α- and δ-tocopherol induce expression of hepatic α-tocopherol-transfer-protein mRNA

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α-Tocopherol transfer protein (α-TTP) supplements nascent very-low-density lipoprotein (VLDL) preferentially with α-tocopherol by selecting the α-isomers against other stereoisomers of tocopherol. It is exclusively expressed in liver. We investigated whether the expression of the hepatic α-TTP can be induced by dietary tocopherols. Vitamin E-depleted rats were fed with a diet containing α- and δ-tocopherol (ratio 1:3). The expression of α-TTP mRNA was measured in liver tissue. The ratio of tocopherol stereoisomers was determined in plasma, plasma lipoproteins and tissues to measure the metabolic action of α-TTP. Refeeding a diet containing either α- or δ-tocopherol, or both, caused a steady increase of the expression of α-TTP mRNA. In parallel the α/δ-tocopherol ratio increased in plasma, VLDL, high-density lipoprotein and low-density lipoprotein as well as in liver tissue, when the diet was fed containing both isomers. The α-tocopherol/δ-tocopherol ratio of heart, kidney, lung, lamellar bodies of lung and in lung lavage showed no or a comparatively low increase. The data show that both tocopherol isomers were able to induce α-TTP mRNA in rat liver and, thus, the ability of liver to select for the α-isomer. On the other hand, tocopherol depletion did not change the expression of hepatic α-TTP mRNA in the rat.

INTRODUCTION

There is mounting evidence that disorders caused by oxidative stress can be treated by tocopherol in high doses [1,2]. Natural plant tocopherols contain mono- (δ-), di- (β- or γ-) and trimethyl (α-) tocols [3]. The biologically most active form is α-tocopherol. In the diet of humans and some animals, however, non-α-tocopherol stereoisomers predominate. The intake of γ-tocopherol in humans, for instance, is about 2–4 times that of α-tocopherol [4]. Although the intestinal absorption of individual tocopherol isomers is similar [5,6], α-tocopherol shows the highest concentration in blood and tissues [5]. After intestinal absorption, the tocopherol isomers occur first in chylomicrons. Chylomicrons are metabolized intravascularly to remnants. These remnants, containing the freshly absorbed species of tocopherol, are taken up by the liver [6,7]. The α-tocopherol transfer protein (α-TTP) supplements nascent very-low-density lipoprotein (VLDL) with α-tocopherol and selects the α-form at the expense of other tocopherol isomers [6,8]. The non-α isomers are excreted with bile.

α-TTP, a 32 kDa protein exclusively expressed in liver, was first identified [9] and characterized [10,11] in rat liver cytosol. The function of this protein is to maintain the plasma level of α-tocopherol. This conclusion is supported by the existence of two human diseases apparently caused by a genetic defect of α-TTP, namely (i) familial isolated vitamin E deficiency ('FIVE') and (ii) ataxia with vitamin E deficiency ('AVED') [12,13]. Recently, it was ascertained that FIVE and AVED seems to be the same disorder [14]. In patients the genetic defect causes impaired incorporation of α-tocopherol into nascent VLDL, leading to very low levels of plasma vitamin E. These results indicate that α-TTP plays a very important role in vitamin E metabolism. Absorption and transport of vitamin E in chylomicrons, however, is normal in this disease. Therefore, it seems to be possible in some cases to prevent symptoms of the disease(s) by high doses of vitamin E.

In the present study we investigated whether or not refeeding of an α- and δ-tocopherol-containing diet to vitamin E-depleted rats induces the expression of the hepatic α-TTP mRNA. The biological function of α-TTP was demonstrated in parallel by measuring the α-tocopherol/δ-tocopherol ratios of plasma, plasma lipoproteins and tissues in the period after refeeding. Furthermore, we investigated which of the tocopherol is isomers induced expression of α-TTP mRNA.

EXPERIMENTAL

Materials

The vitamin E-depleted diet, the diet containing 100 mg of α-tocopherol (Sigma) plus 300 mg of δ-tocopherol (Sigma)/kg, and the diets containing 100 mg of α-tocopherol or 100 mg δ-tocopherol of were purchased from Altromin (Lage, Germany). Using HPLC we confirmed that all tocopherols used for the preparation of the diets were pure isomers. The diets were different only in their vitamin E contents. The chemicals used were obtained from different suppliers in *pro analysi* grade.

Feeding regime

Rats (body mass 100–200 g) were given the vitamin E-depleted diet *ad libitum* for 5 weeks. Thereafter the rats were deprived of food for 24 h and given an α-tocopherol-and-δ-tocopherol-supplemented diet. After 24 h the diet was removed and the content of the vitamin E isomers and of total fatty acids were determined in plasma, VLDL, high-density lipoprotein (HDL), low-density lipoprotein (LDL), liver, heart, kidney, lung lamellar bodies and lung lavage. The determination of these variables

Abbreviations used: α-TTP, α-tocopherol transfer protein; VLDL, HDL and LDL, very-low-, high- and low-density lipoprotein; i.p., intraperitoneal; ss, single-stranded.

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Table 1 Dependence of the expression of the mRNA of α-TTP in rat liver on the feeding state

The α-TTP mRNAs were quantified by Northern blots as described in the Experimental section. The values are means ± S.D. ($n = 4$). * Indicates a significant difference ($P \leqslant 0.01$) from the values of state 1 or state 3 respectively.

Feeding state		αTTP mRNA expression (arbitrary units)
No.	Details	
1	Standard diet	0.394 ± 0.064
2	Vitamin E-depleted diet for 5 weeks	0.368 ± 0.076
3	State 2 followed by 24 h food deprivation	0.405 ± 0.136
4	State 3 followed by 24 h vitamin E refeeding	0.294 ± 0.039
5	State 4 followed by 3 h food deprivation	0.725 ± 0.026*
6	State 4 followed by 12 h food deprivation	1.100 ± 0.093*
7	State 4 followed by 24 h food deprivation	2.160 ± 0.103*

was repeated 3 h, 12 h and 24 h after removing the α-tocopherol-and-δ-tocopherol-containing diet.

Isolation of lipoproteins

A 1.3 g portion of KBr was dissolved in 4 ml of plasma, which was then overlayered by 3.5 ml of KBr ($d = 1.063$) and 3.5 ml of KBr ($d = 1.019$). The tubes were centrifuged for 20 h at 40000 ***g*** in a Beckman ultracentrifuge using a SW 41 rotor. The gradient was collected in 0.5 ml fractions starting from the bottom, and in each fraction the cholesterol content was determined. The fractions representing VLDL ($d < 1.006$), LDL ($d = 1.019$–1.063) or HDL ($d = 1.063$–1.21) were collected and dialysed against 0.9 % NaCl (pH 7.4; containing 1 mM EDTA) at 4 °C overnight.

Preparation of homogenates

Following intraperitoneal (i.p.) injection of 30 mg of pentobarbital, liver, heart, kidney and lung were removed, reduced to small pieces and then homogenized with ice-cold 0.9 % NaCl (1:5, w/v) using a glass/Teflon Potter homogenizer (Braun, Melsungen, Germany).

Isolation of lavage and lamellar bodies from lung tissue

Following i.p. injection of 30 mg of pentobarbital, rat lungs were perfused with 0.9 % NaCl via the right ventricle of the heart. The blood-free lungs were lavaged four times *in situ* with ice-cold 0.9 % NaCl. The lavages were pooled and immediately centrifuged at 150 ***g*** for 10 min. The cell-free lavages were immediately used for the experiments. Lamellar bodies of lung tissue were isolated by density-gradient centrifugation as described by Oosterlaken-Dijksterhuis et al. [15].

Table 2 Content of tocopherol isomers in rat tissues and plasma lipoproteins

Vitamin E-depleted rats were refed a diet containing α- and δ-tocopherol in a ratio of 1:3 for 24 h. After that period rats were deprived of food for the time indicated. Values are means with S.D. (in parentheses) ($n = 4$). * Indicates significant difference of $P \leqslant 0.5$ and ** of $P \leqslant 0.01$ compared with the 0 h value.

Fraction and isomer	Time after feeding of the α- and δ-tocopherol-containing diet (h)…	[Tocopherol] (nmol/μmol of total fatty acids)			
		0	3	12	24
Liver tissue					
α		0.44 (0.12)	0.35 (0.07)	0.47 (0.16)	0.19 (0.05)*
δ		0.27 (0.002)	0.15 (0.005)**	0.017 (0.002)**	0.002 (0.0002)**
VLDL					
α		2.5 (0.23)	15.8 (2.3)**	16.7 (2.1)**	21.4 (4.9)**
δ		1.5 (0.7)	4.2 (0.25)**	0.25 (0.25)*	0.15 (0.05)**
LDL					
α		2.1 (0.02)	6.5 (0.7)**	10.0 (4.2)*	11.9 (7.0)*
δ		1.5 (0.02)	3.7 (0.2)**	3.7 (1.7)	0.75 (0.5)*
HDL					
α		0.9 (0.2)	0.7 (0.2)	27.4 (4.4)*	24.9 (7.0)**
δ		4.0 (0.5)	5.0 (0.7)	3.2 (0.5)	0.7 (0.3)**
Plasma					
α		8.4 (1.9)	12.6 (3.7)	9.5 (1.2)	9.8 (3.3)
δ		5.5 (3.7)	4.7 (1.5)	0.3 (0.05)*	0.1 (0.07)**
Heart tissue					
α		0.56 (0.07)	0.58 (0.16)	0.47 (0.12)	0.60 (0.21)
δ		0.12 (0.005)	0.10 (0.03)	0.07 (0.012)**	0.07 (0.017)**
Kidney tissue					
α		0.35 (0.12)	0.26 (0.07)	0.26 (0.05)	0.53 (0.16)
δ		0.1 (0.02)	0.1 (0.03)	0.05 (0.007)**	0.05 (0.01)
Lung tissue					
α		1.2 (0.12)	1.1 (0.28)	1.0 (0.3)	0.8 (0.05)**
δ		0.4 (0.017)	0.35 (0.1)	0.15 (0.05)**	0.07 (0.01)**
Lamellar bodies					
α		0.58 (0.02)	0.79 (0.12)*	0.67 (0.07)	0.42 (0.05)*
δ		0.15 (0.02)	0.25 (0.03)	0.15 (0.05)	0.05 (0.01)*
Lung lavage					
α		0.16 (0.05)	0.44 (0.21)	0.56 (0.16)**	0.37 (0.12)*
δ		0.1 (0.05)	0.25 (0.05)*	0.35 (0.12)*	0.17 (0.02)

Determination of vitamin E isomers

For extraction of the tocopherol isomers we added to plasma or homogenates 5 vol. of methanol and α-tocopherol acetate as an internal standard for quantification. These mixtures were extracted three times with 5 ml of hexane. The hexane extracts were reduced in volume and separated by HPLC as described by the manufacturer [16] with some modifications. We used a 200 mm-long RP-18 (5 μm pore size) column with an inner diameter of 4 mm. The column was eluted with methanol/water (50:1, v/v) at a flow rate of 1 ml/min. The tocopherol isomers were detected by absorbance at 290 nm.

Determination of the fatty acid content of total lipids

Aliquots of plasma, lipoprotein fractions and tissue homogenates were extracted as described by Bligh and Dyer [17]. To the extracts we added heptadecanoic acid as internal standard and, after methanolysis, we analysed the fatty acid methyl esters by GLC as described previously [18,19].

Determination of the α-TTP mRNA expression of a Northern-blot technique

Generation of radiolabelled single-stranded (ss) DNA probes

After reverse transcription of 1 μg of total liver RNA using Expand™ reverse transcriptase (Boehringer-Mannheim, Mannheim, Germany), PCR was subsequently carried out with rat hepatic α-TTP-specific sense primer (5′-ATGGAGTTCTGAG-GTCC-3′), and antisense primer (5′-CTCATTGGATGGTCT-AGAA-TGC-3′). In a parallel PCR, rat cytoplasmic β-actin-specific sense primer (5′-ATGGATG ACGATATCGCTG-3′) and antisense primer (5′-AGGGACAACACAGCCTGGAT-3′) were used. A 50 ng portion of PCR fragments eluted from the gel was labelled in a 40-cycle PCR reaction as described previously [20], using 0.26 μM [^{32}P]dCTP (ICN-Biomedical G.m.b.H., Eschwege, Germany) and rat hepatic α-TTP and rat β-actin antisense primers in separate reactions. The ratio of labelled to unlabelled dCTP was 1:7. Labelled probes were separated from non-incorporated dNTPs by use of a Sephadex 50 column.

Northern-blot analysis

Total cellular RNA was isolated from frozen liver tissue using a single-step acid guanidinium thiocyanate/phenol/chloroform extraction method. The RNAs were quantified by spectrophotometry, and 10 μg of total cellular RNA was run on a 1.0 %-formaldehyde gel in 20 mM Mops buffer, pH 7.0, blotted by capillary transfer onto a nylon membrane (Amersham, Braunschweig, Germany) using the standard protocol described by Sambrook et al. [21]. Blots were incubated for 2 h with prehybridization buffer [350 mM Na_2HPO_4/7 % SDS/30 % deionized formamide/10 % (w/v) BSA fraction V] and hybridized with radiolabelled (2×10^6 c.p.m./ml) α-TTP-ss-DNA probe. After hybridization overnight at 50 °C, filters were washed twice with 150 mM Na_2HPO_4/0.5 % (w/v) SDS and once with 30 mM Na_2HPO_4/0.1 % (w/v) SDS at 55 °C for 10 min. All blots were stripped and reprobed with a ss-DNA-radiolabelled β-actin probe as internal standard. Filters were subsequently exposed to Kodak BIOMAX™ film (Integra, Bioscience, Fernwald, Germany) and the radioactivity of the signals was quantified using a GS-250 Molecular Imager™ (Bio-Rad, Hercules, U.S.A.).

RESULTS

Rats were fed a vitamin E-depleted standard diet for 5 weeks. During this period their body weight increased from 108 ± 6 g to 286 ± 23 g and the vitamin E level of the plasma decreased from 34.4 ± 5.8 μmol/l ($n = 4$) to 3.4 ± 0.2 μmol/l ($n = 4$). Subsequently, the rats were deprived of food for 24 h and then fed a vitamin E-containing diet for 24 h. During refeeding, the rats took up 9 ± 0.6 g of diet per 100 g body weight. In order to measure redistribution of tocopherol between body compartments, refeeding was followed by another period of food deprivation.

We measured expression of α-TTP mRNA in different feeding states (Table 1). Feeding a vitamin E-depleted diet for 5 weeks did not alter the abundance of α-TTP mRNA significantly. After refeeding of tocopherol-containing diet the expression of α-TTP mRNA increased steadily.

Vitamin E from natural sources consists of different stereoisomers. In the diet used for the present study we chose an α-tocopherol/δ-tocopherol ratio of 1:3. Table 2 shows the level of α- and δ-tocopherol in rat plasma and tissues during food deprivation following the tocopherol re-uptake period. The tocopherol concentrations were expressed as the molar ratio of tocopherol to fatty acid, because virtually all tocopherols are associated with lipids [22]. The total tocopherol content (sum of α- and δ-tocopherol) of tissues was highest after refeeding and did not further increase during the subsequent food-deprivation period, although total tocopherol of lipoproteins, the only source of vitamin E for tissues, increased.

Figure 1 shows the α-tocopherol/δ-tocopherol ratio calculated form the data of Table 2. The α/δ ratio increased in liver and plasma lipoproteins after refeeding of tocopherol-containing diet. The highest increase of that ratio was measured in VLDL, followed by liver tissues, HDL and LDL. The α/δ ratio of non-

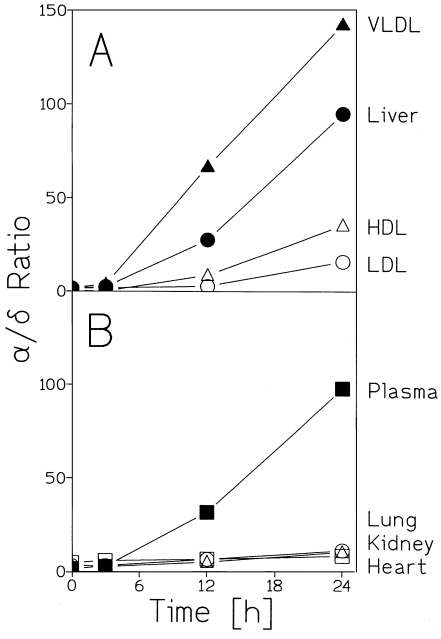

Figure 1 α-Tocopherol/δ-tocopherol ratio in plasma, plasma lipoproteins and tissues

Vitamin E-depleted rats were deprived of food for 24 h and then given an α-tocopherol/δ-tocopherol-supplemented diet. After 24 h the diet was removed (0 h) and the α/δ ratio determined. The determination of the ratio was repeated after 3, 12 and 24 h. The values were calculated from the data given in Table 2.

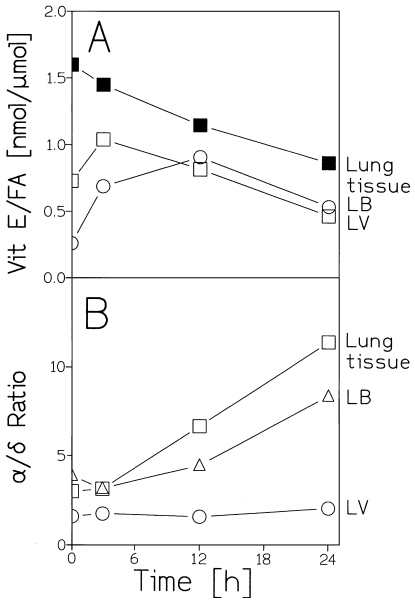

Figure 2 Vitamin concentration and α-tocopherol/δ-tocopherol ratio in lung tissue, lamellar bodies and lavage

Vitamin E-depleted rats were deprived of food for 24 h and then given an α-tocopherol/δ-tocopherol-supplemented diet. After 24 h the diet was removed and the α- and δ-tocopherol concentrations were determined (0 h). The determinations were repeated after 3 h, 12 and 24 h. (**A**) Data are the sum of α- and δ-tocopherol concentrations given in Table 2; (**B**) the α/δ ratios were calculated from data in Table 2. Abbreviations: Vit E, vitamin E; FA, fatty acid; LB, lamellar bodies; LV, lavage.

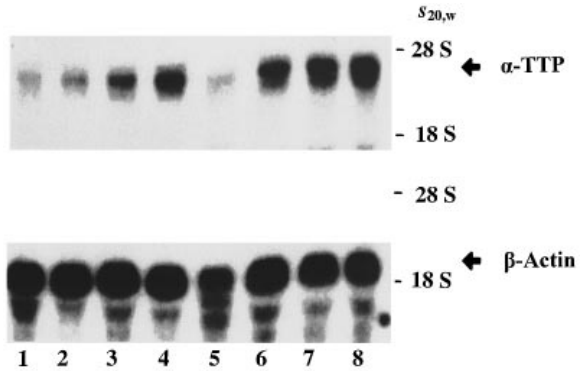

Figure 3 Northern-blot analysis of the α-TTP mRNA isolated from liver tissue after refeeding vitamin E-depleted rats an α- or δ-tocopherol supplemented diet

Vitamin E-depleted rats were deprived of food for 24 h and were then fed either an α- or a δ-tocopherol-supplemented diet. After 24 h the diet was removed (0 h) and the α-TTP mRNA expression determined. The determination of the α-TTP mRNA expression was repeated after 3 h, 12 h and 24 h. Lane 1, refeeding vitamin E-depleted rats an α-tocopherol-supplemented diet (0 h); lane 2, α-tocopherol-supplemented diet 3 h; lane 3, α-tocopherol-supplemented diet 12 h; lane 4, α-tocopherol-supplemented diet 24 h; lane 5, refeeding vitamin E-depleted rats an δ-tocopherol-supplemented diet (0 h); lane 6, δ-tocopherol-supplemented diet 3 h; lane 7, δ-tocopherol-supplemented diet 12 h; lane 8, δ-tocopherol-supplemented diet 24 h; For experimental details of the Northern-blot analysis, see the Experimental section.

hepatic tissues increased less. The factor by which the ratios increased during 24 h after refeeding was 155 for HDL, 85 for VLDL and 58 for liver tissue. In LDL that factor was 11 and for heart kidney and lung tissue it was below 4.

Lung is the only internal organ that has direct contact with oxidants and oxidative pollutants of the air. Thus lung is a primary target of oxidative injury. Lung tissue contained more tocopherol than liver, heart and kidney (Table 2). In liver and lung the tocopherol level decreased during the food-deprivation period after refeeding, whereas in kidney and heart the value increased after 12 h. The decrease of the tocopherol level in lung tissue was accompanied by its time-delayed increase in lamellar bodies and subsequently in lung lavage (Figure 2A). The α/δ ratio of lung tissue and lamellar bodies increased after refeeding, while the ratio in lung lavage did not change significantly (Figure 2B).

Since the diet contained both the α- and δ-isomer, the question was which of the tocopherol isomers caused the induction of α-TTP mRNA expression. Figure 3 shows a representative Northern blot of α-TTP mRNA 24 h after refeeding either α- or δ-tocopherol. Both isomers induced α-TTP mRNA. The increase of mRNA was 6.8-fold and 7.1-fold with α- or δ-tocopherol respectively.

DISCUSSION

The metabolic function of α-TTP is to maintain a sufficient plasma level of vitamin E. Owing to isomer specifity, α-TTP supplements nascent VLDL predominantly with α-tocopherol [6]. Our results show that vitamin E-depletion of rats did not change the RNA expression of α-TTP, whereas refeeding tocopherol to vitamin E-depleted rats increased the expression of α-TTP mRNA about 7-fold (Table 1). Refeeding caused also an increase of the α/δ ratio in VLDL, LDL and HDL, as well as in liver and, to a lower extent, in kidney, heart and lung tissue (Figures 1 and 2B). An increase in the α/δ ratio in individual lipoprotein fractions is evidence of increased α-TTP action concomitantly with the increased α-TTP mRNA expression. Therefore we conclude that the increase of the expression on the mRNA level results in an increased expression on the protein level.

In a previously published report, Traber et al. [23] showed that vitamin E deficiency in dogs did not alter the preferential incorporation of RRR-α-tocopherol compared with all-*rac*-α-tocopherol into plasma. They concluded that vitamin E deficiency in dogs did not increase the apparent metabolic function of α-TTP. From experiments of Yoshida et al. [10] one can draw the same conclusion. During purification of α-TTP from rat liver cytosol they found that vitamin E depletion of rats did not change the [^{3}H]tocopherol-binding capacity of liver cytosolic proteins. These results indirectly show that vitamin E depletion does not lead to significant changes of α-TTP content. This idea is strongly supported by the present findings that vitamin E depletion does not affect the expression of α-TTP mRNA.

The lung is directly exposed to oxidative pollutants of air. Therefore alveolar surfactant is a likely target of air oxidants. Recently we showed that alveolar surfactant is supplemented with vitamin E during its formation in type II pneumocytes [17] and that vitamin E turnover increased in response to hyperoxia in type II pneumocytes, lamellar bodies and lung lavages [24]. The idea that lung tissue has to be protected by vitamin E is supported by the finding that the vitamin E content of lung tissue is about twice that of other tissues (Table 2). Figure 2(B) shows that the α/δ ratio increased fastest in lung tissue, fast in lamellar bodies and slowly in lung lavage. The graded increase of the ratios corroborates the idea [17] that tocopherol, newly taken up, is first incorporated into lamellar bodies before it appears in lung lavage. This is further supported by Figure 2(A): after refeeding

vitamin E, peak levels were first seen in lung tissue, then in lamellar bodies and subsequently in lung lavage.

The expression of the α-TTP mRNA increased after refeeding either α- or δ-tocopherol. We used δ-tocopherol because its biological activity differs strongly from that of the α-isomer. The fact that δ-tocopherol was able to induce α-TTP mRNA expression shows that the stimulatory effect on the expression of α-TTP mRNA is not linked to the biological activity of the individual isomer. Perhaps α-TTP is necessary to remove non-α-tocopherols from the vitamin E pool supplied to nascent lipoproteins. If α-TTP is indeed the intracellular carrier of non-α-tocopherols, it must recognize isomer-specific target sites, as non-α-tocopherols are excreted in the bile fluid.

In summary, we show that vitamin E depletion does not change the expression of α-TTP mRNA. However, refeeding either α- or δ-tocopherol to vitamin E-depleted rats increases the expression of α-TTP mRNA. Refeeding a diet containing α- and δ-tocopherol increases the metabolic action of α-TTP in rat liver.

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