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Modulation of cell proliferation and gene expression by α-tocopheryl phosphates: relevance to atherosclerosis and inflammation

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Abstract

The effect of a mixture of α-tocopheryl phosphate and di-α-tocopheryl phosphate (TPm) was studied in vitro on two cell lines, RASMC (from rat aortic smooth muscle) and human THP-1 monocytic leukaemia cells. Inhibition of cell proliferation by TPm was shown in both lines and occurred with TPm at concentrations lower than those at which α-tocopherol was equally inhibitory. TPm led in non-stimulated THP-1 cells to inhibition of CD36 mRNA and protein expression, to inhibition of oxidized low density lipoprotein surface binding and oxLDL uptake. In non-stimulated THP-1 cells, α-tocopherol had only very weak effects on these events. Contrary to α-tocopherol, TPm was cytotoxic to THP-1 cells at high concentrations. Thus, TPm is able to inhibit the major aggravating elements involved in the progression of atherosclerosis. The higher potency of TPm may be due to a better uptake of the molecule and to its intracellular hydrolysis, providing more α-tocopherol to sensitive sites. Alternatively, a direct effect of the phosphate ester on specific cell targets may be considered.

Keywords: Tocopherol; Tocopheryl phosphate; OxLDL; CD36; Growth inhibition; Apoptosis

A series of studies have drawn attention on the regulatory aspects of α-tocopherol on macrophages and smooth muscle cells. Modulation of signalling cascades and gene expression in these cells is at the basis of the preventive effect of α-tocopherol in atherosclerosis and inflammatory disease. A specific receptor for oxidized LDL (oxLDL) (the CD36 scavenger receptor) is expressed in endothelial cells, monocytes/macrophages, and cultured human aortic smooth muscle cells. A number of studies have indicated that CD36 can transport oxLDL into the cytosol of these cells and that α-tocopherol inhibits oxLDL uptake by a mechanism involving down-regulation of CD36 mRNA and protein expression [1,2]. Considering the central role of foam cells in the progression of atherosclerosis and the fact that they are generated through the uptake of oxLDL via CD36 [3], the possible beneficial effect of α-tocopherol against atherosclerosis may be explained, at least in part, by the down-regulation of CD36, with consequent reduction of foam cell formation [1,2]. A reduction of the scavenger receptor SR-A expression and activity in the presence of α-tocopherol was also observed [4]. The role of α-tocopherol in diminishing scavenger receptor activity (CD36) has been confirmed in vivo. Correspondingly, rats depleted of vitamin E have shown an increased expression of the scavenger receptor CD36. Similarly, rabbits at high cholesterol diet show an induction of CD36 that is prevented by α-tocopherol (Azzi et al., unpublished).

Recently, novel tocopherol derivatives have been described: the tocopheryl phosphate ester (TP) and the bis-tocopheryl phosphate ester, or di-α-tocopheryl phosphate (T_2P). The former compound is the ester

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derivative of phosphate with the hydroxyl group of tocopherol, while the latter is obtained by esterification of two tocopherol moieties with one phosphate molecule [5].

Interest in tocopheryl phosphate derivatives has increased after the discovery that α-tocopheryl phosphate is present in plant and animal tissues as well as in foods [5]. Sometimes the amounts of α-tocopheryl phosphate, such as in chocolate and cheese, are 10- to 30-fold higher than free α-tocopherol. In animal tissues (including humans) the amounts of α-tocopheryl phosphate are of the same order of magnitude as that of α-tocopherol and in some cases it can be decisively higher (rat and pig liver). Furthermore, supplementation of the diet of rats with α-tocopheryl phosphate resulted in an increased deposition of tocopheryl phosphate in liver and adipose tissue and as well in an increase of α-tocopherol [5]. These findings prompt a number of questions, ranging from the possibility that α-tocopheryl phosphate is a reserve form of α-tocopherol to the hypothesis that it may represent an active compound capable of regulatory effects at cellular level.

Given the important effects of α-tocopherol on cells involved in atherosclerosis and inflammation, tocopheryl phosphates have been tested in for their ability to regulate monocyte and smooth muscle cell proliferation, to down-regulate CD36 expression and the CD36 mediated uptake of oxLDL. The data suggest that α-tocopheryl phosphates are not only able to mimic the effects of α-tocopherol, but they appear more effective at lower concentrations. The data are discussed in terms of a facilitated delivery effect of α-tocopherol through its phosphate-ester form or of a specific and direct effect of α-tocopheryl phosphates, independent of that of α- tocopherol.

Experimental procedures

Materials. The mixture of α-tocopheryl phosphates, TPm (*Phospha E*, Phosphagenics, Ltd.) was synthesized as follows. α-Tocopherol (51 g, 0.1 mol; RRR-α-tocopherol (Cognis, LaGrange, IL, USA)) was reacted with P_4O_{10} (8.5 g, 30 mmol) under high shear conditions at 80–90 °C to hydrolyse the residual polyphosphate bonds. Analysis of the resultant product using ^{31}P NMR indicated a mixture of α-tocopheryl phosphate, di-α-tocopheryl phosphate, and some residual inorganic phosphate. The TPm had the following composition: TP, 55.3%; T_2P, 30.6%; α-tocopherol, 5%; inorganic phosphate, 2.6%; water, 1.9%; oleic acid, 1.0%; and impurities (i.e., sterols), 3.57%. Stock solutions of TPm were prepared in ethanol with sonication. The tocopherol equivalents in the TPm is approximately 0.8 (i.e., 1 g TPm is equivalent to 0.8 g α-tocopherol). Stock solutions of α-tocopherol were prepared in ethanol.

The product was also used in some experiments (Fig. 1A) after conversion to the sodium form from the free acid form, as follows. The TPm was dissolved in ethanol and NaOH (in ethanol) was added at a 2:1 molar ratio of sodium to phosphorus. Removal of the ethanol was under reduced pressure. Stock solutions of the sodium form were prepared in 0.1% ethanol with sonication.

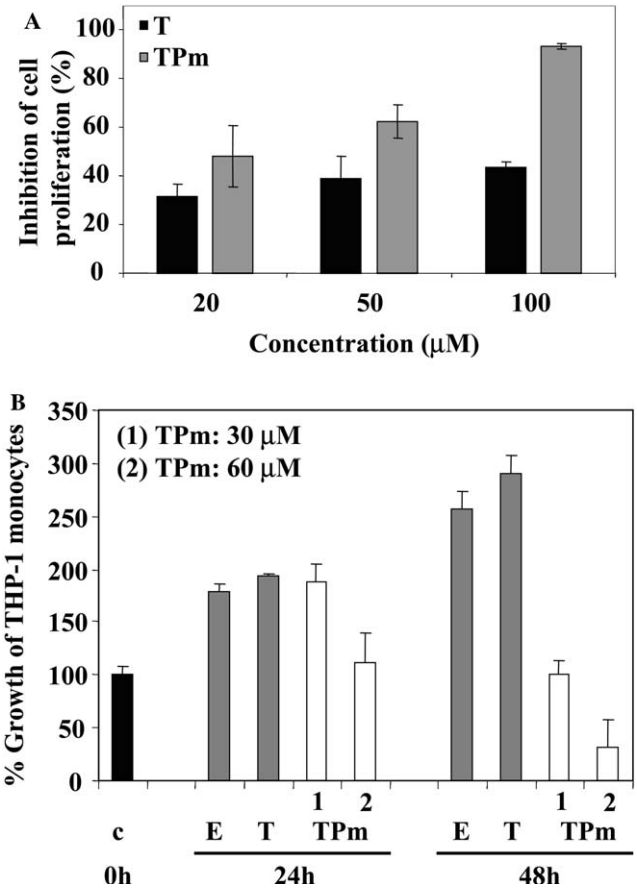

Fig. 1. Effect of TPm (sodium form) and α-tocopherol on the proliferation of (A) rat aortic smooth muscle cells (RASMC) and (B) human THP-1 monocytes. E, ethanol control (0.1%); T, α-tocopherol (50 μ M).

Cell culture. Rat aortic smooth muscle cells (RASMC, Cell Applications, USA) were seeded in growth medium (DMEM/F12 + 10% serum) into 6-well plates (25,000 cells/well). After 24 h, cells were washed twice with Hanks' buffered salt solution and DMEM/F12 + 0.2% serum was added to each well. Cells were serum-starved for 48 h prior to treatments.

Human THP-1 monocytic leukaemia cells (ATCC # TIB-202) were cultured in RPMI/10% FCS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, and 4.5 g/L glucose. THP-1 cells (10^6 per plate) were plated 24 h before treatments with α-tocopherol or TPm at the concentrations indicated.

Proliferation assay. RASMC were grown and serum-starved as above. Treatment solutions containing drugs were then prepared in growth medium and added to each well (3 ml/well). RASMC were plated into 6-well plates at 5×10^4 cells/well and the effect of α-tocopherol and TPm on smooth muscle cell proliferation was tested at three concentrations: 20, 50, and 100 μM. Control treatments included: growth medium and growth medium + vehicles (ethanol did not exceed 0.1%). After an incubation period (72 h at 37 °C, 5% CO_2), cells were trypsinized from the plate, stained with trypan blue, and counted with a hemocytometer. The experiment was performed in triplicate and the mean and standard deviation were calculated for each treatment.

THP-1 cells were plated into 6-well plates at 2×10^5 cells/well and treated with 0.1% ethanol (control), α-tocopherol (50 μM) or TPm at the indicated concentrations. The cells were counted in duplicate with a hemocytometer at 0, 24, and 48 h. The experiment was performed in triplicate and the mean and the standard deviation were calculated for each treatment.

RT-PCR. Total RNA was isolated using a RNA extraction kit from Qiagen. Semi-quantitative assays for CD36 mRNA expression were performed with a RT-PCR kit (Perkin–Elmer), using primer CD36PCRF: 5′-ATCCCATATCTATCAAAATC-3′, which anneals to exon 6, and primer CD36PCRR: 5′-TCGATTATGGCAACTTTAC-3′, which anneals to exon 7, for 30 cycles at 95 °C, 30 s; 48 °C, 30 s; and 72 °C, 30 s. Control reactions were performed with primers specific for the human glyceraldehyde-3-dehydrogenase (GAPDH). GAP1: 5′-AG CCACATCGCTCAGACACC-3′ and GAP2: 5′-TGAGGCTGTTG TCATACTTCTC-3′ for 28 cycles at 95 °C, 30 s; 68 °C, 30 s; and 72 °C, 30 s. The PCR products were loaded on a 2.4% agarose gel and the bands were quantified with a LumiImager (Roche).

Western blots. Western blots were done according to standard methods with monoclonal mouse anti-human CD36 primary antibody (Ancell) and sheep anti-mouse IgG secondary antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech). An anti-β-actin antibody (Sigma) was used as internal control. Proteins were visualized using an ECL detection kit according to the manufacturer's description (Amersham Pharmacia Biotech). Chemiluminescence was monitored by exposure to film (Hyperfilm ECL) and the signals were analysed using a LumiImager (Roche).

FACS analysis. For FACS, the cells were pre-treated for 24 h with 50 μM α-tocopherol, TPm or 0.1% ethanol solvent (control). Thereafter, the cells were washed two times with PBS, once with PBS/1% BSA, and then incubated in 250 μl of anti-CD36 antibodies (1:50 diluted) (Ancell) for 30 min at 4 °C. After that, the cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS. FACS was performed with a FACSscan (Becton–Dickinson).

Uptake and binding of oxLDL-DiO. Labelling of oxLDL with DiO (Intracell corporation) was done basically as previously described [1,6]. Uptake and binding of oxLDL-DiO was studied by FACS. The cells were pre-treated for 16 h with 50 μM α-tocopherol, TPm or 0.1% ethanol solvent (control) and then incubated with oxLDL-DiO (5 μg/ml medium). For the uptake experiments the incubation was done at 37 °C for 6 h, for the binding experiment the incubation was done for 30 min at 4 °C. Thereafter, the cells were washed three times with PBS/3% BSA, once with PBS, and fixed with 4% paraformaldehyde in PBS. FACS was performed with a FACSscan (Becton–Dickinson).

Apoptosis assay. THP-1 cells (2×10^6) were treated with 0.1% ethanol (control), α-tocopherol (50 μM) or TPm for 24 h, harvested, and washed with PBS. Apoptotic DNA laddering was assayed using the apoptotic DNA laddering kit as described by the manufacturer (Roche).

Results

Inhibition of cell proliferation by TPm

The effect of TPm on cell growth was assessed in rat aortic smooth muscle cells (RASMC) and human monocytes (THP-1). The growth of RASMC was significantly inhibited by TPm at concentrations of 20 μM (Fig. 1A).

Similarly, treatment of THP-1 cells with TPm for 24 h led to growth inhibition only with higher TPm concentrations (60 μM). However, for the 48 h treatments TPm at 30 μM inhibited proliferation strongly (Fig. 1B).

Induction of apoptosis only at high concentration of TPm

Higher concentrations of TPm were cytotoxic to THP-1 cells, as judged by trypan exclusion. To assess whether THP-1 cells underwent apoptosis, nuclear DNA laddering was monitored (Fig. 2). THP-1 cells were incubated with increasing concentrations of TPm, genomic DNA extracted, and separated on a 1% agarose gel. Low concentrations (5–46 μM) of TPm had no effect on DNA laddering, whereas high concentrations (46–184 μM) induced significant DNA degradation. These results suggest that growth inhibition with low concentrations of TPm was not the result of apoptosis.

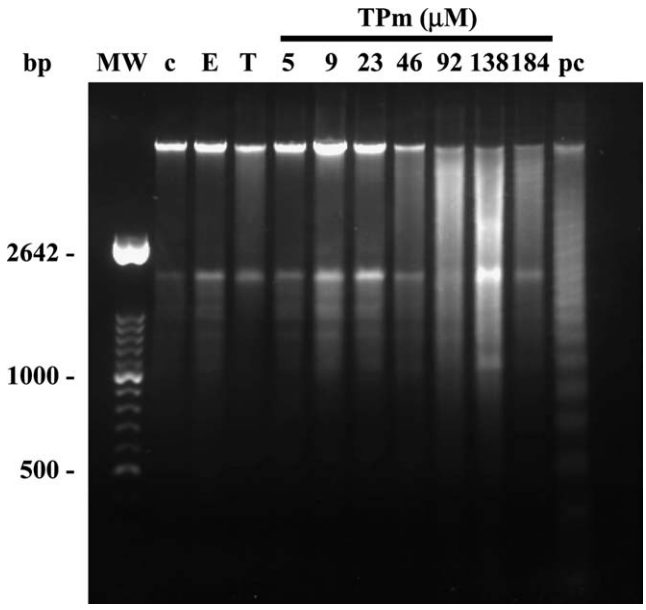

Fig. 2. Effect of TPm and α-tocopherol on THP-1 cells apoptosis monitored by nuclear DNA laddering. THP-1 cells were incubated with control ethanol (E, 0.1%), α-tocopherol (T, 50 μM), or increasing concentrations of TPm for 24 h. The genomic DNA was extracted and run on a 1% agarose gel and apoptosis was assessed by DNA laddering. c, Untreated control; pc, positive control. The experiment was repeated three times with similar results.

Inhibition of CD36 protein expression by TPm

The over-expression of the CD36 scavenger receptor in smooth muscle cells and activated monocytes/macrophages was recently shown to be decreased by treatment with α-tocopherol, in what may represent one of the mechanisms of how vitamin E prevents development of atherosclerosis [1,2]. Therefore, we measured whether and how strongly TPm was able to inhibit CD36 expression. THP-1 monocytes were treated for 24 h with increasing concentrations of TPm (9–46 μM) and CD36 surface expression was assayed by FACS. TPm strongly decreased CD36 expression (Fig. 3), whereas α-tocopherol (50 μM) had no effect on non-stimulated THP-1 cells (not shown).

Total CD36 protein expression in THP-1 cells was assessed by Western blots. Treatment of non-stimulated THP-1 monocytes with increasing TPm concentrations (9–46 μM) led to decreasing levels of CD36

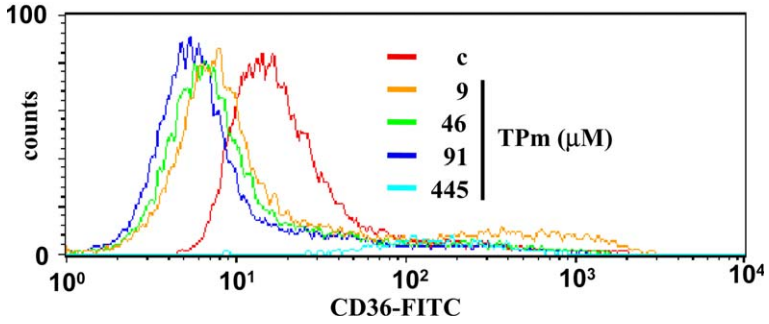

Fig. 3. Inhibition of CD36 protein expression by TPm. THP-1 cells were incubated with increasing concentrations of TPm for 24 h and CD36 expression was quantified by FACS using an anti-human-CD36-FITC antibody. The experiment was repeated three times with similar results.

protein, whereas α-tocopherol (50 μM) had no effect (Fig. 4).

Inhibition of CD36 mRNA transcription by TPm

Vitamin E was shown to modulate the expression of several genes, including the CD36 scavenger receptor gene (reviewed in [7,8]). To assess whether TPm had similar effects, the expression of CD36 was assayed in non-stimulated THP-1 monocytes by semi-quantitative RT-PCR. THP-1 cells were incubated for 24 h with increasing concentrations of TPm (5–46 μM), total RNA was isolated and the expression of CD36 mRNA was quantified by RT-PCR. The expression of CD36 decreased with increasing concentrations of TPm whereas α-tocopherol had no effect (Fig. 5).

Inhibition of oxLDL-DiO uptake and binding by TPm

The CD36 scavenger receptor is responsible for the uptake of oxLDL into monocytes/macrophages, and during the atherosclerotic process the over-expression of

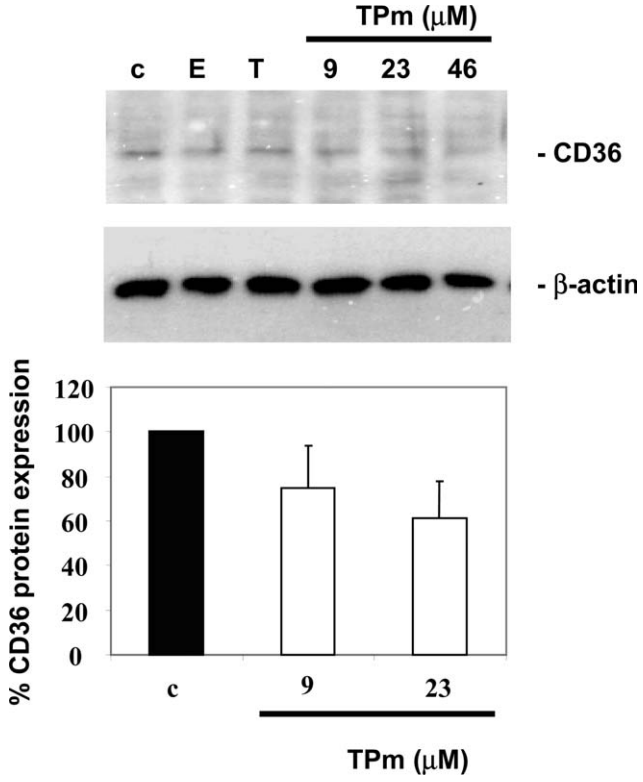

Fig. 4. Inhibition of CD36 protein expression by TPm. THP-1 cells were incubated with solvent control ethanol (E, 0.1%), α-tocopherol (T, 50 μM), or increasing concentrations of TPm for 24 h, and CD36 expression was quantified by Western blot analysis. As internal control β-actin was used. The graph shows the mean and standard deviation from three experiments.

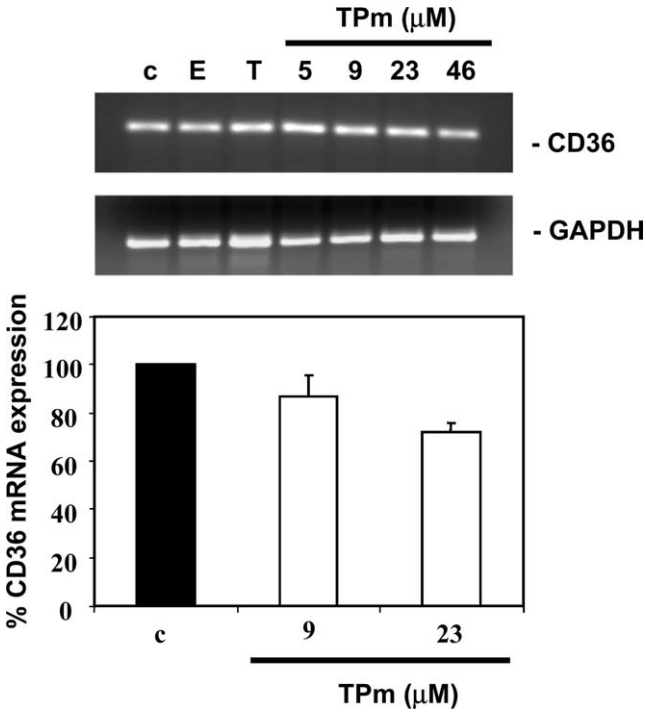

Fig. 5. Inhibition of CD36 mRNA transcription by TPm. THP-1 cells were incubated with solvent control ethanol (E, 0.1%), α-tocopherol (T, 50 μM), or increasing concentrations of TPm for 24 h, and CD36 expression was quantified by RT-PCR. Amplification of GAPDH was used as internal control. The graph shows the mean and standard deviation from two experiments.

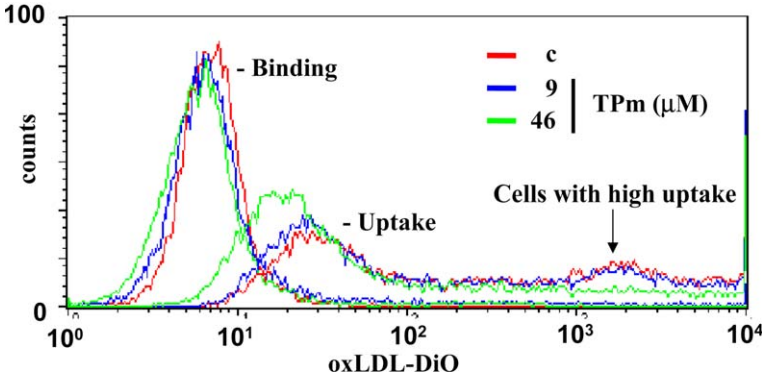

Fig. 6. Inhibition of oxLDL-DiO uptake and binding by TPm. THP-1 cells were incubated with increasing concentrations of TPm for 24 h. The uptake of oxLDL was measured after incubation with oxLDL-DiO (5 μg/ml) at 37 °C for the last 6 h. The binding of oxLDL was measured after incubation with oxLDL-DiO (5 μg/ml) at 4 °C for the last 30 min. Uptake and binding of oxLDL-DiO was quantified by FACS. The experiment was repeated two times with similar results.

CD36 leads to foam cell formation. To assess whether decreased expression of CD36 can reduce binding and uptake of oxLDL, non-stimulated THP-1 monocytes were incubated with increasing concentrations of TPm (9–46 μM), and uptake or binding of fluorescent labelled oxLDL-DiO was analysed by FACS. Parallel to the results of CD36 protein expression, TPm treatment resulted in a decrease of oxLDL-DiO binding and uptake (Fig. 6).

Discussion

Natural vitamin E is a mixture of tocopherols and tocotrienols (α-, β-, γ-, δ-tocopherol, and α-, β-, γ-, δ-tocotrienol). Commercially, vitamin E is available in different forms: a mixture of natural tocopherols and tocotrienols (extracted from natural sources), RRR-α-tocopherol, synthetic α-tocopherol, which is an equimolar racemic mixture of eight stereoisomers (*all rac*-α-tocopherol), or a racemic 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. Once in the gut, the esters of vitamin E are split to their unesterified forms under the action of pancreatic and intestinal esterases and only the non-esterized tocopherols are efficiently taken up [9–12].

Other vitamin E analogues, such as α-tocopheryl succinate, α-tocopheryl polyethylene glycol 1000 succinate, tocopheryl nicotinate, tocopheryl ferulate, tretinoin tocopheril, α-tocopheryl phosphate, and trolox, have also been synthesized and their cellular effects were investigated [11,13–17]. These molecules often act as completely novel compounds, are transported differently, and have their own effects on cellular signalling and apoptosis [13,14,18–22]. Since most of these analogues are modified at the tocopherol-6-O position, they do not have any antioxidant activity before their hydrolysis.

Recently, using a novel isolation method, one of the synthetic analogues of tocopherol, the α-tocopheryl phosphate, was shown to occur naturally in food and tissues [5]. Moreover, it was shown that low amounts of tocopherol can become phosphorylated and dephosphorylated, suggesting that the inter-conversion may serve some cellular functions [23]. Several functions and activities have been suggested for tocopheryl phosphate; induction of hippocampal long term potentiation [24], protection of mouse skin against ultraviolet-induced damage [23], activation of cAMP phosphodiesterase [25], and activation of rat liver phenylalanine hydroxylase [26].

We find here that TPm reduces growth of rat aortic smooth muscle cells and human THP-1 monocytes more efficiently than α-tocopherol, even when using much lower concentrations of TPm compared to α-tocopherol. At these concentrations no evidence of apoptosis was detected, suggesting that growth inhibition is the result of modulation of cellular signalling. Moreover, the expression of total CD36 protein in non-activated THP-1 monocytes and on their surface is decreased by TPm treatment, whereas α-tocopherol had no effect. Decreased expression of CD36 protein led to decreased binding and uptake of oxLDL, suggesting that TPm could reduce the formation of foam cells. The inhibition of CD36 protein expression is at least partially the result of decreased mRNA expression, albeit the degree of reduction of CD36 surface expression appeared to be generally stronger than the one seen at the mRNA level. Since CD36 recognizes and internalizes anionic phospholipids like phosphatidylserine [27], TPm may induce CD36 internalization in addition to the other effects seen.

Taken together, TPm shows activities like growth inhibition, reduction of CD36 mRNA and protein expression that are similar to α-tocopherol, albeit at much lower concentrations. These effects can be

rationalized in different ways. It is conceivable that TPm acts at cellular level in a way which is unrelated to that of α-tocopherol, similarly to what has been shown with the succinate ester. It might have direct effects at the level of signal transduction intermediate steps or in gene regulation. However, it may also be possible that the phosphorylated forms of tocopherol can be transported to the sites of action more efficiently than the non-phosphorylated one and that the effects observed with TPm at lower concentrations relative to α-tocopherol may be due to higher local concentration than the latter. At this time it is unknown whether phosphorylated tocopherol is much better taken up into cells when compared to tocopherol and to what degree it is de-phosphorylated or metabolized. A distinct difference between TPm and α-tocopherol is that TPm can be toxic to THP-1 cells at high concentrations. This effect may be possibly obtained only at pharmacological levels of the compound, which could thus be considered to have effects as a drug to be employed when excessive cell proliferation should be counteracted by an apoptotic stimulus. At lower concentrations TPm usage may result in protection against atherosclerosis and in the defence of skin against UV light damage [23]. Moreover, since CD36 mediates inflammatory changes in response to non-lipid ligands like β-amyloid [28], a reduction of CD36 expression by TPm in macrophages/microglia could decrease inflammatory reactions. Further studies on the activities of TPm on gene expression and cellular signalling are required to solve these issues.

Acknowledgments

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