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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.

Keywords: α-Tocopheryl-phosphate; Transport; Organic anion transporter (OAT); Vitamin E; Tocopherol; Glibenclamide; Probenecid

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 where 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, ATTC (ATCC # TIB-202). RPMI Medium 1640, fetal bovine serum (FBS) and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY). (±)-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% CO_2 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 bioreduced 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 μM of α-T and α-TP for another 48 h. After treatment, 20 μl of the 96 Cell titer solution was added to each well and incubated for 4 h at 37 °C in humidified 5% CO_2 atmosphere and absorbance read at 490 nm with a 96-well plate reader. The total volume of the reaction mixture was 120 μl 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 μl 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 μl 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 μM 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 μM 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 μl 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 μl of 2 N HCl and shaken vigorously for 1 min. α-T and α-TP was extracted with hexane containing 0.02% butylated hdroxytoluene (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 where pooled and dried under nitrogen gas. The dried samples were dissolved in 100 μl of 10% acetic acid in isopropanol and sonicated for 10 min. The dissolved sample was injected into a 150 × 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 μg 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 μM for 24 and 48 h. No α-TP cytotoxicity is observed up to a concentration of 100 μM. Cells do not exhibit any damage. The conclusion can be drawn that, at least at cellular level, α-TP below or up to 100 μM can be used without causing cell damage. Other cell lines such has Hep-G2 and CaCo2 cells (up to 72 h incubation, MTS assay) are not damaged by α-TP concentrations up to 100 μM.

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.