*Free Rad. Res.*, Vol. 28(1), pp. 87–92
Reprints available directly from the publisher
Photocopying permitted by license only



# Effect of α-Tocopherol Status on α-Tocopherol Transfer Protein Expression and Its Messenger RNA Level in Rat Liver

HAN-SUK KIM[a,*], HIROYUKI ARAI[b], MAKOTO ARITA[b], YUJI SATO[b,†], TOHRU OGIHARA[a], KEIZO INOUE[b], MAKOTO MINO[a] and HIROSHI TAMAI[a]

[a]*Department of Pediatrics, Osaka Medical College, Daigaku-machi, Takatsuki, Osaka 569 and* [b]*Department of Health Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo, Tokyo 113, Japan*

Accepted by Prof. E. Niki

*(Received 13 June 1997; In revised form 1 August 1997)*

Studies were designed to explore the role of vitamin E nutritional status in regulating the synthesis of α-tocopherol transfer protein (αTTP) in rat liver. In vitamin E-replete rats, expression of this protein and its specific messenger RNA (mRNA) was decreased by about 300% compared with rats with normal vitamin E levels. In vitamin E-depleted rats, the αTTP-specific mRNA level increased to about 150% of that in the normal vitamin E group. However, protein expression remained constant. These findings indicate that the synthesis of αTTP can be regulated by the vitamin E nutritional status.

*Keywords:* Vitamin E, α-tocopherol transfer protein, messenger RNA, gene expression, effect of nutritional status

*Abbreviations:* αTTP, α-tocopherol transfer protein; mRNA, messenger RNA; FIVED, familial isolated vitamin E deficiency; HPLC, high performance liquid chromatography; cDNA, complementary DNA; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

## INTRODUCTION

α-Tocopherol, the most biologically active form of vitamin E, is a lipid-soluble antioxidant present in the membranes of intracellular organelles, where it plays an important role in suppressing lipid peroxidation.[1] α-Tocopherol transfer protein (αTTP), which specifically binds α-tocopherol and enhances its transfer between membranes, is present in the liver cytosol of animals, including rats[2,3] and humans.[4] Recently, we cloned the gene encoding αTTP from humans and demonstrated it to be the causative gene for familial isolated vitamin E deficiency (FIVED).[5] Patients affected by this disease have remarkably low plasma levels of α-tocopherol.[6] These findings established liver αTTP as a critical factor in determining the plasma α-tocopherol level.

* Corresponding author. Tel.: 0726-83-1221. Fax: 0726-84-5798.
† Present address: Tokyo Metropolitan Issue of Gerontlopy. Tel.: 03-3964-3241. Fax: 03-3579-4776.

It is well known that the plasma vitamin E level is regulated within a narrow concentration range in the biologically system, despite the fact that absorption of vitamin E into the enterocytes occurs passively alongside that of lipids.[6] This suggests that there is a system for regulating the vitamin E concentration in the body.[7]

In the present study, we investigated the effect of vitamin E nutritional status on the expression of αTTP in the rat liver, and the possible role of this protein in the regulatory system.

## MATERIALS & METHODS

### Animals

Male weanling Sprague-Dawley rats were divided into three groups of four rats each (normal vitamin E, vitamin E-depleted and vitamin E-replete). The rats in the vitamin E-depleted group were fed a vitamin E-deficient diet containing less than 1 mg/kg dl-α-tocopherol. The rats in the normal vitamin E and vitamin E-replete groups were fed the same diet as the vitamin E-deficient group, but supplemented with 50 mg and 600 mg dl-α-tocopherol per kg diet, respectively. The rats were fed with each diet for twenty weeks.

### Western Blot Analysis

Rats were sacrificed by decapitation and their livers perfused with ice-cold buffer A (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). The livers were homogenized in 2.3 vols. of buffer A. Cytosol was prepared from homogenates by ultracentrifugation at 100, 000 g for 60 min. 50 μg of protein in cytosol was separated on SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting using the monoclonal antibodies against αTTP(ATR1)[3] was then carried out and, the nitro-cellulose membrane was washed and incubated for 1hr at room temperature in a solution containing goat anti-mouse IgG conjugated to alkaline phosphatase. After washing, the blot was developed by soaking in a buffer (0.1 M $NaHCO_3$ and 1 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml Nitroblue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

### Northern Blot Hybridization Analysis

Total RNA from each rat liver was isolated according to the acid guanidine thiocyanate procedure.[9] Total RNA (10μg) was run on 1.0% agarose gel and transferred to a nylon membrane (Biodyme, PallBioSupport). The membrane containing total RNA was hybridized at 60°C in 5 × Saline-Sodium Citrate solution (SSC), 5 × Denhard's solution, 0.1% SDS, 50 mM sodium phosphate (pH 6.5), 50% foramide, 250 μg/ml salmon sperm DNA, and labeled probe ($10^6$ cpm/ml). The probe was labeled by the random primer labeling method.[3] The blot was washed twice for 5 min at room temperature in 2 × SSC, 0.1% SDS, and then washed twice for 20 min at 65°C in 0.1 × SSC, 0.1% SDS. Autoradiography was performed at –80°C for 5 days with an intensifying screen.

### Determination of Tocopherols in Plasma and Liver

Six hour fasting blood was collected in heparinized tubes. Plasma was separated from whole blood by centrifugation at 800 g for 15 min. After perfusion with buffer A the livers were homogenized and the protein content was determined by Lowry's method. Plasma and liver homogenates (0.5 ml) were subjected to tocopherol determination using high performance liquid chromatography(HPLC) analysis and electrochemical detection. The HPLC system was an IRIKA P-530 (IRIKA, Kyoto) with an IRIKA RP-18 column (4 × 250 mm). The eluent was methanol/water/$NaClO_4$ in a ratio of 1000:2:7 (v/v/w) and a flow rate of 10 ml/min. Detection was performed with an IRIKA Amperometric E-520 detector. The retention time was 6.88 min for tocol, which was used as an internal standard, 9.366 min for γ-, and 10.88 min for α-tocopherol, as previously described.[10]

### Determination of Lipids in Plasma

The remaining plasma was used for the determination of other lipids (cholesterol, triglyceride and phospholipid) by the enzyme assay (the cholesterol-oxidase(COD)) · N-Ethyl-N-(2-hydroxy-3sulfopropyl)-3,5-dimethoxyaniline, sodium salt (DAOS) method, the glycerol-3-phosphate-oxidase (GPD) · DAOS method, and the choline-oxidase · DAOS method) using commercial kit (Wako Co., Ltd., Osaka, Japan).

### Statistical Analysis

Content of α-tocopherol in plasma and liver, and lipids in plasma are presented as mean (standard error, SEM). Statistical significance was estimated by Mann-Whitney U test.

## RESULTS

Table I shows the plasma and liver vitamin E (α-tocopherol) concentrations for each group. The plasma α-tocopherol concentration of the vitamin E-depleted group was less than 0.5% that of the normal group. The liver α-tocopherol concentration was extremely low in this group. In the vitamin E-repleted group, although they had taken 50 times more vitamin E than the normal vitamin E group, their plasma and liver α-tocopherol concentrations were only about 250% higher. The plasma levels of other lipids were not affected by vitamin E nutritional status, consistent with previous reports.[11–13]

The expression of αTTP in rat liver was studied by both northern and western blot analysis (Fig. 1). It was found that the expression of both mRNA and protein were significantly suppressed in the rats of the vitamin E-replete group. Densitometric analysis of each band revealed a decrease of approximately 300% in the expression of mRNA and protein in this group. In the vitamin E-depleted status, the αTTP-specific mRNA level was increased by about 150% compared with the normal vitamin E group. However, western blotting showed no difference between vitamin E-depleted and the normal vitamin E groups in the expression of this protein.

## DISCUSSION

The plasma α-tocopherol concentration rarely increases more than three-fold in response to vitamin E supplementation, irrespective of dose, suggesting that there is a limitation on plasma α-tocopherol concentration. αTTP present in the liver appears to be critical for the regulation of

TABLE I Plasma and liver α-tocopherol levels and plasma lipids contents of each group

| | VE-depleted | VE-repleted | normal VE |
|---|---|---|---|
| (plasma) | | | |
| VE(mg/dl) | 91.7*(11.4) | 2270.9*(405.5) | 869.8 (54.0) |
| t-lipids(mg/dl) | 277.3 (18.6) | 251.7 (18.9) | 261.8 (12.6) |
| VE/t-lipids(mg/g) | 0.4* (0.1) | 9.1* (1.7) | 3.3 (0.3) |
| (liver) | | | |
| VE(μg/g · protein) | 3.6* (1.9) | 133.5* (44.2) | 38.1 (16.8) |

Values represent the mean (S. E. M)
*: $p < 0.05$
Content of α-tocopherol (VE) in plasma and liver, the total amount of cholesterol, triglyceride, and phospholipid (t-lipids) in plasma, and the plasma ratio of α-tocopherol to total lipids (VE/t-lipids) in each group. Data are presented as mean (standard error, SEM). Comparison between groups was made by Mann-Whitney U test. * symbol indicates significant difference from control subjects ($p < 0.05$).

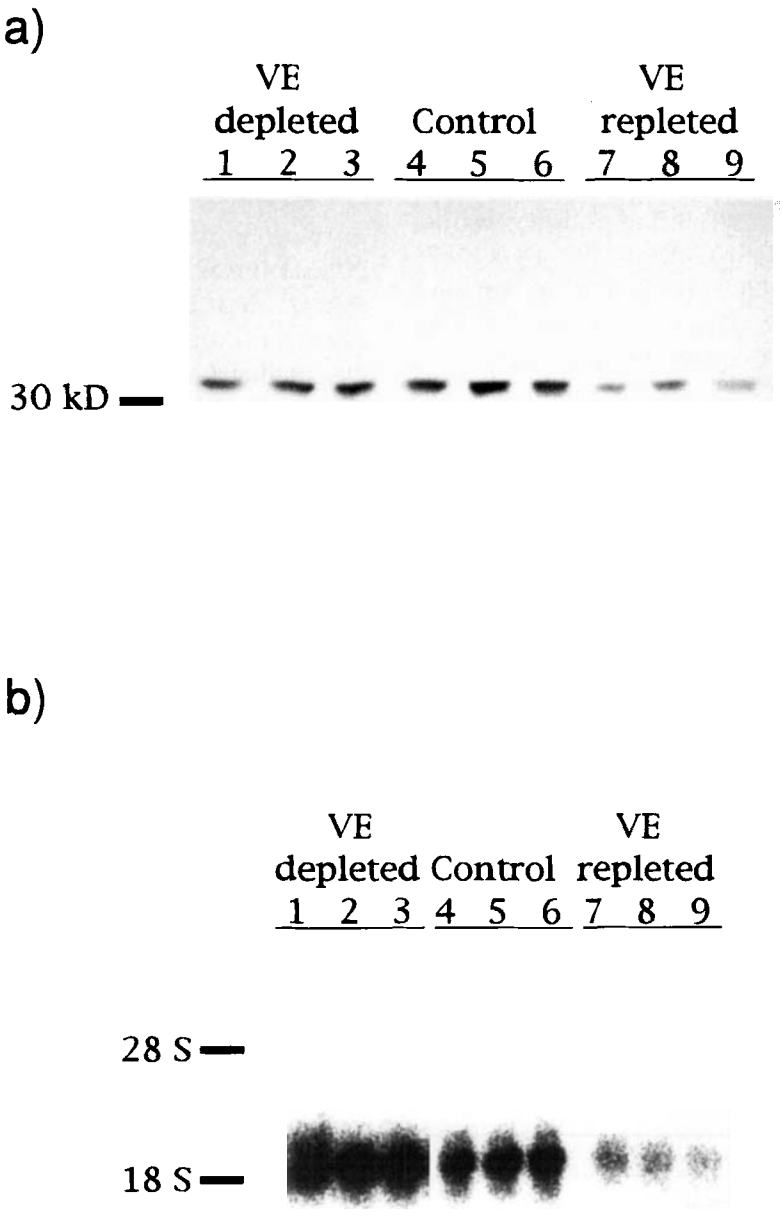


FIGURE 1 Western and Northern blot analysis of αTTP. Lanes 1, 2 and 3: vitamin E (VE) -depleted group; lanes 4, 5, and 6: normal VE group; lanes 7, 8, and 9: VE-replete group. **a)** Western blot analysis of αTTP expression in rat liver in each group. Densitometric analysis revealed that protein expression in the VE-replete group was decreased by about 300% compared with that in the normal VE group. In the VE-depleted group, αTTP expression was constant. **b)** Northern blot analysis of αTTP-specific mRNA level in each group. Analysis with the Bass 2000 system showed that the αTTP-specific mRNA level was reduced by about 300% in the VE-replete group. On the other hand, the mRNA level in VE-depleted rats was increased to about 150% of normal.

plasma α-tocopherol within a narrow range of concentration. Tocopherols taken up by the intestine are incorporated into chylomicrons, and delivered to the liver. Tocopherols in circulating lipoproteins such as LDL are also endocytosed to a great extent by the liver. In the liver cells, the endocytosed lipoproteins are hydrolyzed in the lysosomes, and free tocopherols are released.

αTTP in the cytosol traps α-tocopherol and incorporates it into nascent VLDL for re-secretion into the plasma. Excess absorbed tocopherols, which do not bind to αTTP, may be readily excreted in bile.[14,15] Therefore, the total amount of α-tocopherol delivered to VLDL is limited by the amount of αTTP in the liver. Studies in FIVED patients have demonstrated that, in the absence of αTTP, plasma α-tocopherol concentrations fall rapidly.[16] Thus, the protein is also necessary to maintain minimal plasma levels of α-tocopherol.

We found that expression of αTTP mRNA was significantly suppressed in vitamin E-replete rats, indicating the presence of a mechanism for down-regulating αTTP in response to overdosage of vitamin E. This mechanism may also help to keep the plasma α-tocopherol concentrations within a narrow range. On the other hand, in vitamin E-depleted rats, the αTTP protein level was not significantly altered, although its mRNA levels were slightly up-regulated. This suggests a possibility that the turnover of αTTP in the livers of vitamin E-deficient rats is faster than that in normal rats. In our preliminary observations, we found that α-tocopherol-bound αTTP is more stable than α-tocopherol-depleted αTTP *in vitro*.

It is unclear at present whether there is any benefit in limiting plasma vitamin E concentrations. In some in vitro systems, high concentrations of vitamin E are known to act as a pro-oxidant.[17,18] Indeed, in the absence of aqueous antioxidants such as vitamin C, LDL-associated α-tocopherol acts as a chain transfer agent during free radical production *in vitro*. An α-tocopheroxyl radical formed on the surface of the LDL then abstracts hydrogen from a polyunsaturated fatty acid to form a peroxyl radical. In this way, α-tocopherol does not act as a chain-breaking antioxidant but propagates lipid peroxidation. Plasma vitamin E level may have to be kept within a narrow concentration range in order to prevent this types of reaction.

It has now become evident that αTTP plays an important role in determining the plasma α-tocopherol level. However, the biological factors which regulate the expression of αTTP have not been identified. Previous studies have demonstrated that αTTP expression decreases dramatically within 24 h when rat liver parenchymal cells are transferred to *in vitro* primary culture.[19] In addition, we have observed that αTTP expression levels in some liver tumor cell lines such as Hep G2 and McARH7777, both of which have the biosynthetic capabilities of normal liver parenchymal cells,[20] are extremely low (unpublished observations). These data indicate that the expression of αTTP is influenced by the differentiation state of hepatocytes. The present paper demonstrates for the first time that the plasma vitamin E level affects the expression of the αTTP gene at the transcriptional level.

## *References*


[1] Kayden, H. J. and Traber, M. G. (1993). Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. *Journal of Lipid Research*, **34**, 343–358.

[2] Sato, Y., Hagiwara, K., Arai, H. and Inoue, K. (1991). Purification and characterization of α-tocopherol transfer protein from rat liver. *FEBS Letters*, **288**, 41–45.

[3] Sato, Y., Arai, H., Miyata, A., Tokita, S., Yamamoto, K., Tanabe, T. and Inoue, K. (1993). Primary structure of α-tocopherol transfer protein from rat liver—Homology with cellular retinaldehyde-binding protein-. *Journal of Biological Chemistry*, **268**, 17705–17710.

[4] Yoshida, H., Yusin, M., Ren, I., Kuhlenkamp, J., Hirano, T., Stolz, A. and Kaplowitz, N. (1992). Identification, purification, and immunochemical characterization of a tocopherol-binding protein in rat liver cytosol. *Journal of Lipid Research*, **33**, 343–350.

[5] Arita, M., Sato, Y., Miyata, A., Tanabe, T., Takahashi, E., Kayden, H. J., Arai, H. and Inoue, K. (1995). Human α-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochemical Journal*, **306**, 437–443.

[6] Ouahchi, K., Arita, M., Kayden, H. J., Hentanti, F., Hamida, M. B., Sokol, R., Arai, H., Inoue, K., Mandel, J. L. and Koenig, M. (1995). Ataxia with isolated vitamin E deficiency is caused by mutations in the α-tocopherol transfer protein. *Nature Genetics*, **9**, 141–145.

[7] Traber, M. G., Ramakrishnan, R. and Kayden, H. J. (1994). Human plasma vitamin E kinetics demonstrate rapid recycling of plasma *RRR*-α-tocopherol. *Proceedings of National Academy of Sciences of the United States of America*, **91**, 10005–10008.

[8] Traber, M. G. (1994). Determinants of plasma vitamin E concentrations. *Free Radical Biology and Medicine*, **16**, 229–239.

[9] Chomczinski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, **162**, 156–159.
[10] Tamai, H., Manago, M., Yokota, K. and Mino, M. (1988). Determination of α-tocopherol in baccal mucosal cells using an electrochemical detector. *International Journal of Vitamins and Nutrition Research*, **58**, 202–207.
[11] Farrell, P. M. and Bieri, J. G. (1975). Megavitamin E supplementation in man. *American Journal of Clinical Nutrition*, **28**, 1381–1386.
[12] Dimitrov, N. V., Meyer, C., Gilliland, D., Ruppenthal, M., Chenoweth, W. and Malone, W. (1991). Plasma tocopherol concentration in response to supplemental vitamin E. *American Journal of Clinical Nutrition*, **53**, 723–729.
[13] Dieber-Rotheneder, M., Ruhl, H., Waeg, G., Striegl, G. and Esterbauer, H. (1991). Effect of oral supplementation with D-α-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *Journal of Lipid Research*, **32**, 1325–1332.
[14] Bjørneboe, A., Bjørnboe, G-E. A. and Drevon, C. A. (1987). Serum half-life, distribution, hepatic uptake and biliary excretion of α-tocopherol in rats. *Biochimica et Biophysica Acta*, **921**, 175–181.
[15] Lee-Kim, Y. C., Meidani, M., Kassarjian, Z., Blumberg, J. B. and Russell, R. M. (1988). Enterohepatic circulation of newly administered α-tocopherol in the rat. *International Journal of Vitamin and Nutrition Research*, **58**, 284–291.
[16] Traber, M. G., Sokol, R. J., Burton, G. W., Ingold, K. U., Papas, A. M., Huffaker, J. E. and Kayden, H. J. (1990). Impaired ability of patients with familial isolated vitamin E deficiency to incorporate α-tocopherol into lipoproteins secreted by the liver. *Journal of Clinical Investigation*, **85**, 397–407.
[17] Nagaoka, S., Okauchi, Y., Urano, S., Nagashima, U. and Mukai, K. (1990). Kinetic and abinitio study of prooxidant effect of vitamin-E—hydrogen abstraction from fatty acid esters and egg york lecithin. *Journal of American Chemical Society*, **112**, 8921–8924.
[18] Mukai, K. (1993). Synthesis and kinetic study of antioxidant and prooxidant actions of vitamin E derivatives. In *Vitamin E in health and disease* (ed.) L. Packer, (Marcel Dekker, Inc., New York, NY) pp. 97–119.
[19] Kim, H., Arai, H., Arita, M., Sato, Y., Ogihara, T., Tamai, H., Inoue, K. and Mino, M. (1996). Age-related changes of α-tocopherol transfer protein expression in rat liver. *Journal of Nutritional Science and Vitaminology*, **42**, 11–18.
[20] Knowles, B. B., Howe, C. C. and Aden, D. P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*, **209**, 497–8.