

Molecular mechanism of α -tocopheryl-phosphate transport across the cell membrane

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Abstract

α -Tocopheryl-phosphate (α -TP) is synthesized and hydrolyzed in animal cells and tissues where it modulates several functions. α -TP is more potent than α -T in inhibiting cell proliferation, down-regulating CD36 transcription, inhibiting atherosclerotic plaque formation. Administration of α -TP to cells or animals requires its transfer through membranes, via a transporter. We show here that α -TP is passing the plasma membrane via a system that is inhibited by glibenclamide and probenecid, inhibitors of a number of transporters. Glibenclamide and probenecid prevent dose-dependently α -TP inhibition of cell proliferation. The two inhibitors act on ATP binding cassette (ABC) and organic anion transporters (OAT). Since ABC transporters function to export solutes and α -TP is transported into cells, it may be concluded that α -TP transport may occur via an OAT family member. Due to the protection by glibenclamide and probenecid on the α -TP induced cell growth inhibition it appears that α -TP acts after its uptake inside cells.

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α -Tocopherol (α -T) is a typical antioxidant when studied in a test tube. However, like other antioxidants, *in vivo* this molecule has different properties, which are used to trigger signal transduction and modulate gene transcription [1]. The unique capacity of the human body to take up α -T indicates that this molecule has special properties [2]. The detailed mechanism of α -T function is however not fully clarified. We have shown that α -T is able to affect a master switch in cells, protein kinase C [3]. Protein kinase C and the associated cell proliferation inhibition are specific for α -T while β -tocopherol (β -T) is not producing a significant inhibition of either of them [4]. This finding supports the hypothesis that α -T is not acting as an antioxidant. Inhibition by α -T of protein kinase C results in a number of cellular events like the inhibition of O_2^- production in macrophages and neutrophils [5]. This is due to the NADPH oxidase inhibition, whose assembly is prevented by the lack of P47 phosphorylation, of one of the oxidase

subunits [5]. Another effect which we have clarified is the inhibition by α -T of several steps of the PI3 kinase cascade [6]. Independent of the inhibition of protein kinase C, we have described another function of α -T, the transcriptional down-regulation of the gene coding for the scavenger receptor CD36 [7]. The uptake of oxidized lipoproteins by this receptor is an early event in the progression of atherosclerosis, and consequently its regulation by α -T may be of remarkable pathophysiological significance. After this initial discovery, a number of studies, carried out in tocopherol transfer protein knockout mice or in animals treated with a low α -T diet, have shown that several genes are regulated by α -T, but none of them are genes that code for antioxidant enzymes [8–11]. An overexpression of antioxidant enzymes would be expected as a compensatory mechanism consequent to α -T diminution, in case its action were that of an antioxidant.

Although a number of groups have been searching for an α -T receptor, capable of distinguishing α -T from similar molecules, no such a protein could be detected till now. The tocopherol associated proteins (TAPs) are apparently

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involved in α -T trafficking, however their direct role in cell signalling and gene transcription remains to be clarified [12].

More recently, we have searched for an α -T derivative, acting at lower concentrations than α -T. Such a molecule could also have the receptor recognition properties that have not been found for α -T. α -TP has been found in normal tissues [13]. This ester of phosphoric acid with the tocopherol hydroxyl group can also be synthesized by isolated cells [14]. In this study, the following questions will be dealt with: is α -TP, when added to cells, toxic? Is it going to produce, when added to cells, the same effects as α -T? If α -TP is active, does it need to penetrate into cells in order to exhibit its activity? Is the activity of α -TP due to its hydrolysis to α -T?

Materials and methods

Human THP-1 monocytic leukaemia cell line was obtained from American Type Culture Collection, ATCC (ATCC # TIB-202). RPMI Medium 1640, fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). (\pm)- α -Tocopherol phosphate disodium salt (T2020), α -tocopherol, glibenclamide, and taurocholate were from Sigma Chemicals (St. Louis, MO). Stock solutions of α -tocopherol were prepared in ethanol. Glibenclamide was dissolved in DMSO.

Cell culture. Human THP-1 monocytic leukaemia cells were cultured in RPMI 1640 medium with 10% FBS, 25 mM Hepes, L-glutamine, 1.0 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin. Cell culture was maintained at 37 °C in a humidified incubator supplied with a 95% air and 5% CO₂ atmosphere. THP-1 cells were plated 24 h before treatments.

Proliferation assay. Cell proliferation was determined by a MTS assay with CellTiter 96[®] Aqueous One Solution Reagent (Promega Corp., Madison, WI) which is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The MTS tetrazolium compound is bio-reduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. THP-1 cells were plated in 96-well microplates at a concentration of 10,000 cells per well and incubated for 24 h. After that, cells were incubated with 0.5% ethanol (vehicle), 10, 25, and 50 μ M of α -T and α -TP for another 48 h. After treatment, 20 μ l of the 96 Cell titer solution was added to each well and incubated for 4 h at 37 °C in humidified 5% CO₂ atmosphere and absorbance read at 490 nm with a 96-well plate reader. The total volume of the reaction mixture was 120 μ l for the proliferation assay. The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment.

Treatment the cells with glibenclamide and proliferation studies. THP-1 cells were plated in 96-well plates (10,000 cells/100 μ l medium) and were pre-incubated with indicated concentrations of glibenclamide for 3 h, after that, α -TP was added to wells treated with or without glibenclamide. Cells were incubated for another 24 h and at the end of the incubation period, the proliferation of cells were measured with MTS assay as described above.

Cytotoxicity assay. Cytotoxicity was evaluated by lactate dehydrogenase (LDH) release into culture medium. THP-1 cells were plated (5000 cells/100 μ l medium) and incubated with medium (without pyruvate) containing 2.5% FBS for 24 h. Cells were incubated with indicated concentrations of α -TP for 24 and 48 h. After the incubation period, LDH release was measured CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega). The assay measures the release of LDH from cells with damaged membranes by fluorimetry. LDH released into the culture

medium is measured with a coupled enzymatic assay that results in the conversion of resazurin into a fluorescent product (resorufin). The amount of fluorescence produced is proportional to the number of lysed cells. The experiments were performed in quadruplicate and the mean and standard deviation were calculated for each treatment.

Determination of cellular α -T and α -TP content. Cells (2×10^6) were incubated with 10, 25, and 50 μ M α -T or α -TP for 24 h. After the incubation, in order to eliminate the adsorbed tocopherol, cells were washed in ice-cold PBS containing 5 mM taurocholate twice and once in PBS alone and were suspended in 1 ml PBS. Aliquots of cell samples were taken to measure protein concentration.

Treatment with glibenclamide for uptake studies. Cells (2×10^6) were pre-incubated with the indicated concentrations of glibenclamide for 3 h; after the treatment, 50 μ M of α -TP was added to the wells treated with or without glibenclamide and incubated for another 24 h. Control experiments were performed on ice.

α -T and α -TP extraction. Cell suspensions were taken into screw-cap tubes, 1 ml ethanol containing 1.2% pyrogallol (prepared freshly) was added and kept at 70 °C for 2 min, afterwards 100 μ l of 30% KOH was added and kept at 70 °C for 30 min. After the samples were cooled down to room temperature, the extract containing α -TP was acidified with 800 μ l of 2 N HCl and shaken vigorously for 1 min. α -T and α -TP was extracted with hexane containing 0.02% butylated hydroxytoluene (BHT), 2 ml of hexane was added, shaken vigorously for 2 min and centrifuged at 3000 rpm for 3 min. The upper phase was moved to another tube and extracted twice with hexane. The hexane phases were pooled and dried under nitrogen gas. The dried samples were dissolved in 100 μ l of 10% acetic acid in isopropanol and sonicated for 10 min. The dissolved sample was injected into a 150 \times 4.60 mm Phenomenex Luna C8 5 μ column using a Waters 2695 Separation module, Waters 2487 Dual Absorbance detector, Hewlett Packard 1100 series Fluorescence detector with the column heated to 40 °C. Mobile phase A was 0.2% phosphoric acid in isopropanol and B was water. The flow rate was 0.4 ml/min with the gradient beginning with 60% A to 100% A over 20 min, 100% A maintained for 10 min, then to 60% A over 4 min, and maintained at 60% A for 15 min before the next injection. Detection was by UV at 286 nm, and by fluorescence with excitation at 297 nm and emission at 319 nm. Concentration of α -TP and α -T were expressed per μ g of total cellular protein, as determined by BSA protein assay kit (Pierce, Rockford, IL).

Results and discussion

Is α -tocopheryl-phosphate toxic when added to cells?

To the question posed above, the answer is given by a toxicity analysis in isolated cells (Fig. 1). In this experiment, α -TP cytotoxicity is measured as LDH release from THP-1 cells treated with increasing concentrations of α -TP up to 200 μ M for 24 and 48 h. No α -TP cytotoxicity is observed up to a concentration of 100 μ M. Cells do not exhibit any damage. The conclusion can be drawn that, at least at cellular level, α -TP below or up to 100 μ M can be used without causing cell damage. Other cell lines such as Hep-G2 and CaCo2 cells (up to 72 h incubation, MTS assay) are not damaged by α -TP concentrations up to 100 μ M.

Does α -tocopheryl-phosphate produce the same effects as alpha-tocopherol when added to cells?

A number of cellular reaction (cell proliferation and gene expression) has been previously shown to be more sensitive to α -TP than to α -T.

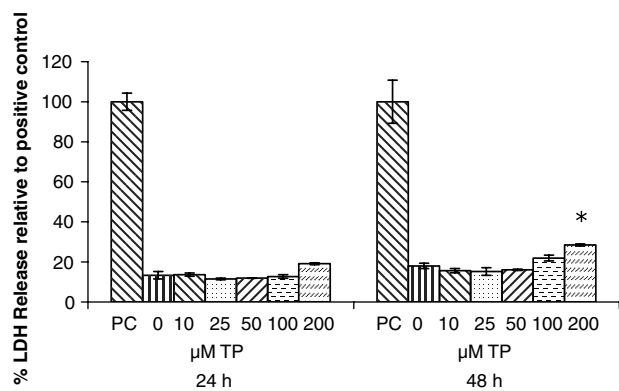


Fig. 1. Effect of α -TP on LDH release in THP-1 cells. Cells were treated with increasing concentrations of α -TP for 24 and 48 h and LDH activity was measured and LDH release was expressed as % of control. The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment. PC, positive control (complete Triton X-100 lysis). A statistically significant difference of $p < 0.05$ (two-tailed unpaired Student's t -test) in the comparison of any given sample series with the one without added α -TP (0 μ M, 48 h control) is marked (*).

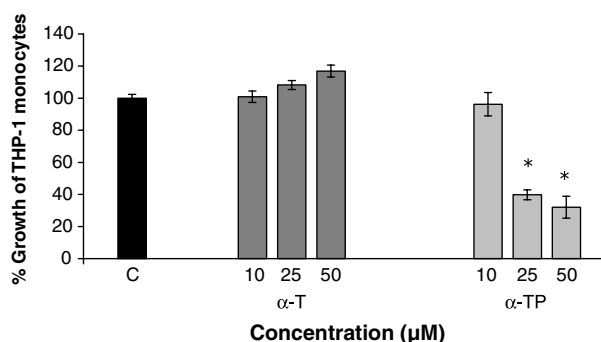


Fig. 2. Effect of α -T and α -TP on the proliferation of THP-1 monocytes. Cells were treated with increasing concentrations of α -T and α -TP for 48 h and cell proliferation was measured with an MTS assay kit (see Materials and methods). Control (C) refers to the 48 h value. The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment. A statistically significant difference of $p < 0.05$ (two-tailed unpaired Student's t -test) in the comparison of any given sample series to the one without added α -TP (control, C) is marked (*).

In this study α -TP has been shown (Fig. 2) to be capable, in isolated THP-1 cells, to inhibit their proliferation. Cell proliferation is measured spectrophotometrically as absorbance increase consequent to the metabolic reduction of the yellow tetrazolium salt (MTS) to form purple formazan crystals by living cells. It is evident that α -TP inhibits cell growth and that, under these conditions, α -T does not produce any significant effect.

Is the activity of α -tocopheryl-phosphate due to its hydrolysis to α -tocopherol?

Having established that α -TP is a potent modulator of several cell functions, we have asked the question whether this effect was related to the hydrolysis of α -T from the pre-

Table 1
 α -Tocopheryl-phosphate uptake and α -tocopherol hydrolysis by THP-1 cells

THP-1 cells treated with α -TP	pmol α -TP/ μ g cell protein taken up	pmol α -T/ μ g cell protein hydrolyzed
Control	0.00	0.00
10 μ M α -TP	10.62 \pm 1.93	1.89 \pm 0.40
25 μ M α -TP	16.99 \pm 0.08	2.83 \pm 0.00
50 μ M α -TP	23.99 \pm 1.62	3.59 \pm 0.07

Cells were treated with increasing concentrations of α -TP for 24 h, incorporated α -TP and hydrolyzed α -T content of the cells was determined by HPLC. The values show the mean and the standard deviation from triplicate values of two separate experiments.

cursor molecule. In the experiment presented in Table 1 it is shown that α -TP is taken up by cells- left column- in amounts which are increasing at higher concentration of added α -TP; parallelly, the amount of α -T produced in the cells has been measured and results to be about 15% of the α -TP taken up by cells (right column) Consequently, we can conclude that α -TP is transported into THP-1 cells, and that the amount of α -T which is liberated from it is small.

In the experiments shown in Table 2 the amount of α -T which is associated to cells after addition of α -T has been measured. The experiment shows that the more α -T is added to cells (10, 25, and 50 μ M) the more it becomes associated with them (2.3, 5.1, and 11 pmol/ μ g protein, respectively). The amounts of α -T associated with cells, when cells were treated with α -T, are bigger than the amounts hydrolyzed from α -TP. However, the effects produced by α -TP (compare Fig. 3) are much more significant, indicating that α -T is not responsible for the modulation of these cellular events but rather the intact α -TP.

Does tocopheryl-phosphate need to penetrate into cells in order to exhibit its activity?

If α -TP acts at cellular level as we have shown, by producing inhibition of cell proliferation (Fig. 2), and inhibition of CD36 expression [15], how is α -TP acting? Does it act at the surface of cells or after its penetration into cells?

An experiment to show penetration of α -TP into cells is presented in Table 3. The association of α -TP to THP-1 cells appears to be temperature dependent as it occurs for carrier mediated transport of solutes. Furthermore, the presence of glibenclamide [16–18] inhibits the uptake of

Table 2
Amount of α -tocopherol associated to THP-1 cells

α -T added to THP-1 cells	pmol α -T/ μ g cell protein
Control	0.03
10 μ M α -T	2.31 \pm 0.38
25 μ M α -T	5.16 \pm 2.38
50 μ M α -T	11.03 \pm 3.18

Cells were treated with increasing concentrations of α -T for 24 h; α -T content of the cells was determined by HPLC. The values show the mean and the standard deviation from triplicate values of two independent experiments.

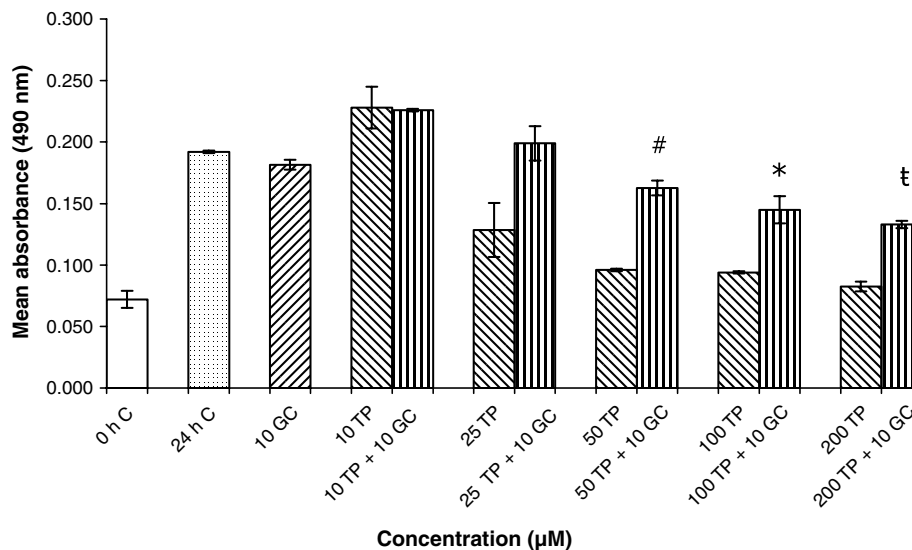


Fig. 3. Abrogation of α -TP inhibition by 10 μ M glibenclamide. THP-1 cells are incubated with increasing concentrations of α -TP alone and with 10 μ M glibenclamide (GC). Cell proliferation is measured with MTS assay after 24 h (see Materials and methods). The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment. A statistically significant difference of $p < 0.05$ (two-tailed unpaired Student's t -test) in the comparison of a given sample series with the one where 50, 100, 200 μ M α -TP and no glibenclamide were present are marked (#), (*), and (†), respectively.

Table 3
Temperature dependence of α -TP uptake by THP-1 cells and inhibition by glibenclamide

α -T and/or glybenclamide added to THP-1 cells	pmol α -TP/ μ g cell protein 4 °C	pmol α -TP/ μ g cell protein 37 °C
50 μ M α -TP	2.86	16.72 \pm 0.32
50 μ M α -TP + 10 μ M GC	4.81	17.97 \pm 0.48
50 μ M α -TP + 50 μ M GC	2.00	12.43 \pm 0.30
50 μ M α -TP + 100 μ M GC	3.85	8.42 \pm 0.83

For the experimental conditions see Materials and methods. GC, glibenclamide.

α -TP at 37 °C, in a dose-dependent concentration. The residual amount of α -TP present at 4 °C can be attributed to non-specific binding. Glibenclamide (GC), an oral anti-diabetic drug, is also a general inhibitor of a number of carriers from the ABC transporters to the organic anion transporters (OAT) [19–25]. Probenecid, another OAT inhibitor was also tested (data not shown). Probenecid appears to inhibit α -TP transport although at higher concentrations and after longer pre-incubation times. Inhibita-

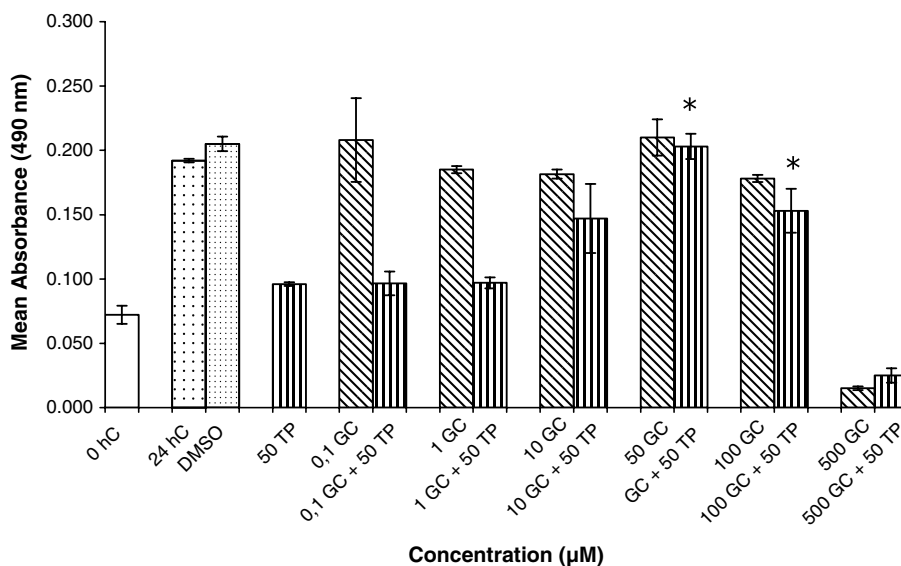


Fig. 4. Abrogation of α -TP inhibition by increasing amounts of glibenclamide (GC). THP-1 cells are incubated with increasing concentrations of glibenclamide (GC) alone or with 50 μ M α -TP. Cell proliferation is measured with MTS assay after 24 h (see Materials and methods). DMSO (solvent for glibenclamide, was less than 0.5%). The experiment was performed in quadruplicate and the mean and standard deviation were calculated for each treatment. A statistically significant difference of $p < 0.05$ (two-tailed unpaired Student's t -test) in the comparison of a sample series with the one treated with 50 μ M α -TP is marked (*).

indicative of α -TP mediated transport across the cell membrane.

In the experiment shown in Fig. 3, the effect of α -TP at different concentration has been tested in the cell proliferation assay; it shows, as in Fig. 2, that the more α -TP is added to cells the more their proliferation is inhibited; however, if in the presence of α -TP also glibenclamide is added, the inhibition by α -TP is significantly diminished; in other words, glibenclamide, by preventing α -TP penetration, diminishes its effects on cell proliferation. This phenomenon is also clearly visible when, at a fixed α -TP concentration, increasing amounts of glibenclamide are added to the cells. In this case, higher amounts of glibenclamide are able to completely suppress the inhibition of cell proliferation by α -TP (Fig. 4). Such a result indicates that α -TP is not acting at the cell surface but, in order to produce its biological effects, its internalization is required. Probenecid, also an inhibitor of α -TP transport, shows as well the ability to prevent the α -TP induced inhibition of proliferation, although at higher concentrations and after longer pre-incubation times (data not shown).

Conclusions

In conclusion, we have shown in this study and previous ones that, at cellular level, α -TP is acting in a way similar to α -T but more potently. How can the data reported be rationalized? Being α -TP more active than α -T and given the molecular structure of the two compounds it appears possible that the conversion of α -T to α -TP produces a more active compound. Such a conversion requires phosphorylation of the OH group of α -T and therefore must involve a kinase. A phosphatase should be also necessary to maintain a given level of α -TP in cells. These reactions are part of current investigations. α -TP, added to cell media is capable of producing effects, such as modulation of proliferation and gene expression [15]. Without the experimental data, it could be assumed that α -TP acts at the cell membrane, possibly modulating surface receptors; alternatively one can suppose that α -TP is internalized by the cell and that its action occur within the cytosol. A specific action of α -TP in organelles such as mitochondria, Golgi apparatus or nucleus appears also possible, but would possibly require intracellular transport proteins such as TAP, which in fact is increasing transport of the synthetic vitamin E derivative, α -tocopheryl succinate, into cells [26,27]. This study has produced evidence that α -TP penetrates across the cell membrane and that this event is mediated by a carrier, possibly of the family of the organic anion transporters. The transport inhibitors glibenclamide and probenecid prevented, dose-dependently, α -TP transport and α -TP inhibition of cell proliferation. Both, the ATP binding cassette (ABC) transporters and the organic anion transporters (OAT) are sensitive to these two inhibitors [28–30]. However, since ABC transporters function to export cell solutes [28–30] and α -TP is transported into cells, it may be concluded that α -TP transport may occur via an OAT

family member. Once in the cytosol, we have given evidence that α -TP is not significantly hydrolyzed in THP-1 cells, but instead it acts as such. As to the partners with whom it possibly interacts, no experimental data are available at the present time. We can only speculate at this time that it may bind to a specific protein, capable of recognizing α -TP as a unique signal and able to modulate cellular and enzymatic events accordingly. α -TP is therefore worth of further studies, both looking at its detailed mechanism of action and its effect in animals and humans.

Acknowledgments

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