Vitamins and Regulation of Gene Expression

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Vitamins and Regulation of Gene Expression

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A number of vitamins have been reported to affect the regulation of gene expression. The most recent results on this subject will be critically reviewed with special focus on the effective dose in vivo, that may be extrapolated from in vitro studies.

Carotenoids, Vitamin A and Retinoids

all-trans-Retinoic acid (RA) is the major biologically active form of vitamin A, and nuclear retinoid receptors are the major mediators of *all-trans*-RA actions [1, 2]. RA metabolites of vitamin A are key regulators of gene expression involved in embryonic development and maintenance of epithelial tissues [3]. The absence of retinoic acid receptor (RAR) γ is associated with loss of the RA-inducible expression of the Hoxa-1, Hoxa-3, laminin B1, collagen IV (α_1), GATA-4, and BMP-2 genes. [4] Furthermore, the loss of RAR-γ is associated with a reduction in the metabolism of *all-trans*-RA to more polar derivatives, while the loss of RAR-α is associated with an increase in metabolism of RA [4]. RA also induces osteopontin gene expression in concert with vitamin D [5] and *all-trans*-RA 4-hydrolase [6] and suppresses that of collagenase [7]. Carotenoids both with and without provitamin A upregulate gap junctional communication and connexin43 gene expression in human dermal fibroblasts and inhibit carcinogen-induced neoplastic transformation [8, 9].

Vitamin C

The expression of alkaline phosphatase, type I collagen and osteocalcin are increased by ascorbic acid and are diminished in scurvy [10]. Calcineurin (protein phosphatase 2B) is an Fe^{2+} enzyme, that is inhibited by superoxide radicals and activated by ascorbate [11]. 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 at the basis of an immune response enhancement by vitamin C [12].

Vitamin D

1,25 Dihydroxyvitamin D_3 interaction with the vitamin D receptor results not only in calcium homeostasis regulation, but has numerous other functions including inhibition of proliferation of cancer cells, effects on hormone secretion and suppression of T-cell proliferation and cytokine production. These effects are possibly related to gene expression regulation [13].

Vitamins K

The vitamins K were found to increase the expression of prothrombin and carboxylase messenger RNA and c-myc messenger RNA, but had no effect on the expression to TGF-β_1 messenger RNA [14, 15].

Vitamin E

Effect of α- and β-Tocopherol on Smooth Muscle Cell Proliferation

α-Tocopherol at concentrations between 10 and 50 μ*M* (comparable to the physiological range of 25–40 μ*M*), inhibits rat A7r5 smooth muscle cell proliferation (fig. 1A), while β-tocopherol is ineffective. When α- 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 [16]. The oxidized product of α-tocopherol, α-tocopheryl quinone, and several other water-and lipid-soluble antioxidants are not inhibitory, indicating that the effects of α-tocopherol are not related to its antioxidant properties [16]. Inhibitory effects of α-tocopherol are also observed in primary human aortic smooth muscle cells (hAOMSC from Clonetics Corp.,

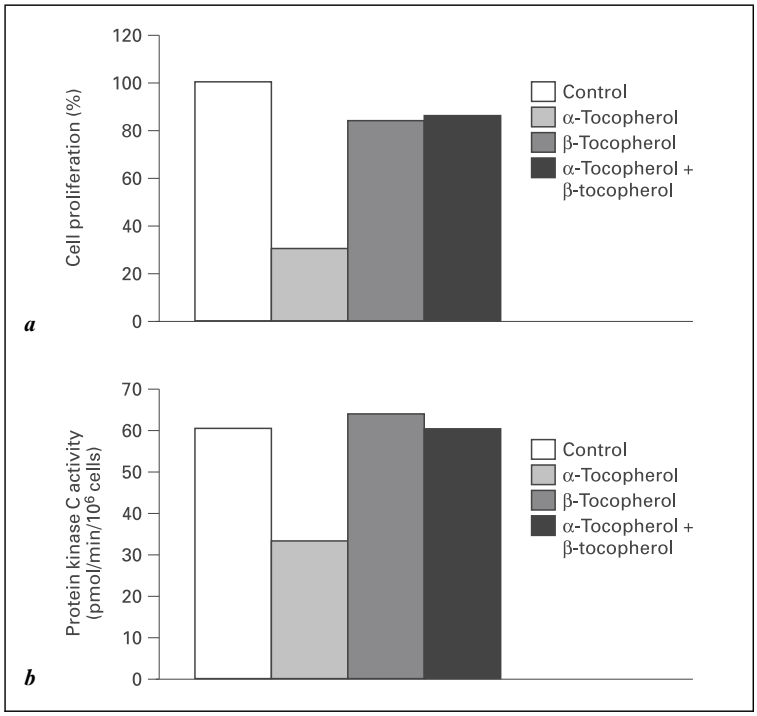

Fig. 1. Differential effects of α- and β-tocopherol on smooth muscle cell proliferation and protein kinase C activity. Quiescent cells are restimulated to grow with FCS (10%) in the presence of α-tocopherol and/or β-tocopherol (50 μ*M*). ***a*** After completion of the cell cycle (30 h) cells are counted with a hemocytometer. Viability is greater than 95%. Control (100%) represents $289 \pm 12 \times 103$ cells. ***b*** After 7 h restimulation, cells are permeabilized and protein kinase C is measured as described in Materials and Methods. Phorbol myristate acetate (100 n*M*) is added 60 min before assaying activity. The basal kinase activity is subtracted in all samples and only the PMA-stimulated activity is shown.

San Diego, Calif., USA. Other cell types such as Balb/3T3 mouse fibroblast and NB-2a mouse neuroblastoma are α-tocopherol-sensitive whereas CHO, Saos-2 human osteosarcoma cells, and P388 mouse macrophages are insensitive (table 1).

Effect of α-Tocopherol on Protein Kinase C Activity

The differential inhibition of protein kinase C by α-tocopherol and β-tocopherol is shown in figure 1B. As can be seen, β-tocopherol is much less effective and, as in the proliferation studies, prevents the inhibition of protein kinase C by α-tocopherol. The experiment reported in figure 2 shows that

Table 1. Antiproliferative effect of α-tocopherol is cell-specific

Tissue of origin	Sensitive	Insensitive
Rat aorta smooth muscle	A10/A7r5	
Human aorta smooth muscle	hAI	
Human Tenon's fibroblasts	hTF	
Human skin fibroblasts	CCD-SK	
Mouse neuroblastoma	NB2A	
Human pigmented		
Retinal epithelial cells	hPRE	
Human leukemia	U937	
Human prostate tumor	DU-145	
Mouse fibroblast	Balb/c-3T3	
Glioma	C6	
		Hela
Chinese hamster lung		LR73
Chinese hamster ovary		CHO
Human osteosarcoma		Saos-2
Mouse macrophage		P388 D1

Cells were made quiescent and restimulated with fetal calf serum in the presence or absence of α-tocopherol (50 μ*M*). Cells were counted after the completion of a cycle.

inhibition of protein kinase C and of proliferation occurs at the same range of α-tocopherol concentrations.

In the experiment shown in figure 3, the inhibition of protein kinase C activity by α-tocopherol has been studied as a function of the smooth muscle cell cycle. When α-tocopherol is added to G_0 cells, inhibition of protein kinase C activity is found when measured 7 h after fetal calf serum restimulation (fig. 3, inset). However, if α-tocopherol is added to G_1 cells and protein kinase C activity measured 7 h later, no inhibition is seen [17]. This result indicates that there is not direct interaction between α-tocopherol and protein kinase C and that α-tocopherol affects early events during the G_0/G_1 transition of the cell cycle. No changes at the level of transcription (not shown) or of protein kinase C expression during the G_1 phase in the absence or presence of α-tocopherol are observed (fig. 3) indicating that the oscillations of protein kinase C activity are the consequence of an alteration on the phosphorylation state of the enzyme. Related to this, α-tocopherol may act by inhibiting protein kinase C phosphorylation or stimulating its dephosphorylation (shown and discussed below).

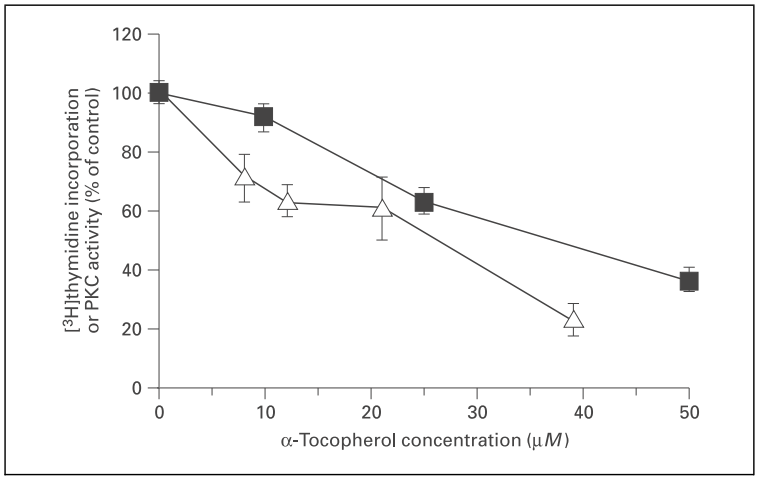

Fig. 2. Parallel inhibition of protein kinase C activity and of proliferation in smooth muscle cells. Quiescent cells are restimulated with FCS in the presence or absence of the indicated concentrations of α-tocopherol. After 7 h (cells in G_1-phase), protein kinase C is measured (■). Control (100%) corresponds to 138.6 ± 4.9 pmol/min/106 cells. For DNA synthesis determination (△), [^{3}H]thymidine is given to the cells in the S-phase. Control represents 84,332 ± 5,150 cpm. Results are expressed as mean ± SD of triplicate determinations.

α-Tocopherol Inhibits Selectively Protein Kinase C-α

Gö 6976 at nanomolar concentrations inhibits protein kinase C-α and -βI, without affecting the other isoforms [18]. As can be seen in figure 4, when protein kinase C-α and -β are completely inhibited by Gö 6976, the residual activity is not sensitive to α-tocopherol indicating that the protein kinase C-δ, -ε, -ζ and -μ isoforms are not involved in the α-tocopherol-induced protein kinase C inhibition.

Taken together, the above experiments indicate that protein kinase C-α (and possibly -β) are the specific target for α-tocopherol action, a conclusion in complete agreement with the studies described below done by immunoprecipitating individual protein kinase C isoforms and measuring their activity.

Effect of α-Tocopherol on Protein Kinase C Isoforms Autophosphorylation

Autophosphorylation has been found to correlate with protein kinase C activity in immunoprecipitates. Cells are incubated in the absence or presence of α- or β-tocopherol for 7 h during the G_1 phase. Then, extracts are prepared and immunoprecipitation of the individual protein kinase C isoforms is performed. Cellular phosphorylation, autophosphorylation and histone phos-

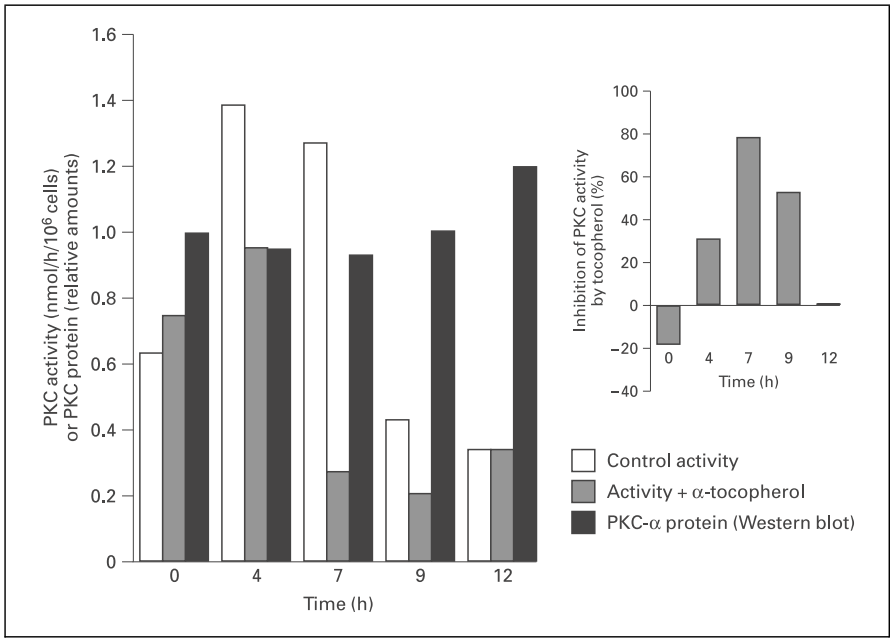

Fig. 3. Inhibition of protein kinase C activity by α-tocopherol is a function of the cycle. Qiescent cells (G_0) are stimulated for different times with FCS in the absence or presence of 50 μ*M* α-tocopherol. At the indicated points, PMA-stimulated protein kinase C activity is measured. Protein kinase C-α immunoblots are scanned by densitometry and signals are expressed as arbitrary units of absorbance.

phorylation are determined. As can be seen in table 2, protein kinase C-α from α-tocopherol-treated cells is inhibited. The activity of all other protein kinase C isoforms is not affected (not shown). On the other hand, β-tocopherol is without effect in all four protein kinase C isoforms studied.

Protein Phosphatase PP_2A Inhibition Abolishes the Effect of α-Tocopherol

When protein kinase C activity is measured in permeabilized cells in the presence of 2 n*M* okadaic acid (fig. 5) capable of inhibiting only protein phosphatase 2A (PP_2A), the effect of α-tocopherol disappears, showing that α-tocopherol affects the phosphorylation state of protein kinase C-α by changing the activity of PP_2A.

Effect of α-Tocopherol on Gene Expression

By differential display analysis it has been found that several genes of smooth muscle cells are differentially transcribed in the presence of α-tocoph-

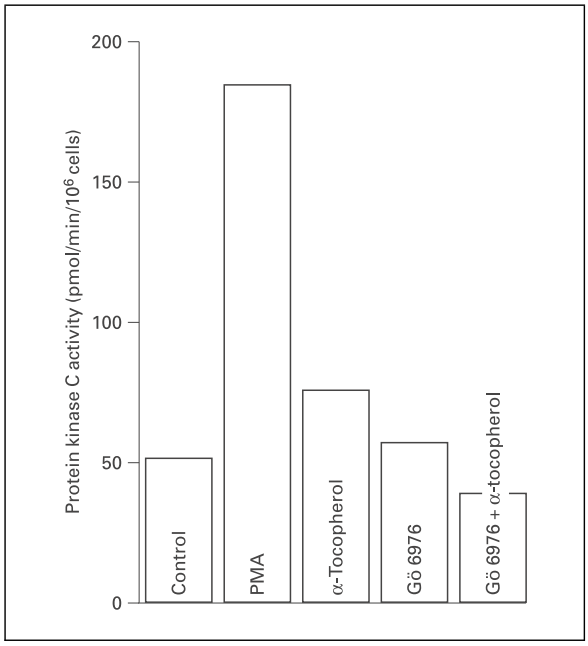

Fig. 4. Effect of α-tocoherol in the presence of protein kinase C-α inhibitors. Quiescent cells are restimulated for 7 h with FCS in the absence or presence of 50 μ*M* α-tocopherol. Gö 6976 (20 n*M*) was added to the permeabilized cells and protein kinase C is measured as described.

erol but not β-tocopherol. In particular, the gene of α-tropomyosin shows a transient enhancement of transcription as a function of the cell cycle time. α-Tropomyosin translation is also increased by α-tocopherol and not by β-tocopherol. Since no changes of mRNA stability can be observed in the presence of α-tocopherol, the data support the conclusion of a transcriptional control exerted by α-tocopherol on α-tropomyosin (data not shown).

Discussion

We have observed inhibition of smooth muscle cell proliferation in vitro by α-tocopherol at physiological concentrations. This could explain the notion that in vivo smooth muscle cells are quiescent and only multiply under stress conditions [19, 20]. Depletion of α-tocopherol, as a consequence of dietary or oxidative diminution, thus can lead to cell growth stimulation and develop-

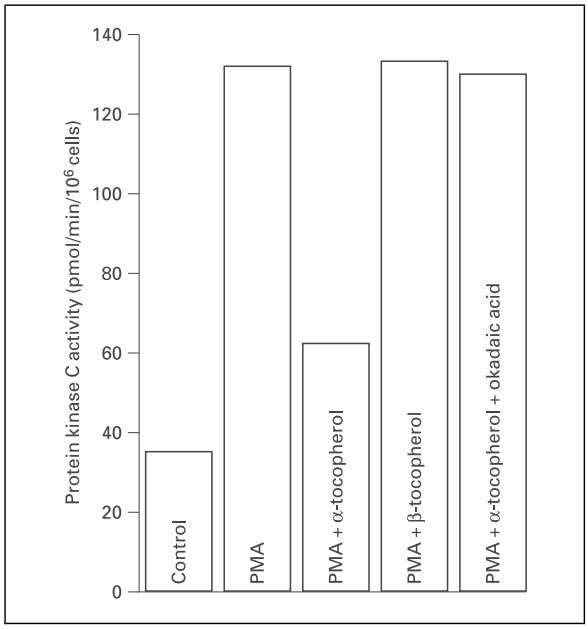

Fig. 5. Involvement of protein phosphatase 2A on the inhibition of protein kinase C by α-tocopherol. Cells are restimulated for 7 h with FCS in the presence or absence of 50 μ*M* α- or β-tocopherol. Okadaic acid (2 n*M*) is added 1 h before the protein kinase C assay. Values are determined using the phosphatase-insensitive peptide $MBP_{(4\text{-}14)}$ as a substrate.

ment of atherosclerosis. β-Tocopherol, an antioxidant almost as potent as α-tocopherol, does not show any effect at the level of cell proliferation or protein kinase C activity. Since β-tocopherol prevents and does not potentiate the effects of α-tocopherol, it appears justifiable to conclude that the mechanism of action of α-tocopherol as a regulator of smooth muscle cell proliferation cannot be associated with its antioxidant properties. Moreover, the above results can be the consequence of the existence of a common intermediate, a putative α-tocopherol-binding protein, that should bind α- and β-tocopherol at the same site, α-tocopherol acting as an agonist and β-tocopherol as an antagonist.

Protein kinase C inhibition appears to be necessary to cause smooth muscle cell proliferation inhibition in vitro but how this enzyme is inhibited by α-tocopherol is not fully clarified. A direct interaction of α-tocopherol with protein kinase C has been excluded on the basis of the cell cycle dependence and cell type specificity. Moreover, no changes in the expression of the protein in the presence of α-tocopherol has been observed. By using isoform-specific

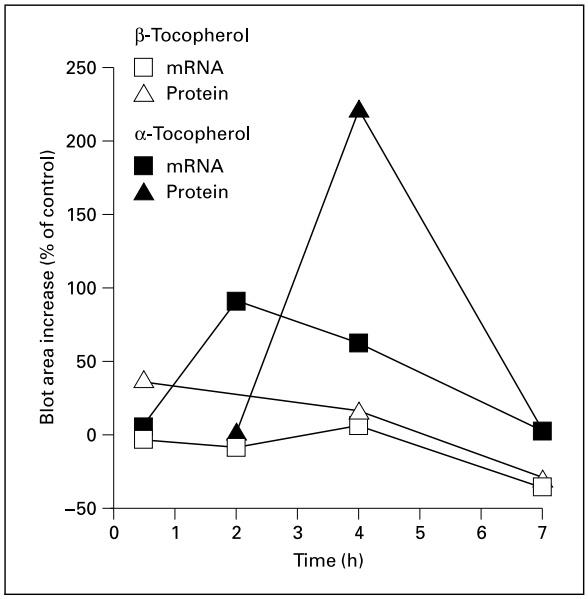

Fig. 6. Effect of α-tocopherol on gene expression. Cells are restimulated for 7 h with FCS in the presence or absence of 50 μ*M* α- or β-tocopherol. From cell extracts at different incubation times, Northern and Western blots are made using an exon 12 α-tropomyosin probe and antibodies against human α-tropomyosin.

inhibitors and activators (Gö 6976 and C_2-ceramide) it is found that the protein kinase C-α isoform is the target of α-tocopherol action.

It is known that protein kinase C becomes active as a consequence of a permissive phosphorylation and an autophosphorylation at discrete sites [21, 22]. Thus, a lack of phosphorylation of protein kinase C by α-tocopherol may be involved in its inhibitory effects. By incubating smooth muscle cell with $^{32}P_i$ it is found that protein kinase C-α phosphorylation is strongly diminished by α-tocopherol. To address further this question, the autophosphorylation activity of individual immunoprecipitated protein kinase C isoforms has been studied. A specific inhibition by α-tocopherol in the autophosphorylation of protein kinase C-α but not in the other protein kinase C isoforms is found.

Accordingly, the following scenario can be imagined: in the presence of α-tocopherol and during the transition $G_0 \rightarrow G_1$, an inhibition of protein kinase C-α phosphorylation or activation of its dephosphorylation occurs. This posttranslational change results in a less active enzyme. The effect of α-tocopherol disappears when the cells are treated with okadaic acid,

Table 2. Effect of α- and β-tocopherol on protein kinase C-α phosphorylation state, autophosphorylating activity and activity towards histone III-S

	^{32}P-protein kinase C-α %	Autophosphorylating activity of protein kinase C-α, %	Histone activity %
PMA	100	100	100
α-Tocopherol	18.5	36.4	56.0
β-Tocopherol	74.1	84.9	79.0

Quiescent A7r5 cells were incubated in phosphate-free medium for 48 h. Cells received 0.25 mCi/ml ^{32}Pi for the last 14 h. Cells were restimulated for 7 h with fetal calf serum in the presence of 50 μ*M* α-tocopherol or β-tocopherol. PMA (100 n*M*) was added for the last hour as indicated. Cell extracts were prepared and proteins resolved by SDS-PAGE. For activity, after treatment of cells, extracts were prepared, protein kinase C-α was immunoprecipitated and kinase reactions in the absence (autophosphorylation) or presence (kinase activity) of histone III-S were performed. Proteins were resolved in a 10% SDS-PAGE and radioactive bands were quantified with a BioRad Molecular-Analyst software. Protein amount was estimated by staining the gel with the SYPRO kit or by immunoblots with the MC5 antibody. Data are expressed as percent of maximal protein kinase C phosphorylation or activity obtained in the presence of PMA alone.

suggesting that the inhibition of protein kinase C phosphorylation may occur by activation of a phosphatase of the 2A type. Indeed the activity of this phosphatase is increased in vitro by α-tocopherol. The question remains open as to the mechanism of α-tocopherol activation of PP_2A. Studies in progress will clarify if in the G_0 to G_1 transition, activation of a pre-existing phosphatase or its new expression is caused by the presence of α-tocopherol.

In conclusion, the specificity of α-tocopherol effects are indicative of a site-directed recognition mechanism and at physiological concentrations the following events may take place: binding of α-tocopherol to a 'receptor protein', activation (or expression) of a protein phosphatase (PP_2A), dephosphorylation of protein kinase C resulting in its inhibition, effects at the level of gene transcription such as the transient increase in α-tropomyosin, followed by final inhibition of cell proliferation. On the other side, if the amount of cellular α-tocopherol content is too low, due to diet, malabsorption or high oxidative stress, the above sequence of events may result into a high level of cell proliferation.

Finally, the epidemiological information linking a decrease of plasma α-tocopherol with an increased risk of ischemic heart disease finds in these studies a molecular counterpart.

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