chemokines, IL-8 and MCP-1 will recruit new monocytes.

As mentioned above, transcription of ICAM, VCAM-1, and MCP-1 is partly dependant on NF-κB activation. Studies carried out on cultures of endothelial cells treated with oxLDL have shown an increase at the levels of gene and protein expression of ICAM and VCAM-1 adhesion molecules [89]. Previous treatment with α-tocopherol reduced the endothelial cellular adhesion proteins expression.

Similar observations have been made in the case of monocytes and polymorphonuclear leukocytes, whose adhesion (induced by oxLDL or agonist stimulation) to vascular endothelia was inhibited as a consequence of enrichment of their α-tocopherol content, both *in vitro* as well as *in vivo* [90-92]. Similarly, the α-tocopherol induced diminution of monocytes adhesion is dependent on inhibition of adhesion molecules expression [93-95]. The reduction of IL-1β synthesis by activated monocytes [64], and the decrease in MCP-1 and IL-8 production in human aortic endothelial cells [93] has also been reported to be under the influence of α-tocopherol.

Modulation of apoptosis

Apoptosis may play an important role in atherogenesis. Oxidized low-density lipoprotein (oxLDL) promotes apoptosis in the arterial wall in addition to several other proatherogenic effects. α-Tocopherol and γ-tocopherol significantly reduced oxLDL induced apoptosis in smooth muscle cells and cell death effectors caspase-3 and -8 [96]. α-Tocopherol was significantly more effective than γ-tocopherol in preventing induction of oxLDL-mediated apoptosis. The antiatherogenic effects of tocopherols were at least in part mediated by reduction of the MAPK and JunK cascade together with a protective profile of apoptotic genes of the Bcl-2 family. Oxysterols, particularly those oxidised at position 7 (7 beta-hydroxycholesterol), are toxic to cells in culture and have been shown to induce apoptosis in cell types such as vascular endothelial cells, smooth muscle cells and monocytes. 7 beta-hydroxycholesterol is one of the most commonly detected oxysterols in foods and its level in plasma has been positively associated with an increased risk of atherosclerosis. The precise mechanism by which oxysterols induce apoptosis is unknown but it may involve the generation of oxidative stress. α- and not γ- tocopherol or α-tocopheryl-acetate was effective at inhibiting 7 beta-hydroxycholesterol induced apoptosis in human U937 moncytes, suggesting for this effect a non-antioxidant mechanism [97].

Modulation of gene transcription

Recently, the capacity of α-tocopherol to modulate gene expression has been investigated (Table 2) [98, 99]. Inhibition of scavenger receptors SR-A and CD36 expression at the transcriptional level by α-tocopherol in aortic SMC [100] and monocytes/macrophages [100-102], followed by a decreased uptake of oxLDL in these cells, can prevent foam cells formation *in vitro,* and thus inhibit atherosclerosis progression. This hypothesis is sustained by the fact that ApoE$^{-/-}$ mice, that are prone to develop atherosclerosis, do not develop atherosclerotic lesion if the CD36 scavenger receptor is absent [103]. The expression of CD36 mRNA is correlated with the lipid peroxide content in peritoneal macrophages during mice aging, and this is accompanied by an age-dependent increase in the cellular uptake of oxLDL. Treatment with vitamin E decreased the amount of cellular lipid peroxides and resulted in inhibition of macrophage uptake of oxLDL and in cellular CD36 mRNA expression [104].

Inhibiting effects of α-tocopherol at the level of gene transcription has been shown for over 30 genes (Table 2) [99]. Many of these genes play an important role in atherosclerosis, such as the cellular adhesion molecules induced by cytokines inside the human vascular endothelia [105, 106]; VCAM-1 expressed at the macrophages surfaces level [107]; L-selectin from pulmonary macrophages [107]; or Mac-1 (CD11/CD18) induced by oxLDL within monocytes [108]. Moreover, the expression level of hepatic α-TTP gene in rats can be upregulated by vitamin E [12, 109], a correct regulation of α-TTP may be important for the maintenance of adequate α-tocopherol levels in the plasma and the consequent reduced risk for atherosclerosis development. Moreover, several tocopherol binding proteins (TAPs, TBP) have recently

been discovered, that may play a role in cellular signalling possibly by mediating the intracellular tocopherol distribution [19].

In all the observed vitamin E effects, the involvement of PKC and other enzymes has not always been studied and it remains to be investigated whether the regulation of transcription of certain genes is the consequence of PKC inhibition or possibly mediated by tocopherol binding protein, such as TAPs or TTP. The results presented here support non-antioxidant action mechanisms of α-tocopherol, resulting from tocopherol specific interactions with regulatory proteins and enzymes, ultimately leading to precise effects seen at the level of signal transduction and gene expression [2, 98, 99].

Conclusions

Although the studies carried out with cell culture and animal models prove promising anti-atherosclerotic effects of α-tocopherol, the results of clinical trials are contradictory. It is possible that inadequate subjects selection (by sex, vitamin E status, genetic polymorphism), the presence of advanced lesions, the dosage and chemical form of vitamin E administered in each of the studies partly explain the incongruence between the reported data. It seems that the α-tocopherol treatment should be started at early atherosclerotic stages, maintained over a long period of time, and possibly be associated with vitamin C administration. Individuals with higher risk factors may benefit most from a preventive vitamin E supplementation. The protective mechanisms of tocopherol are correlated with prevention of LDL oxidation and with non-antioxidant functions, such as the anti-inflammatory action, the inhibition of smooth muscle cells proliferation inside the arterial wall, and platelets aggregation. These events are the results of specific effects of α-tocopherol on signal transduction and gene expression, which are often not related to the antioxidant properties of vitamin E. Identifying some of the new cellular reactions as well as the mechanism of modulation of gene expression by vitamin E will probably help to better link molecular and clinical events.

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