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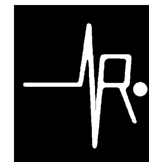

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Original Contribution

α-Tocopheryl phosphate: A novel, natural form of vitamin E

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Abstract

We have detected α-tocopheryl phosphate in biological tissues including liver and adipose tissue, as well as in a variety of foods, suggesting a ubiquitous presence in animal and plant tissue. α-Tocopheryl phosphate is a water-soluble molecule that is resistant to both acid and alkaline hydrolysis, making it undetectable using standard assays for vitamin E. A new method was therefore developed to allow the extraction of both α-tocopheryl phosphate and α-tocopherol from a single specimen. We used ESMS to detect endogenous α-tocopheryl phosphate in biological samples that also contained α-tocopherol. Due to the significance of these findings, further proof was required to unequivocally demonstrate the presence of endogenous α-tocopheryl phosphate in biological samples. Four independent methods of analysis were examined: HPLC, LCMS, LCMS/MS, and GCMS. α-Tocopherol phosphate was identified in all instances by comparison between standard α-tocopheryl phosphate and extracts of biological tissues. The results show that α-tocopheryl phosphate is a natural form of vitamin E. The discovery of endogenous α-tocopheryl phosphate has implications for the expanding knowledge of the roles of α-tocopherol in biological systems.
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RRR-α-tocopherol is the most active form of vitamin E. It is absorbed by the human body as efficiently as the δ and γ forms of the vitamin but after 24 h the α form is preferentially enriched in the plasma. α-Tocopherol has the highest biological activity based on fetal resorption assays and is less susceptible to degradation, compared to the other forms [1,2]. Tocopherol transfer and associated proteins have been suggested to act as transport/chaperone proteins between cellular compartments, but the mechanisms of tocopherol uptake, storage, and transfer remain unclear [3,4]. The unique gene regulatory properties of α-tocopherol suggest the existence of a receptor, but despite intensive research, such a protein has yet to be identified. To date, only free and protein-bound or associated α-tocopherols have been detected in biological tissues [5]. An attempt has also been made to identify active metabolites possibly involved in α-tocopherol signaling functions [6].

The present report confirms and extends the findings described in our earlier reports [7–9], that α-tocopherol also exists in the previously undetected natural form, α-tocopheryl phosphate (TP). Significantly, TP contains a chroman OH group esterified by phosphoric acid, making the molecule an ideal candidate for a number of cellular functions, such as oxidant-protected intracellular transport,

Abbreviations: TP, α-tocopheryl phosphate; DCM, dichloromethane; THF, tetrahydrofuran.

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enzymatic regulation of its concentration, and cell signaling [8].

Our previous findings [7] were determined using an electrospray mass spectrometry (ESMS) protocol. The present paper provides additional evidence for the existence of endogenous TP in biological tissues, using independent detection by HPLC (high-performance liquid chromatography), LCMS (liquid chromatography mass spectrometry), LCMS/MS (liquid chromatography tandem mass spectrometry), and GCMS (gas chromatography mass spectrometry).

Materials and methods

Reagents

HPLC-grade chloroform, methanol, dichloromethane (DCM), tetrahydrofuran (THF), acetic acid, phosphoric acid, petroleum ether, and hexane were obtained from BDH (Melbourne, Australia). Medium-chain triglyceride was from Abitec Corp. (IL, USA). Acetonitrile was from EMD (NJ, USA). Formic acid was from Ajax Chemicals (Sydney, Australia). Isopropanol was from Lab Scan (Bangkok, Thailand). Ammonia was from Rhone-Poulenc Ltd. (Manchester, England). TP was prepared from α-tocopherol and P_4O_{10} [7], as described below. Disodium tocopherol phosphate (disodium tocopheryl phosphate) is available from Sigma–Aldrich (Castle Hill, NSW, Australia). [^{14}C]Tocopherol was from Amersham (55 mCi/mmol; Sydney, Australia).

Synthesis of TP

In brief, tocopherol (51 g, 0.1 mol) was reacted with P_4O_{10} (8.5 g, 30 mmol) under high shear conditions at 80–90°C for 2 h. Water (2.5 ml) was then added and the reaction stirred for a further 1 h at 80–90°C to hydrolyze the residual polyphosphate bonds. Analysis of the resultant product using ^{31}P NMR and HPLC indicated a mixture of α-tocopheryl phosphate, unreacted tocopherol, and some residual inorganic phosphate. The product was dissolved in 380 ml ethanol and NaOH (4.8 g dissolved in 100 ml ethanol) was added to precipitate out the inorganic phosphate. After filtration the filtrate was again treated with NaOH (5 g) in 100 ml ethanol to form disodium α-tocopheryl phosphate, which is insoluble in ethanol. Filtration gave pure α-tocopheryl phosphate as the disodium salt.

Biological tissues

Rat liver and adipose tissues were obtained from 12- to 18-week-old, male, Sprague–Dawley rats. The animals were purchased from Monash University Animal Services and were acclimatized in the animal house of the Department of Biochemistry and Molecular Biology, Monash University, for at least 2 weeks. The animals were killed by decapitation before the collection of organs, which were frozen in liquid nitrogen and stored at −80°C until use.

Extraction of TP

Standard analytical methods for the extraction of α-tocopherol often involve a hydrolysis step employing sodium or potassium hydroxide. The hydrolysis ensures that α-tocopheryl esters, such as added α-tocopherol acetate, are converted to free α-tocopherol and made soluble in organic solvents. However, this step does not result in the hydrolysis of the phosphate group of TP, but simply converts the molecule into the disodium or dipotassium salt. These sodium and potassium salts of TP are highly water soluble and relatively insoluble in organic solvents, which results in TP remaining in the aqueous layer after extraction of the free α-tocopherol. It was therefore necessary to develop an effective extraction protocol for TP. The method for TP extraction has been described previously and uses a preparation of lipids from tissue initially extracted with DCM [7]. The method, as described below, allows for an acidification step of the aqueous layer, thereby converting the TP salt back to the free acid, which is readily extracted into hexane.

A 1-g sample was placed into a 50-ml centrifuge tube and homogenized in 10 ml of DCM for 30 s (or until the tissue was reduced to a liquid) with an Omni tissue homogenizer (5 × 95-mm probe). Samples were centrifuged at 2000*g* for 10 min to separate the DCM from the denatured and precipitated protein. The DCM phase was transferred to 50-ml vials and dried down under nitrogen gas. Nine milliliters of 2 M KOH was added and incubated at 80°C for 40 min. After the samples were cooled to room temperature, 10 ml hexane was added to each tube, shaken vigorously, and centrifuged as above. The upper hexane layers were discarded. The alkaline layer containing the TP was acidified to pH ~1 by addition of 2 M HCl (10 ml). Hexane (10 ml) was added to the acidified samples and shaken vigorously, and the phases were separated with centrifugation, as above. The upper hexane layer was removed to a fresh vial and the hexane extraction repeated twice more. The hexane phases were pooled and dried down under nitrogen gas for analysis by ESMS. For analysis using HPLC, LCMS, or LCMS/MS, several grams of tissue were required to detect endogenous TP. The extraction procedure was therefore scaled up accordingly.

Sample analysis using ESMS

Samples containing extracted TP were analyzed by ESMS using a Micromass platform mass spectrometer (Waters, Sydney, Australia). The samples were dissolved in 1 ml 90% aqueous THF containing 1% NH_3 solution and 20 μl was injected into the sample loop. The sample was eluted with THF:H_2O (9:1) at 20 μl/min. The samples were analyzed in negative ion mode with the cone voltage set at

40 V. Spectra were recorded between 450 and 1000 Da/e in scan mode and with selected ion monitoring (SIR) at *m/z* 509.5 (TP).

Sample analysis using HPLC

Acetic acid (16 μl) and isopropanol (144 μl) were added to the sample vial and sonicated for 10 min. The dissolved sample was injected into a 150 × 4.6-mm Phenomenex Luna C8 5-μm column (Lane Cove, Australia) using a Waters HPLC system (600 controller, 717 autosampler, 486 detector, 2475 fluorescence detector, Empower Pro software) (Sydney, Australia) 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, maintaining 100% A for 10 min, then to 60% A over 4 min, and maintaining 60% A for 15 min before the next injection. Detection was by fluorescence with excitation at 297 nm and emission at 319 nm. The fluorescence excitation and emission maxima were predetermined for TP using standard in a Cary Eclipse spectrofluorimeter (Varian, Australia).

Sample analysis using LCMS

The dried sample was dissolved in 0.3 ml of 10% acetic acid in isopropanol and injected into a 4.6 × 50-mm Phenomenex Luna C8 5-μm column at room temperature. Mobile phase A was acetonitrile:water (75:25) containing 0.1% formic acid, and B was isopropanol containing 0.1% formic acid. The flow rate was 1.1 ml/min with a gradient from 30 to 90% B over 12 min. The pumping system was Gilson (306 pumps, 811C dynamic mixer; OH, USA) with 700-μl chamber and 215 autosampler with 819 injection module. A Micromass ZMD quadrupole mass analyzer with electrospray source was used with detection in SIR mode for TP, at 30 V cone voltage and with MassLynx version 3.5 software. An Agilent 1100 diode array detector (Melbourne, Australia) was also inline.

Sample analysis using LCMS/MS

Mass spectrometry of the TP standard was performed by infusion into a Waters Quattro Micro triple quadrupole mass spectrometer in positive ESI mode. The molecular ion $M^{+}H$ 511.4 was optimized for intensity, and scans of daughter ions revealed the transition ion *m/z* 245.1, which was used for multiple reaction monitoring experiments. A diode array set at 280 nm was also used to monitor the LC. A Waters Acquity UPLC was used for LCMS/MS experiments, in which a sample of standard solution of TP was injected into a 2.1 × 50-mm C18 1.7-μm bridged ethane-linked hybrid column (Waters). The LC solvent was methanol with the pH adjusted to 9.5 using Na_2CO_3, using isocratic flow at 0.3 ml/min and column temperature at 40°C. A sample of liver extract was then analyzed after dissolving it in 0.4 ml of 0.05% ammonia in isopropanol:methanol (1:1) and 5 μl injected into the LCMS using the same conditions.

Radiolabeling studies in vivo and in vitro

A young, male rat was dosed orally with [^{14}C]tocopherol (1.1 mg/kg) mixed with medium-chain triglyceride and sacrificed 24 h later. α-Tocopheryl phosphate was extracted from the liver as described above. Fat cells in culture (3T3 L1 adipocytes) were grown as previously described [10] to 80% confluence and then placed in medium supplemented with [^{14}C] tocopherol (6 μg/ml; 0.07 μCi/ml) for 24 h. Cells were washed four times with PBS, scraped, and extracted for TP as described above. Extracts containing TP were analyzed by HPLC. The peak containing TP from the fat cells was collected, dried, and reinjected in order to obtain a cleaner separation.

Results

Endogenous TP can be detected using ESMS

α-Tocopheryl phosphate is readily ionizable and displays good solubility in water at high pH and in organic solvents at low pH. These properties make TP ideally suited to analysis using ESMS. Standard (synthetic) TP was analyzed first to optimize the conditions needed for the detection of TP using the electrospray. A single peak was evident in the ESMS spectra (Fig. 1A) with a molecular mass of 509.5. This peak corresponds to the theoretical mass of TP run in negative ion mode (509.5), confirming the identity of the molecule.

Liver and adipose tissue are known to have roles in both the storage and the distribution of cellular tocopherols [4]. As such, liver and adipose tissue were considered likely locations where endogenous TP might be found. Preparations of liver and adipose tissue were analyzed for the presence of TP using ESMS. Spectra from both liver (Fig. 1B) and adipose tissue (Fig. 1C) contain a peak of mass (509.5) identical to that obtained from standard TP, providing initial evidence for the presence of endogenous TP in both liver and fat. The mass spectra also showed the presence of other lipids. The 511.4 signal may be explained by the ability of palmitic acid to dimerize and be detected in negative ion mode. We demonstrated this by addition of palmitic acid to extracts of TP from liver, which resulted in an increase in the 511.4 signal. The association of two palmitic acid molecules was very strong and could not be disrupted by changes in ESMS conditions (e.g., changing the cone voltage did not remove it, nor did changing the amounts of ammonia in the sample or replacing it with trimethylammonium hydroxide, diethylamine, triethylamine, trichloroacetic acid, or trifluoroacetic acid and replacing the THF pump solvent with acetonitrile). The association of free fatty acids that were liberated by the

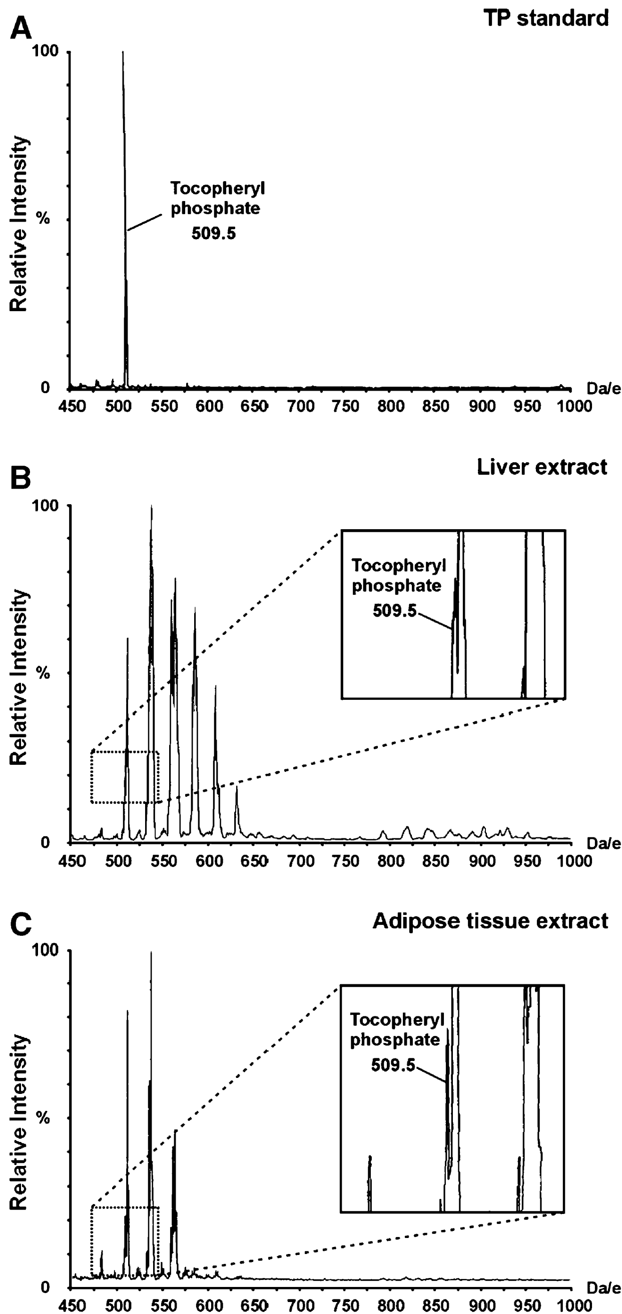

Fig. 1. ESMS scan of TP. (A) TP standard and TP extracted with other lipids from (B) liver and (C) adipose tissue were analyzed by ESMS, as described under Materials and methods. The TP standard (MW 509.5) produced the same signal as that found in extracts of both liver and adipose tissue.

alkaline hydrolysis may also be responsible for other signals in the ESMS spectra. For example, 563.4 may be a product of two oleic acid molecules.

Endogenous TP can be detected using HPLC

Having identified the signals by ESMS in liver and fat that corresponded to that expected for TP, it was necessary to demonstrate that additional methods of detection could also identify TP in biological samples. HPLC is routinely used for the detection of α-tocopherol, based upon both the retention time and the fluorescence characteristics of the molecule. Fluorescence analysis of standard TP suggested that detection using fluorescence during HPLC would also be possible (data not shown). Standard TP was analyzed by HPLC using the excitation and emission wavelengths of 297 and 319 nm, respectively, and its chromatographic characteristics were determined (Fig. 2A). Using fluorescence detection, HPLC proved sensitive enough to detect quantities of standard TP as low as 10 ng/ml.

Samples extracted from liver were subjected to the HPLC analysis and a peak with a retention time identical to that of synthetic TP was present (Fig. 2B), in addition to a number of other peaks. Extracts of TP from tissues were analyzed first without any spiking with synthetic TP and then were rerun with an amount of synthetic TP in order to show that the two peaks had the same retention times. HPLC therefore provides strong supporting evidence to the ESMS data for the existence of endogenous TP.

Endogenous TP can be detected using LCMS

Finally, LCMS and LCMS/MS were used as further methods of detection. Analysis by LCMS of liver extracts was performed, as shown in Fig. 3A, with detection by MS in SIR mode at *m/z* 509.5. Standard TP and TP present in

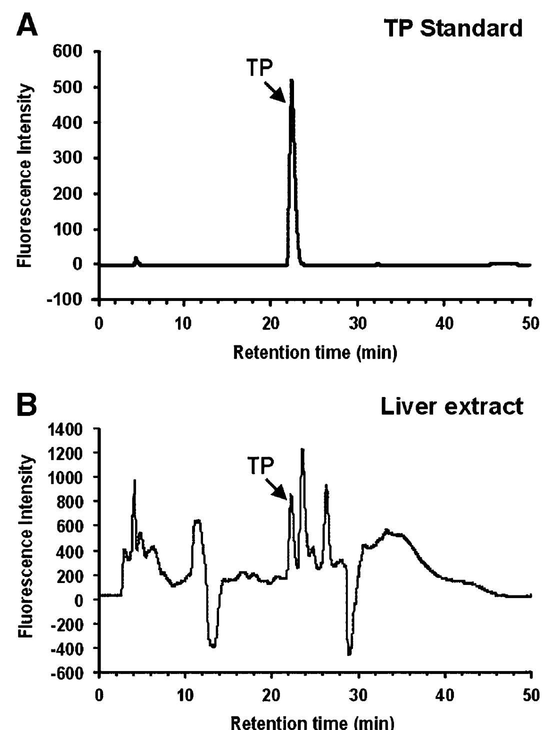

Fig. 2. HPLC analysis of TP. (A) TP standard and (B) TP extracted from liver were analyzed by HPLC with detection by fluorescence as described under Materials and methods. The identity of TP within the liver extract was confirmed as it coincided with the retention time for the synthetic TP standard.

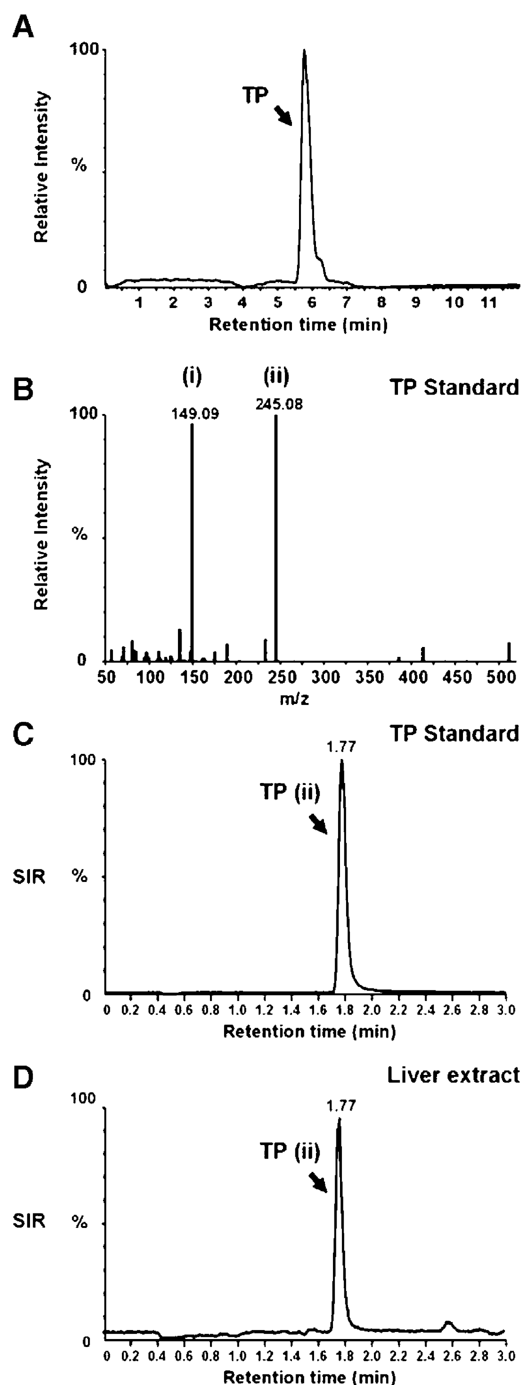

Fig. 3. LCMS and LCMS/MS analysis of TP. (A) LCMS analysis of TP extracted from liver using detection with selected ion monitoring, SIR at 509.5. The HPLC conditions in the LCMS and LCMS/MS were different, as described under Materials and methods. The TP peak is indicated by the arrow. (B) Fragmentation of synthetic TP standard (*m/z* 511.38 in positive ion mode) results in the daughter products, *m/z* 245.08 and 149.09. (C) LCMS/MS of synthetic standard TP was monitored by SIR at *m/z* 245.08. The 245.08 daughter product has a retention time of 1.77 min. (D) LCMS/MS of TP extracted from liver monitored at *m/z* 245.1 showed that the 245.08 daughter product was also present with the same retention time, thereby indicating the presence of TP in the liver extract.

the liver extract appear at the identical position (indicated by the arrow).

Analysis by LCMS/MS of liver extracts was also performed, as further proof of its identity. First, standard TP was fragmented by MS/MS, which produced two daughter products (Fig. 3B; *m/z* 149.09 and 245.08). Second, the chromatography was introduced (i.e., LCMS/MS) and the behavior of standard TP was determined, with the daughter product 245.08 monitored in SIR and UV modes (Fig. 3C; a column different from that in the LCMS was used). Finally, the liver extract was found to contain a peak with the same retention time (1.77 min) as that of the standard TP (Fig. 3D). This indicated the presence of endogenous TP.

Collectively, these analytical results demonstrate that TP is indeed a naturally occurring compound found in biological tissues.

GCMS is a powerful tool for analysis of lipids and we have been able to detect TP using GCMS after derivatizing TP to its dimethyl ester using diazomethane. However, we have not found it useful for routine work because the method proved to be unreliable because of the difficulties in the derivatization. We used it to provide an additional, albeit tentative, method to show the presence of endogenous TP in tissue samples (results not shown). The endogenous material had the same retention time, molecular ion (*m/z* 538), and base peak (*m/z* 273) as the synthetic TP.

TP can be synthesized in vivo and in vitro from α-tocopherol

Having confirmed the existence of TP in biological tissue, we sought to establish its origin. Two main possibilities exist. First, TP is ingested as part of the diet, as is α-tocopherol. Second, it should be possible for TP to be produced in vivo by phosphorylation of α-tocopherol, which, intriguingly, implies the existence of a kinase. To investigate the possible conversion of α-tocopherol to TP, radiolabeled α-[^{14}C] tocopherol was incubated with 3T3-L1 fat cells and was also given orally to a rat. Twenty-four hours later, the fat cells and rat liver were extracted to search for the presence of [^{14}C] TP produced in vitro and in vivo, respectively.

Preparations of fat cell extracts were subjected to analysis by HPLC. The fraction corresponding to the retention time of TP was collected and rerun in order to remove surplus lipid. A main peak was evident in this second run (Fig. 4A). This peak was measured by scintillation counting and found to contain the majority of the radioactive counts (i.e., [^{14}C]TP), evidence for the production of TP in vitro. Production of TP in vivo was also suggested when the rat liver samples were also found to contain a peak high in radioactivity. The peak had the same retention time as synthetic TP. Synthetic TP was also spiked into the liver sample and then rerun on the HPLC to confirm the coelution of the synthetic with what is likely to be the metabolically labeled TP (although these results do not demonstrate the

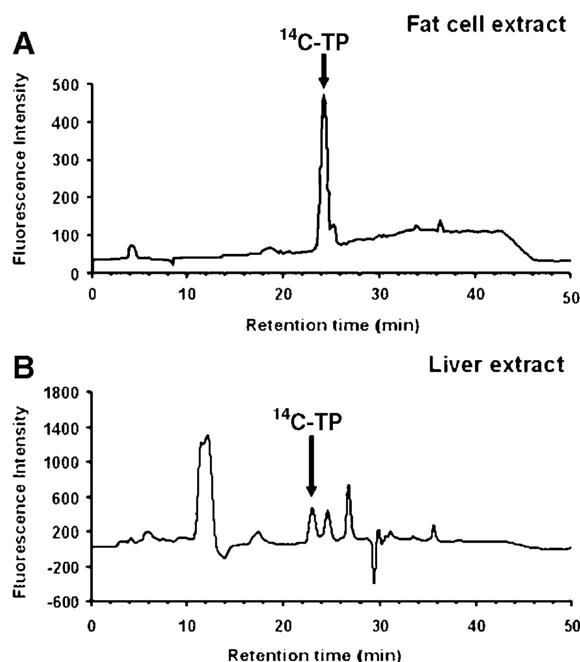

Fig. 4. HPLC analysis of [^{14}C]TP extracted from 3T3-L1 fat cells and liver. (A) Fat cells were grown in the presence of [^{14}C] tocopherol for 24 h and then TP extracted and analyzed as described under Materials and methods. (B) A rat was treated with one oral dose of [^{14}C] tocopherol for 24 h and then TP was extracted and analyzed as described under Materials and methods. The radiolabeled TP was found within the peak eluting with the same retention time as standard TP, as indicated by the arrow.

chemical identity of the labeled TP). Preliminary data indicate that the amount of [^{14}C]TP represented about 1% of the TP pool.

Discussion

The standard analytical methods for the detection of α-tocopherol in tissue samples and foodstuffs normally include a hydrolysis step as part of the extraction process. The hydrolysis ensures that α-tocopheryl esters, such as added α-tocopherol acetate, are converted to free α-tocopherol before analysis.

Surprisingly, α-tocopheryl phosphate is resistant to the alkaline conditions encountered in these procedures and is consequently not included with free α-tocopherol in typical analyses. Indeed, refluxing α-tocopheryl phosphate under strongly alkaline or acidic conditions for extended periods in excess of 24 h does not lead to cleavage of the phosphate bond (unpublished data). However, alkaline hydrolysis converts TP to a salt form, rendering it relatively insoluble to the organic solvents commonly used for α-tocopherol extractions. Therefore, TP is not detected by standard assays for α-tocopherol.

Acidification of the aqueous layer converts the TP salt back to the free acid, which is readily extracted into hexane. Using this method, we have previously used ESMS to identify a molecule of molecular mass identical to that of TP in samples of biological tissue [7]. In the present study, we have positively identified this molecule as being TP by using HPLC, LCMS, and LCMS/MS. α-Tocopheryl phosphate is therefore a natural form of vitamin E.

Surprisingly, the conversion of TP to a salt form during the initial hydrolysis step was found to be critical for its extraction, as TP could not be directly extracted in the water phase without the alkali step. This suggests that in nature it may exist in a form by which its negative phosphate group charges are neutralized by positive ones embedded in a hydrophobic moiety (phosphatidylethanolamine or a hydrophobic protein), and that dissociation is required to liberate TP.

The extraction protocol for extracting both α-tocopherol and TP from the same sample has been used in a wide range of biological samples [7]. The content of TP in the extracts was determined by ESMS with an internal calibrant, such as dicetyl phosphate. The quantities of TP in tissues in that report were obtained by using the only practical method at the time (ESMS). We are now reviewing the values in that report by using the HPLC and LCMS methods described in the current study. Using the extraction protocol described here, our present indication is that the TP content of liver is in the submicrogram range, i.e., in the order of 0.1 μg/g liver. The low amount is in contrast to the concentration of α-tocopherol in rat liver, which we found to be on the order of 10 μg/g liver.

We have previously hypothesized that TP may represent the storage form of α-tocopherol [7]. However, considering the small amounts of TP reported here by using several detection techniques, we consider this unlikely. If α-tocopheryl phosphate is not a storage form then it may represent an absorption form of the vitamin. The possibility remains that TP is a signaling molecule, this being compatible with its very low amounts found in tissues, on the same order of magnitude as known signaling molecules such as inositol phosphate.

Should TP operate in a signaling context, hydrolysis of the phosphate group by a phosphatase may represent a mechanism for regulating the levels of the “active” signal. However, the activity of such a phosphatase must be regulated, to prevent rapid and total hydrolysis of TP. Alkaline phosphatase is a possible candidate as the dephosphorylating agent, as it has been shown to hydrolyze the phosphate group from TP in vitro [11,12].

Equally important is the existence (for the time being only postulated) of a kinase. The shielding of the OH group of α-tocopherol by three methyl groups makes it difficult to have α-tocopherol phosphorylated by a tyrosine kinase. However, such a kinase may still be a good candidate, especially in view of the fact that in some situations δ-tocopherol and γ-tocopherols (with a lower number of methyl groups in the chroman ring) have been described to be more potent than α-tocopherol in vitro. One may speculate that the cellular concentration of their phosphate esters reaches higher values due to their higher affinity for the kinase. Analogously the extremely high in vitro potency

of tocotrienols can hardly be explained by their unsaturated side chain and can rather be considered the consequence of their faster phosphorylation. If considered a signaling molecule the obvious target of TP can be enzymes or transcription factors. This possibility is open to experimental test and is an obvious alternative to the elusive search for α-tocopherol receptors. Finally, tocopheryl succinate has been also shown to have cell properties far stronger than those of α-tocopherol. As a working hypothesis it can be suggested that tocopheryl succinate may substitute for TP at the level of a receptor causing a permanent activation of cellular signals. The existence of a phosphatase(s) and kinase(s), which may be involved in interconverting α-tocopherol and TP, is now being explored.

In conclusion, the demonstration of the existence of TP in tissues is not only an interesting observation but lends itself to a series of experimental, mechanistic studies currently being carried out in our laboratories.

Acknowledgments

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