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Review

Regulation of gene expression by α-tocopherol

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Abstract

Several genes are regulated by tocopherols which can be categorized, based on their function, into five groups: genes that are involved in the uptake and degradation of tocopherols (Group 1) include α-tocopherol transfer protein (α-TTP) and cytochrome P450 (CYP3A); genes that are associated with lipid uptake and atherosclerosis (Group 2) include CD36, SR-BI and SR-AI/II. Genes that modulate the expression of extracellular proteins (Group 3) include tropomyosin, collagenα1, MMP-1, MMP-19 and connective tissue growth factor (CTGF). Genes that are related to inflammation, cell adhesion and platelet aggregation (Group 4) include E-selectin, ICAM-1, integrins, glycoprotein IIb, Il-2, IL-4 and IL-β. Group 5 comprises genes coding for proteins involved in cell signaling and cell cycle regulation and consists of PPAR-γ, cyclin D1, cyclin E, Bcl2-L1, p27 and CD95 (Apo-1/Fas ligand).

The expression of P27, Bcl2, α-TTP, CYP3A, tropomyosin, Il-2, PPAR-γ, and CTGF appears to be up-regulated by one or more tocopherols whereas all other listed genes are down-regulated. Several mechanisms may underlie tocopherol-dependent gene regulation. In some cases protein kinase C has been implicated due to its deactivation by α-tocopherol and its participation in the regulation of a number of transcription factors (NF-κB, AP-1). In other cases a direct involvement of PXR/RXR has been documented. The antioxidant responsive element (ARE) appears in some cases to be involved as well as the transforming growth factor β responsive element (TGF-β-RE). This heterogeneity of mediators of tocopherol action suggests the need of a common element that could be a receptor or a co-receptor, able to interact with tocopherol and with transcription factors directed toward specific regions of promoter sequences of sensitive genes. Here we review recent results of the search for molecular mechanisms underpinning the central signaling mechanism.

Keywords: α-tocopherol (tocopherols, tocotrienols); cell regulation; gene expression; inhibition of cell proliferation.

Introduction

For a number of years the action of α-tocopherol has been ascribed to its capacity to chemically act as a lipid-based (lipoprotein and membranes) free radical chain-breaking molecule, thereby defending the organism against radical-induced damage. We have proposed an alternate function for α-tocopherol, specifically that of a 'regulator of gene expression' (Marilley et al., 1996; Fazzio et al., 1997; Azzi et al., 1998b). This function, independent of the radical chain-breaking ability attributed to tocopherols, has been confirmed in a number of experiments, both *in vitro* and *ex vivo* (Prasad et al., 1990, 1993; Houglum et al., 1991; Stauble et al., 1994; Marilley et al., 1996; Fazzio et al., 1997; Chojkier et al., 1998; Aratri et al., 1999; Ricciarelli et al., 2000; Davies et al., 2001; Fischer et al., 2001a; Gysin et al., 2002; Gohil et al., 2003; Landes et al., 2003). This may either result from the regulation of gene transcription or from affecting mRNA or protein stability.

The basis for distinguishing antioxidant vs. non-antioxidant effects of α-tocopherol has been methodological, experimental and evolutionary. If the mechanism by which α-tocopherol alters cellular events was related to its known radical chain-breaking properties the following corollaries would be expected. Firstly, other lipid soluble radical chain-breaking molecules should act in the same way, especially if they are highly related molecules, such as the four tocopherols and the tocotrienols. This similarity of function, despite very close physico-chemical properties, has been shown not to apply in a number of cases. Secondly, the 'radical scavenger' α-tocopherol (McCay et al., 1971; Urano and Matsuo, 1976) would be used by cells as a regulator. This would imply that regulation of certain cellular functions is entrusted to a controlled production and elimination of lipid soluble free radicals. With knowledge of the difficulty of controlling the propagation of radical chain reactions, such a regulatory mechanism would be unthinkable. If this, however, were the case, all lipid soluble radical scavengers should act similarly as cell regulators, an event that has been disproved in several cases (Ricciarelli et al., 2001).

A number of genes, possibly involved in cell proliferation functions and neural signal transduction, were found to be modulated by the absence of α-tocopherol. Unfortunately, the type of experiment carried out (90 days of α-tocopherol deficiency) leaves a certain degree of ambiguity between primary events caused by the absence of tocopherol and secondary ones. One possible confounding factor is that lack of α-tocopherol is associated with an increased activity of protein kinase C (Boscoboinik et al., 1991; Ricciarelli et al., 1998) and this event in turn is associated with the change in expression of a number of genes under the control of the TRE (TPA responsive ele-

ment). Furthermore, activation of protein kinase C is associated with an increased production of superoxide in monocytes, macrophages and neutrophils (Cachia et al., 1998). Although not all genes are under the control of the system tocopherol-PKC-TRE, all these intermingled phenomena make it more difficult, in the animal model described by Gohil et al. (2003), to precisely analyze the genes or events under the direct control of α-tocopherol. They do, however, demonstrate that no oxidative stress is associated with tocopherol deficiency.

The experimental arguments come, among others, from the recent data of Gohil et al. (2003). Deletion of the α-TTP gene in mice results in systemic deficiency of α-tocopherol and neurological dysfunctions similar to symptoms described in patients with a mutated α-TTP gene. mRNAs from brain cortex and liver of α-TTP gene deficient and wild-type mice (Gohil et al., 2003) show up-regulation or down-regulation of a number of genes after three months with an α-tocopherol-deficient diet. Remarkably, none of the classical antioxidant genes were upregulated in the α-TTP gene deficient mice, indicating that the absence of α-tocopherol did not result in an oxidative stress situation with consequent increase of superoxide dismutase, catalase or peroxidases and no evident signs of oxidative stress were present, like an increase of heme oxygenase (Tyrrell and Basu-Modak, 1994). The evolutionary argument can be summarized as follows. The α-tocopherol transfer protein (α-TTP), a cytosolic liver protein, plays a central role in determining the α-tocopherol concentration in plasma. This protein is specifically responsible for the retention of α-tocopherol in the body and is highly conserved in a number of species up to *Homo sapiens*. It is counterintuitive that the specificity for α-tocopherol selection, through protein structural conservation, has been maintained over time without the existence of an evolutionary pressure for the uptake of a unique molecule, α-tocopherol, rather than a generic antioxidant. In fact, α-TTP is not able to efficiently transport other tocopherols and tocotrienols (Hosomi et al., 1997; Panagabko et al., 2003). As discussed in a number of studies and summarized below, α-tocopherol is entrusted with high priority cellular functions, such as signal transduction, regulation and gene expression and it would be uneconomical to consume it as a radical scavenger (Azzi et al., 2002a). Rather, it would be essential to protect α-tocopherol through a network of cellular antioxidant defenses, such as catalases, superoxide dismutases, ascorbate, glutathione, α-lipoic acid etc., similarly to what occurs with proteins, nucleic acids and lipids. However, it is clear that diminution of α-tocopherol, due to excess radical production or insufficient dietary intake, may be the cause of disease. In the following paragraphs a number of genes will be described that appear to be under the control of α-tocopherol (Table 1).

Group 1: genes that are involved in the uptake and degradation of α-tocopherol

α-Tocopherol transfer protein (α-TTP), a cytosolic liver protein, plays an important role in determining the plasma α-tocopherol concentration by selective retention and incorporation of α-tocopherol into VLDL. The relative affinities of α-TTP for substrates calculated from the degree of competition are as follows: RRR-α-tocopherol 100%; β-tocopherol 38%; γ-tocopherol 9%; δ-tocopherol 2%; α-tocopherol acetate 2%; α-tocopherol quinone 2%; SRR-α-tocopherol 11%; α-tocotrienol 12%; trolox 9% (Hosomi et al., 1997). Presumably, α-TTP functions in the intracellular transport of α-tocopherol in hepatocytes and in the secretion of cellular α-tocopherol into extracellular compartments, a reaction that utilizes a novel non-Golgi-mediated pathway (Oram et al., 2001). Protein levels of α-TTP in the liver are lowered by α-tocopherol deficiency and α- and δ-tocopherol supplementation is known to induce expression of hepatic α-TTP mRNA (Fechner et al., 1998) which is most strongly induced by α- and γ-tocotrienol followed by rifampicin, δ-, α- and γ-tocopherol.

Up-regulation of endogenous CYP3A4 and CYP3A5 mRNA was observed with γ-tocotrienol, the most potent activator of PXR. A less prominent response was observed when co-transfection with PXR was omitted from the experimental protocol. This points to a potential interference of individual forms of α-tocopherol with the metabolism and efficacy of drugs (Landes et al., 2003).

Group 2: regulation of genes that are involved in lipid uptake and atherosclerosis

A series of studies have pointed to the effects of α-tocopherol on macrophages and smooth muscle cells with possible relevance to atherosclerosis and inflammatory events. CD36 scavenger receptor (a specific receptor for oxidized LDL) is expressed in macrophages and cultured human aortic smooth muscle cells. Studies indicate that CD36 transports oxidized LDL into the cytosol and that α-tocopherol inhibits oxidized LDL uptake by a mechanism involving down-regulation of CD36 mRNA and protein expression. Therefore, the beneficial effect of α-tocopherol against atherosclerosis can be explained, at least in part, by its effect of lowering the uptake of oxidized lipoproteins, with consequent reduction of foam cell formation (Ricciarelli et al., 2000; Azzi et al., 2002b). A reduction of the scavenger receptor SR-A expression and activity in the presence of α-tocopherol was also observed (Teupser et al., 1999). The α-tocopherol role of diminishing scavenger receptor activity has been confirmed *in vivo* (Devaraj et al., 2001). Rats depleted of α-tocopherol show an increased expression of the scavenger receptor SR-B1 (Kolleck et al., 1999). It is known that scavenger receptors SR-A and SR-B1 play a role in atherosclerosis, by allowing the uptake of oxidized LDL and the initiation, through foam cell formation, of the atherosclerotic process. The abolition of CD36 expression has been shown in apo-E null mice to inhibit plaque formation (Febbraio et al., 2000). α-Tocopherol may have, in susceptible human and animal individuals, a similar effect. In fact, in cholesterol fed rabbits α-tocopherol is able to suppress the cholesterol-induced increase of CD36 in aorta (Azzi et al., unpublished results).

Table 1 The effect of tocopherols on the expression of several genes.

Gene	Pathway	Cell line	Effect
Group 1			
α-TTP		Liver	↑ αT, δT
Cytochrome P450 (CYP3A)	PXR/RXR	HepG2	↑ βT,γT, δT, δTT
Group 2			
CD36		Smooth muscle cells, monocytes/macrophages	↓ αT
SR-BI		Monocytes/macrophages	↓ αT
SR-AI/II		Monocytes/macrophages	↓ αT
Group 3			
Tropomyosin		Smooth muscle cells	↑ αT
Collagen a 1(1)	ARE	Liver stellate cells	↓ αT
MMP-1	PKC	Fibroblasts	↓ αT
MMP-19	PKC	THP-HL-60	↓ αT
Group 4			
E-selectin	NF-κB	Human endothelial cells	↓ αT
ICAM-1		Keratinocytes, neutrophils, endothelial cells, monocytes	↓ αT
VCAM-1		THP-1 monocytes	↓ αT
Integrins		Human erythroleukemia cells	↓ αT
Glycoprotein IIb	PKC	Platelets	↓ αT
CTGF	TGF-β-RE	SMC, fibroblasts	↑ αT
Il-2		Mouse T-cells	↑ αT
IL-4	NF-κB, AP1	Human T-cells	↓ αT
IL-1β	NF-κB, TLR4	THP-monocytes, neutrophils	↓ αT
Group 5			
Cyclin D1		DU-145	↓ αT, γT
Cyclin E		DU-145	↓αT, γT
Bcl2-L1		Rat liver	↑ αT
p27		LNCaP, PC-3	↑ αT
CD95L (CD95 APO-1/Fas ligand)	NF-κB, AP-1	T-cells	↓ αT
PPARγ	SW480, LoVo		↑αT, γT

αT, δT and γT are abbreviations for the corresponding tocopherols. An arrow pointing up indicates an increase, and pointing down a decrease of gene expression. For further explanations and references, see the text.

Group 3: regulation of genes that are implicated in the modulation of extracellular proteins

α-Tropomyosin shows an increased transcription caused by α-tocopherol treatment. Northern and Western blot analyses revealed a time-dependent transient up-regulation of the amount of mRNA and protein in α-tocopherol-treated cells. No effect on the transcription of α-tropomyosin is observed in cells treated with β-tocopherol, indicating that this activity of α-tocopherol was independentent of its antioxidant properties (Azzi et al., 1998a; Aratri et al., 1999). Furthermore, a number of studies have linked the effects of tocopherol with events related with the regulation of connective tissue proteins, collagen expression and fibrogenesis. In human skin fibroblasts, PKC-α protein expression increases during *in vivo* aging as a function of the donor's age. Concomitant with the increase in PKC-α, also collagenase (MMP-1) gene transcription and protein expression increases with age. α-Tocopherol is able to diminish collagenase gene transcription without altering the level of its natural inhibitor, tissue inhibitor of metalloproteinase, TIMP-1 (Ricciarelli et al., 1999). In primary cultures of quiescent stellate cells, inhibition of collagen α1(I) transactivation by α-tocopherol requires only -0.44 kb of the 5′ regulatory region. Transfection of stellate cells with collagen-LUC chimeric genes allowed the localization of an 'antioxidant'-responsive element (ARE) at approx. -0.22 kb of the 5′ region, excluding the first intron (Houglum et al., 1997; Chojkier et al., 1998). Long- and short-term supplementation of mice with α-tocopherol selectively decreases liver collagen mRNA by approximately 70% (Chojkier et al., 1998). At the human level, α-tocopherol treatment of patients affected by chronic hepatitis and hepatic fibrogenesis prevents the fibrogenesis cascade (Houglum et al., 1997). MMP-19 is a metalloproteinase whose production can be up-regulated by phorbol esters (PMA) or by adhesion. The adhesion-dependent expression was down-regulated or even abrogated by using α- but not β-tocopherol (Mauch et al., 2002).

The effect of α-tocopherol treatment on gene expression in human aortic vascular smooth muscle cells was analyzed by gene expression arrays. The expression of the connective tissue growth factor (CTGF) gene was induced by α-tocopherol 1.8-fold in gene array experiments, and similar results were also obtained by RT-PCR (1.7-fold) and at the protein level (1.4-fold). The antioxidants β-tocopherol and N-acetylcysteine did not induce CTGF gene expression, suggesting a non-antioxidant

mechanism for α-tocopherol action. PKC down-regulation did not prevent CTGF induction by α-tocopherol. Since CTGF stimulates the synthesis of extracellular matrix, the normalization of CTGF gene expression by α-tocopherol may accelerate wound repair and tissue regeneration during atherosclerosis (Villacorta et al., 2003).

Group 4: regulation of genes that are related to inflammation, cell adhesion and platelet aggregation

Diminution of integrin expression has been found to be induced by α- but not β-tocopherol, thus negatively conditioning cell adhesion, an important event both in inflammation and atherosclerosis (Breyer and Azzi, 2001). The decrease is specific for some but not all integrins, for α-tocopherol and occurs at the transcriptional and translational levels (T. Visarius, unpublished results). α-Tocopherol supplementation decreases the expression of plasminogen activator inhibitor-1 and P-selectin levels in type 2 diabetic patients (Devaraj et al., 2002). α-Tocopherol significantly decreases adhesion of activated monocytes to endothelial cells by decreasing expression of CD11b and VLA-4 on monocytes, possibly by inhibiting the activation of NF-κB (Islam et al., 1998).

α-Tocopherol increases both cell-dividing and IL-2-producing capacity of naive T cells from old mice, with no effect on memory T cells. These data indicate that naive T cells exhibit the greatest age-related defect and show for the first time that supplemental α-tocopherol has direct immuno-enhancing effect on naive T cells from old mice (Adolfsson et al., 2001). Furthermore, foreign body-type multinucleated giant cell formation is potently induced by α-tocopherol (McNally and Anderson, 2003).

Several other events that are associated with inflammation appear to be regulated by α-tocopherol. The cytokine interleukin-1-β (IL-1β) was found to be decreased by α-tocopherol. mRNA levels reveal that the inhibitory effect is exerted at the level of IL-1 β gene expression (Akeson et al., 1991). Glycoprotein IIb is the α-subunit of the platelet membrane protein glycoprotein IIb/IIIa, which functions as a specific receptor for platelet aggregation. In cells pre-treated with various concentrations of α-tocopherol, TPA-mediated glycoprotein IIb promoter activity is down-regulated by α-tocopherol. This event may result in a reduction of glycoprotein IIb protein expression and thus contribute to anti-platelet aggregation (Chang et al., 2000). Combined α-tocopherol and selenium deficiency is characterized by alterations in the expression level of genes encoding for proteins involved in inflammation (multispecific organic anion exporter, SPI-3 serine protease inhibitor) and acute phase response (α-1 acid glycoprotein, metallothionein 1) (Fischer et al., 2001a). α-Tocopherol has an inhibitory effect on LDL-induced production of adhesion molecules and adhesion of monocytes to endothelial cells possibly *via* a direct regulatory effect on ICAM-1 expression (Martin et al., 1997).

Co-incubation of neutrophils with α-tocopherol and pre-treatment of HUVEC with α-tocopherol significantly reduces PAF-induced CD11b/CD18 expression and IL-1-β-induced up-regulation of ICAM-1 and VCAM-1, respectively. These findings indicate that α-tocopherol may work as an anti-inflammatory agent by inhibiting neutrophil-endothelial cell adhesive reactions (Yoshikawa et al., 1998).

From all the results reported above it clearly appears that a number of genes involved in acute inflammation and adhesion are down-regulated by α-tocopherol. Due to the intrinsic inflammatory nature of the atherosclerotic process it is conceivable that the regulation of these genes by α-tocopherol may play a role also in the prevention of plaque formation.

Group 5: regulation of genes involved in cell signaling and cell cycle control

α-Tocopherol increases the *de novo* synthesis of protein kinase C (Azzi et al., 1998a) although little is known about the molecular mechanism of this event. Additionally, a significant down-regulation in the expression level of genes important in the inhibition of apoptosis (defender against cell death 1 protein, Bcl2-L1), cell cycle (G1/S-specific cyclin D1) and up-regulation of genes involved in glutathione synthesis (γ-glutamylcysteine synthetase catalytic subunit) has been described (Schwartz et al., 1993a; Wu et al., 1997, 1998; Fischer et al., 2001a).

Additionally, a preventive role of tocopherols against tumours has been described (Schwartz et al., 1993a). Tumours produced in animal by 7,12 dimethylbenz(a)anthracene (DMBA) develop significantly less after α-tocopherol supplementation and are characterized by a notable increased expression of p53 (Schwartz et al., 1993b).

γ-Tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulating cyclins (Gysin et al., 2002). Some studies have demonstrated that at physiological concentrations α-tocopherol induces profound cell cycle arrest mediated by up-regulation of p27. This observation provides a theoretical basis for the putative chemopreventive effect of α-tocopherol (Venkateswaran et al., 2002).

Flow cytometry analysis of γ-tocopherol-treated prostate carcinoma DU-145 (a prostate cancer-derived cell line) showed decreased progression into the S-phase. This effect was associated with reduced DNA synthesis as measured by 5-bromo-2′-deoxy-uridine incorporation. Furthermore, Western blot analysis of γ-tocopherol-treated cells showed decreased levels of cyclin D1 and cyclin E (Gysin et al., 2002).

Pre-treatment with α-tocopherol induces a striking reduction of liver mass recovery and nuclear bromodeoxyuridine labeling, and also leads to a decreased expression of cyclin D1 and of the proliferating cell nuclear antigen after partial hepatectomy. *In vivo* treatment with α-tocopherol could promote an early termination of preparative cell events, which lead to the replicative phase, during partial hepatectomy-promoted liver proliferation. The latter could have a significant implication in the antitumorigenic effect ascribed to the treatment with α-tocopherol (Trejo-Solis et al., 2003).

The most effective inhibitors of PC-3 (a prostate cancer-derived cell line) proliferation were γ-tocopherol and γ-carboxyethyl hydroxychroman. Their effect was discernable at 1 μM and reached a plateau at concentrations equal to or higher than 10 μM. The maximal inhibition values ranged between 70 and 82% (Galli et al., 2004).

γ-Tocopherol up-regulates peroxisome proliferator activated receptor γ (PPAR-γ) expression in SW 480 human colon cancer cell lines. Up-regulation of PPARγ by the tocopherols and in particular by γ-tocopherol may have relevance not only to cancer prevention but also to the management of inflammatory and cardiovascular disorders (Campbell et al., 2003). Down-regulation in the expression of genes important in apoptosis inhibition (defender against cell death 1 protein, Bcl2-L1) has been also described (Fischer et al., 2001b).

In conclusion, the expression of a number of cell cycle related proteins (cyclins D, E1, P27, P53) is affected by α- or by γ-tocopherol. Inhibition of cell cycle and of cell proliferation may be related to these molecular effects. Further knowledge of the detailed mechanism of α-tocopherol and γ-tocopherol molecular action may lead to a possible use of these compounds or of more potent analogs as adjuvants in tumor prevention or progression inhibition strategies.

Molecular basis of α-tocopherol-induced gene regulation

Positive and negative regulation of a number of genes by α-tocopherol has been confirmed in a number of different laboratories. Different arguments (logical and experimental) point to a ligand-induced modulation of specific proteins as being responsible for the α-tocopherol effects that appear to be unique, and not mimicked by structurally related molecules.

The search for transcription factors or receptors apt to convey α-tocopherol-dependent nuclear signaling has started with early experiments in our laboratory. By transfecting smooth muscle cells with a construct containing three TRE (TPA responsive elements), the thymidine kinase promoter and the reporter gene chloramphenicol acetyltransferase a modulation of gene expression by α-tocopherol was observed (Azzi et al., 1999). More recently, a mutation analysis of the promoter region of CTGF has indicated that a TGF-β-RE is capable of activating, in the presence of α-tocopherol, the expression of the mRNA for CTGF (Villacorta et al., 2003).

The search for a receptor has yielded the discovery of three tocopherol-associated proteins (TAP1,2,3) that bind tocopherols as well as other hydrophobic ligands (Stocker et al., 1999; Zimmer et al., 2000; Kempná et al., 2003). It has been suggested that tocopherol-associated protein-1 (TAP1) is a ligand-dependent transcriptional activator (Yamauchi et al., 2001). Using a GFP fusion protein expression system, it was reported that TAP-1 translocates from cytosol to nuclei in α-tocopherol-dependent fashion (Yamauchi et al., 2001). However, whether the TAP proteins can regulate the expression of specific genes in a tocopherol-specific manner remains to be confirmed.

A recent observation has indicated that α-tocopherol activates the human pregnane X receptor-mediated gene expression in HepG2 cells. Tocopherols and tocotrienols enhance pregnane X receptor-mediated gene expression with different efficiency, whereas the tocopherol metabolic products do not (Landes et al., 2003). The question arises whether the activation of genes, via the pregnane X receptor, is restricted to those involved in the drug hydroxylation and elimination pathways [cytochromes P450 (CYP), e.g. CYP3A (Oram et al., 2001; Landes et al., 2003)] or if a similar class of receptors may be involved in the regulation of a number of genes discussed in this study. The question arises whether PXR is activated directly by binding tocopherol, or whether the observed effects were indirect. It can be speculated that the role of TAPs and similar proteins may be that of conferring, through recognition and transport of tocopherol to transcription factors, specificity to the action of the different tocopherols.

The 13-fold decrease in the expression of retinoic acid receptor-related orphan receptor-α mRNA in brains of α-tocopherol α-TTP null mice may indicate that this receptor is involved in mediating α-tocopherol molecular functions (Gohil et al., 2003).

The results reported in this review support two important new concepts regarding α-tocopherol. There is no obvious correlation between the described regulatory functions of α-tocopherol and its free radical chain interrupting properties established to take place only within a lipid phase. α-Tocopherol, in comparison with the other tocopherols, has a unique cellular function which is associated with the regulation of gene expression, most likely by interaction with specific transcription factors or receptors. The detailed mechanism by which binding of α-tocopherol to a receptor or a transcription factor modulates gene transcription is currently under investigation in a number of laboratories.

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