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ORIGINAL CONTRIBUTION

Age-related changes of vitamin E: α-tocopherol levels in plasma and various tissues of mice and hepatic α-tocopherol transfer protein

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Abstract
Purpose Despite numerous studies on the RRR- and all-*rac*-α-tocopherol isoform of vitamin E (VE) during aging, this relationship has not been examined in specific tissues. Since α-tocopherol is the most abundant of VE's eight isoforms, and VE is an important antioxidant that impacts the aging process, we analyzed α-tocopherol levels in plasma and tissues of mice at progressive ages. Moreover, we examined protein and mRNA expression levels of hepatic α-tocopherol transfer protein (α-TTP), which specifically binds α-tocopherol, during aging.
Methods The α-tocopherol levels in plasma, liver, cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum from male C57BL/6NCr mice at 3, 6, 12, 18, and 24 months of age were determined by HPLC and fluorescence detection. Also, hepatic α-TTP protein and mRNA expression levels were analyzed by Western blot and qPCR, respectively.
Results Tissue-specific, age-related changes of α-tocopherol levels normalized by tissue weight were observed in the liver, cerebrum, hippocampus, cerebellum, heart, kidney, and epididymal adipose tissue. Specifically, α-tocopherol levels in epididymal adipose tissue increased greatly as mice aged from 6 to 24 months. Although hepatic α-TTP protein levels also showed age-related changes, α-TTP mRNA expression levels measured after overnight fasting were not altered.
Conclusions In this study, we determined that α-tocopherol levels and hepatic α-TTP protein levels of mice undergo significant tissue-specific, age-related changes. This is the first report to investigate VE in terms of the α-tocopherol levels in plasma and various tissues of mice and hepatic α-TTP protein levels during aging.

Keywords Vitamin E · α-Tocopherol · α-Tocopherol transfer protein · Aging · Liver

Abbreviations

ABCA1	ATP-binding cassette subfamily A, member 1
ANOVA	One-way analysis of variance
C/EBPα	CCAAT/enhancer binding protein α
Cyp4f14	Cytochrome P450, family 4, subfamily f, polypeptide 14
EDTA	Ethylenediaminetetraacetic acid
6-FAM	5′-carboxyfluorescein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HPLC	High-performance liquid chromatography
qPCR	Quantitative polymerase chain reaction
PIP_2	Phosphatidylinositol bisphosphate
PPARγ	Peroxisome proliferator-activated receptor γ
SEM	Standard error of the mean
TG	Triglyceride
TNFα	Tumor necrosis factor α
α-TTP	Alpha tocopherol transfer protein
VE	Vitamin E

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VLDL Very low density lipoprotein
ZEN *N*,*N*-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine

Introduction

Vitamin E (VE) is a lipid-soluble antioxidant that localizes mainly at cell membranes [1]. VE consists of eight isoforms: α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol. In animals, dietary VE is absorbed from the small intestine accompanied by other lipophilic nutrients and integrated into chylomicrons [2]. Chylomicrons containing VE eventually reach the liver, where they are incorporated by endocytosis into hepatocytes. Then, only the α-tocopherol component is selectively secreted from the liver with the help of α-tocopherol transfer protein (α-TTP). In hepatocytes, α-TTP selectively binds α-tocopherol and moves to the cell membrane [3]. Afterward, α-tocopherol is transported into plasma lipoproteins by ATP-binding cassettes, subfamily A, member 1 (ABCA1), and then distributed to peripheral tissues [4, 5]. Consequently, α-tocopherol becomes most abundant in peripheral tissues.

α-TTP is a member of the Sec14 superfamily and also binds phosphatidylinositol bisphosphate (PIP_2) [6]. When α-TTP moves to cells' membranes with α-tocopherol, α-tocopherol is exchanged with PIP_2 on the cell membrane accompanying conformational changes of the α-TTP protein. Impaired α-TTP caused by genetic mutation in the binding domain of α-tocopherol or PIP_2 is responsible for ataxia by VE deficiency [7]. Therefore, α-TTP functions to systematically maintain the α-tocopherol level in peripheral tissues.

Reactive oxygen species are known to oxidize DNA, protein, and lipids and are thought to be one cause of aging [8]. Hydroxyl radicals, which are members of the reactive oxygen species, oxidize polyunsaturated fatty acids and produce lipid peroxyl radicals. In turn, lipid peroxyl radicals react with another fatty acid to produce lipid peroxide [9]. Since VE, as an antioxidant, has the ability to reduce lipid peroxide content and to eliminate lipid peroxyl radicals [1], many investigators have attempted to clarify the relationships between blood VE level and age-associated disease. For example, serum VE levels were lower in the sera of patients with age-related macular degeneration than that of age-matched, normal controls [10].

Frequently, hospitalized elderly patients have reduced plasma α-tocopherol levels [11]. Additionally, aged persons with even mild cognitive impairments or the more debilitating neurodegenerative disorders such as senile dementia or Alzheimer's disease have low α-tocopherol levels in plasma [12, 13]. Moreover, large doses of VE supplementation have prolonged the life spans of mice [14], although others reported, conversely, that substantial VE supplementation lacked any effect on the duration of life [15]. Thus, whether VE has the power to affect longevity is still unclear, as reviewed by Ernst et al. [16].

Since VE levels have not been measured in multiple tissues during aging, this study was performed to determine the VE level by calculating α-tocopherol levels in plasma, liver, cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum of mice during aging. Simultaneously, we investigated the hepatic α-TTP protein and mRNA expression levels during aging, because their levels affect VE levels in plasma and tissues. Our results demonstrated that marked tissue-specific, age-related changes of α-tocopherol and hepatic α-TTP protein levels occur in mice as they advance in age.

Materials and methods

Animals

Male mice of the C57BL/6NCr strain were obtained at 3, 6, 12, 18, and 24 months of age from the animal facility at Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). All mice were fed a CRF-1 solid diet (Oriental Yeast, Tokyo, Japan), which contained 20.1 mg VE (α-tocopherol: 16.5 mg, β-tocopherol: 0.6 mg, γ-tocopherol: 2.5 mg, δ-tocopherol: 0.5 mg) per 100 g. After overnight fasting (18 h), mice were killed, and blood was collected from the right atrium. Blood was gently mixed with ethylenediaminetetraacetic acid (EDTA) and centrifuged at 880 g for 15 min at 4 °C. The resulting supernatants were used as plasma for further analysis. Afterward, mice were perfused systemically with ice-cold phosphate-buffered saline through the left ventricle to wash out the remaining blood cells. Liver, cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum were collected and stored at −80 °C until use. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

Determination of α-tocopherol in plasma and tissues

Liver, cerebrum, hippocampus, cerebellum, epididymal adipose tissue, testis, and pancreas were homogenized with a teflon pestle homogenizer in phosphate-buffered saline. Heart, kidney, soleus muscle, plantaris muscle, and duodenum were crushed with Cryo-Press (Microtech, Chiba, Japan) and suspended in phosphate-buffered saline. The homogenates and plasma were mixed with 5 % ascorbic

acid, 70 % ethanol, and *dl*-tocol (Tama Biochemical Co., Ltd., Tokyo, Japan). The mixture was incubated with *n*-hexane and centrifuged at 21,000 g. The upper *n*-hexane layer, which contained α-tocopherol and *dl*-tocol, was removed. Hexane was evaporated and the residue was redissolved in ethanol. Both α-tocopherol and *dl*-tocol content were determined by using high-performance liquid chromatography (HPLC) (Waters 2695 separations module, Nihon Waters, Tokyo, Japan) and a fluorescence detector (Waters 2475 Multi λ fluorescence detector, Nihon Waters) (Supplementary Figure S1). Separation was achieved on a cadenza CD-C18 column (3 μm, 4.6 × 150 mm, Imtakt Corporation, Kyoto, Japan) combined with a cadenza CD-C18 guard column (3 μm, 2.0 × 5.0 mm, Imtakt Corporation). The mobile phase was 90 % ethanol, and flow rate was 0.7 mL/min. Temperatures for the column and autosampler were set at 40 and 4 °C, respectively. Fifteen μL of each sample was injected into the HPLC and fluorescence detector system. Both α-tocopherol and *dl*-tocol were detected by excitation wavelength at 298 nm and emission wavelength at 325 nm (Supplementary Figure S1). The level of α-tocopherol was normalized by *dl*-tocol as an internal standard and determined from serial-diluted RRR-α-tocopherol standard (Tama Biochemical Co., Ltd.) curve (Supplementary Figure S1).

Determination of triglyceride (TG)

Total lipid was extracted by the Folch method [17] as described previously [18]. Tissues were homogenized with a teflon pestle homogenizer in ultrapure water, and homogenates were incubated with chloroform–methanol (2:1) at 37 °C for 1 h. After incubation, samples were centrifuged at 21,000 g, and precipitates were washed with chloroform–methanol (2:1) at 37 °C for 2 h. Total lipid was obtained by evaporating the supernatant to dryness at 55 °C in N_2 gas and dissolution in 2-propanol. TG content was determined by using enzymatic assay kits (Wako Pure Chemical, Osaka, Japan).

Western blot analysis of α-TTP

Livers were homogenized with buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % IGEPAL® CA-630 (Sigma-Aldrich, Tokyo, Japan), 1 % sodium deoxycholate, 0.1 % SDS, complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan) using a polytron homogenizer. Protein concentrations were measured by the Lowry method [19]. Each sample (10 μg protein) was electrophoresed on 12 % polyacrylamide gel by the method of Laemmli [20] with some modifications. After electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes (Immobilon®-FL, Merck Millipore, Billerica, MA, USA). The primary antibodies used were rat anti-α-TTP monoclonal antibody (A8-F1) (1:2000) generated as described previously [21] and rabbit anti-β-actin polyclonal antibody (1:1000) purchased from Santa Cruz Biotechnology (Texas, USA). The secondary antibodies were IRDye 680RD goat anti-rat IgG (1:15,000) and IRDye 800CW goat anti-rabbit IgG (1:15,000) purchased from LI-COR Biosciences (Lincoln, NE, USA). Secondary antibodies were detected as fluorescence with the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analyses were performed by Image Studio, version 2.1.10 (LI-COR Biosciences). Protein levels of α-TTP were evaluated relative to the β-actin levels and expressed as ratios when compared to 3-month-old mice.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted by using ISOGEN® (Wako Pure Chemical) [22]. Tissues were homogenized with a teflon pestle homogenizer and polytron homogenizer in ISOGEN, and total RNA was extracted according to the manufacturer's protocol. The final RNA pellet was dissolved in RNase-free sterile water. RNA concentrations were determined by measuring absorbance at 260 nm and confirmed as free from protein contamination by measuring absorbance ratios of 260 and 280 nm. Then, cDNA was synthesized using SuperScript III Reverse transcriptase (Invitrogen) following the manufacturer's protocol. The cDNA was stored at −80 °C until use.

Quantitative polymerase chain reaction (qPCR)

Using the qPCR super mix-UDG-with ROX (Invitrogen), qPCR was performed following the manufacturer's protocol. The primers and 5′-carboxyfluorescein (6-FAM)/*N*,*N*-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN)/Iowa Black® FQ-3′ double quenched probes of α-TTP, cytochrome P450, family 4, subfamily f, polypeptide 14 (Cyp4f14), ABCA1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA) [23]. The reactions were performed by using the real-time PCR equipment (StepOne Plus, Applied Biosystems, Foster City, CA, USA). The amplification protocol consisted of denaturation at 95 °C for 2 min, 45 cycles of 95 °C for 15 s, and 60 °C for 30 s. For quantitative analysis of each mRNA expression level, a standard curve method was designed; that is, an aliquot from each experimental sample was used to generate standard curves. The α-TTP, Cyp4f14, and ABCA1 mRNA expression levels in liver from mice of various ages were expressed as the ratio when compared to 3-month-old mice. The α-TTP mRNA expression levels in

Table 1 Body and tissue weights of mice at 3, 6, 12, 18, and 24 months of age

Age (months)	3	6	12	18	24
Body weights (g)	24.0 ± 0.4	33.8 ± 1.0*[3]	37.8 ± 1.1*[3]	35.0 ± 1.7*[3]	29.3 ± 2.1*[12]
Liver weights (g)	0.95 ± 0.02	1.17 ± 0.03*[3]	1.29 ± 0.05*[3]	1.31 ± 0.07*[3]	1.38 ± 0.07*[3]
Brain weights (mg)	449.6 ± 7.3	443.5 ± 3.2	453.8 ± 5.4	452.2 ± 6.4	443.6 ± 13.6
Heart weights (mg)	108.8 ± 4.1	129.2 ± 1.9	146.2 ± 4.6*[3]	148.2 ± 11.3*[3]	163.4 ± 9.8*[3,6]
Kidney weights (mg)	305.2 ± 7.1	349.4 ± 13.6	404.8 ± 15.7*[3]	417.0 ± 19.9*[3]	390.0 ± 34.2*[3]
Epididymal adipose tissue weights (g)	0.45 ± 0.03	1.54 ± 0.12*[3]	1.97 ± 0.14*[3]	1.33 ± 0.11*[3,12]	0.37 ± 0.08*[6,12,18]
Testis weights (mg)	201.9 ± 3.8	214.6 ± 6.1	198.3 ± 12.1	196.8 ± 12.2	175.1 ± 6.5*[6]
Pancreas weights (mg)	136.1 ± 15.5	137.0 ± 7.4	138.2 ± 5.7	172.8 ± 16.3	148.7 ± 10.8

Values are expressed as mean ± SEM of five animals. Asterisks at months *[3] , *[6], *[12], *[18], and *[24] indicate significant differences between groups, $P < 0.05$

various tissues were expressed as the ratio when compared to livers from 12-month-old mice.

Fasting study

Male mice of C57BL/6NCr strain at 3 months of age were fasted for 3 and 18 h. After fasting, mice were killed and perfused systemically with ice-cold phosphate-buffered saline through the left ventricle to wash out the remaining blood cells. Livers were collected and stored at −80 °C until use. We used non-fasted mice as controls.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). The probability of statistical differences between each experimental groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). Statistical differences were considered significant at $P < 0.05$.

Results

Body and tissue weights of mice at progressive ages

Mean body and individual tissue weights of mice at 3, 6, 12, 18, and 24 months of age are shown in Table 1. Mean whole body weights increased from 3 to 12 months of age and then decreased until 24 months of age. Mean liver and heart weights increased from the 3 to 24 months of age. Mean kidney weights increased from 3 to 18 months of age, and epididymal adipose tissue weights increased from 3 to 12 months of age and then decreased until 24 months of age. Mean testis weights increased from 3 to 6 months and then decreased until 24 months of age. Mean pancreas weights increased from 12 to 18 months of age and then decreased at 24 months of age. No such differences were observed in the mean brain weights at progressive ages.

Age-related change of α-tocopherol levels in plasma and various tissues normalized by tissue weight

To investigate the age-related change of α-tocopherol levels, we examined plasma, liver, cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum of mice at 3, 6, 12, 18, and 24 months of age. Values for each of those tissues were normalized with tissue weight excluding plasma (Fig. 1). In the liver and heart, α-tocopherol levels increased from 3 to 6 months of age and then decreased from 6 to 24 months (Fig. 1b, f). The α-tocopherol levels in the liver and heart of mice at 24 months of age were 45 and 27 % lower than those of mice at 6 months of age, respectively. On the other hand, α-tocopherol levels in the cerebrum and hippocampus increased from 3 to 12 months of age (Fig. 1c, d). The α-tocopherol levels in the cerebrum and hippocampus of mice at 12 months of age were 85 and 89 % higher than that of mice at 3 months of age, respectively. In the cerebellum, α-tocopherol increased slightly from 3 to 18 months of age until becoming 30 % higher at the 18 month mark than that at 3 months of age (Fig. 1e). Notably the kidney contained 43 % more α-tocopherol in 24-month-old mice than those at 3 months of age (Fig. 1g), but no significant difference was observed between 18 and 24 months of age. In epididymal adipose tissue, the amount of α-tocopherol rose dramatically from 6 to 24 months of the animals' ages to a final 25-fold higher content (Fig. 1h). There were no significant differences of α-tocopherol levels in plasma, which were not normalized with tissue weight, testis, pancreas, soleus muscle, plantaris muscle, or duodenum during aging (Fig. 1a, i–m).

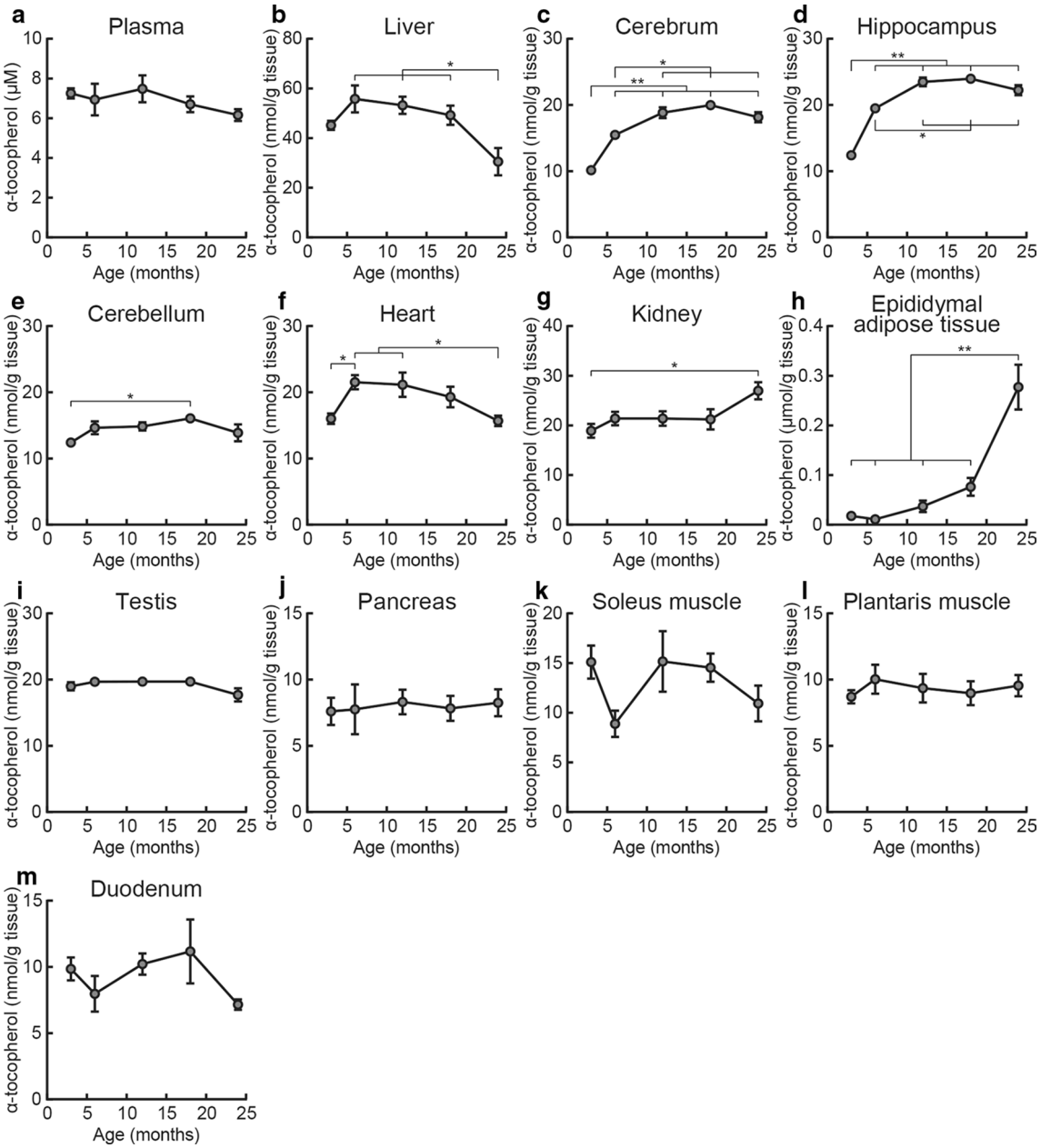

Fig. 1 Age-related changes of α-tocopherol levels in plasma and multiple tissues; values were normalized with tissue weight excluding plasma of mice. The α-tocopherol levels in **a** plasma, **b** liver, **c** cerebrum, **d** hippocampus, **e** cerebellum, **f** heart, **g** kidney, **h** epididymal adipose tissue, **i** testis, **j** pancreas, **k** soleus muscle, **l** plantaris muscle, and **m** duodenum of male mice at 3, 6, 12, 18, and 24 months of age were determined by HPLC and fluorescence detection as described in "Materials and methods" section. Values are expressed as mean ± SEM of five animals. $*P < 0.05$, $**P < 0.01$

Age-related change of α-tocopherol levels normalized with TG

Since α-tocopherol is a lipid-soluble antioxidant, α-tocopherol levels in tissues might be influenced by their lipid content. Therefore, we used enzymatic assays to assess TG in plasma, liver, and epididymal adipose tissue of mice at 3, 6, 12, 18, and 24 months of age, and α-tocopherol levels were normalized with TG (Fig. 2). In the liver, TG content increased profoundly from 3 to 6 months of age and then decreased from 6 to 24 months of age (Fig. 2b). That is, 6-month-old mice had 229 % more liver TG than the 3-month-olds, and those at 24 months of age had 43 % less liver TG than the 6-month-olds. In epididymal tissue, TG content appeared to increase from 18 to 24 months of age, but

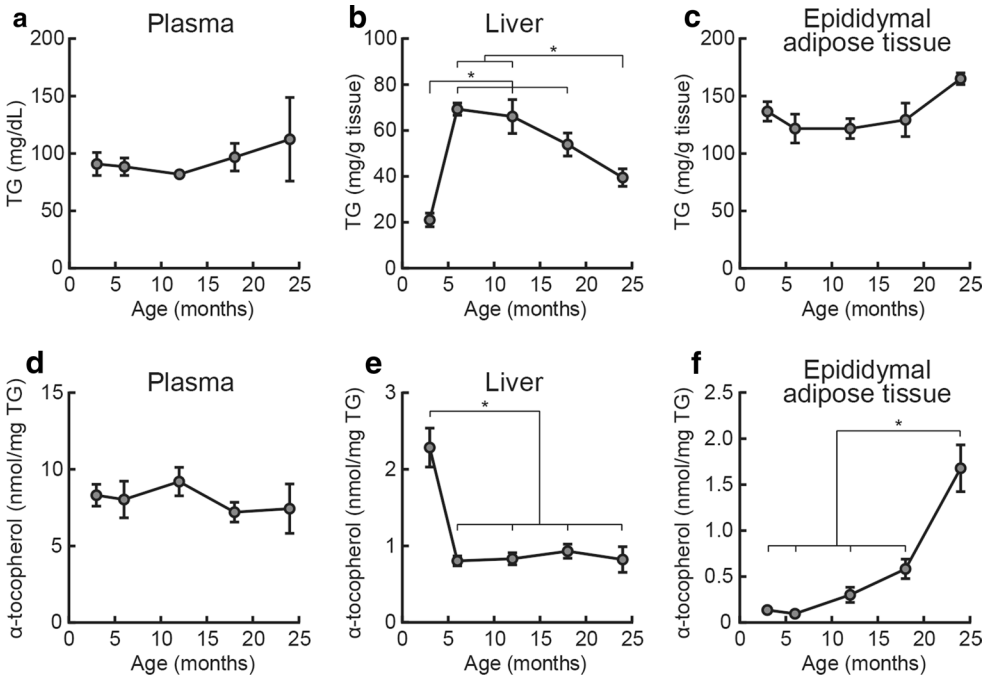

Fig. 2 Age-related changes of triglyceride (TG) and α-tocopherol levels normalized with TG. TG contents in **a** plasma, **b** liver, and **c** epididymal adipose tissue of male mice at 3, 6, 12, 18, and 24 months of age were determined by enzymatic assay as described in "Materials and methods" section. The α-tocopherol levels in **d** plasma, **e** liver, and **f** epididymal adipose tissue of male mice at 3, 6, 12, 18, and 24 months of age were normalized with TG. Values are expressed as mean ± SEM of five animals. $^*P < 0.01$

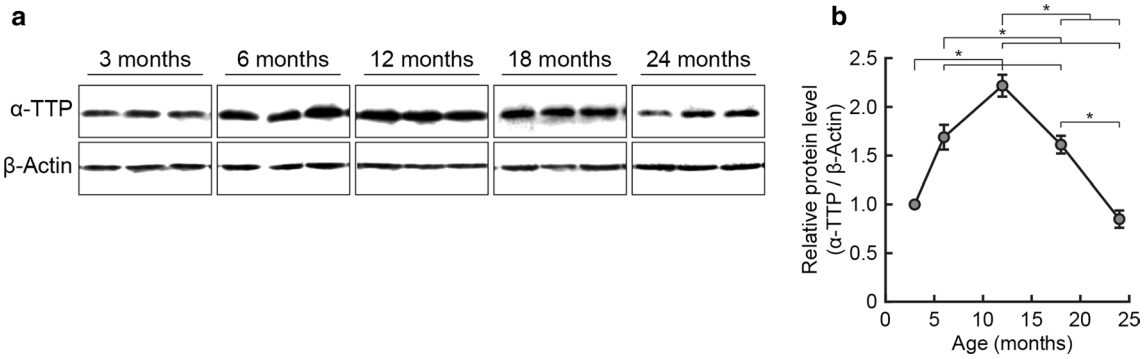

Fig. 3 Age-related changes of hepatic α-TTP protein levels. **a** The α-TTP and β-actin proteins in liver of mice at 3, 6, 12, 18, and 24 months of age were detected by Western blot analysis. **b** Hepatic α-TTP protein levels were quantified as described in "Materials and methods" section. Protein levels of α-TTP were evaluated relative to the β-actin levels and are expressed as the ratios when compared to 3-month-old mice. Values are expressed as mean ± SEM of five animals. $^*P < 0.01$

the difference was not statistically significant (Fig. 2c). In plasma, TG content did not change during aging (Fig. 2a).

We then normalized α-tocopherol levels with TG in plasma, liver, and epididymal adipose tissues (Fig. 2d–f). In the liver, α-tocopherol levels decreased greatly from 3 to 6 months of age (the latter was 65 % lower) and then remained almost stable from 6 to 24 months of age (Fig. 2e). Again, epididymal adipose tissue gained a notable 18-fold increase of α-tocopherol by the time mice aged from 6 to 24 months (Fig. 2f). There was no significant difference of α-tocopherol level in plasma during aging (Fig. 2d).

Age-related changes of α-TTP protein and mRNA expression levels in liver

We next examined α-TTP protein and mRNA expression levels in the liver, because α-TTP is a major determinant of α-tocopherol levels of plasma and tissues [24]. As shown in Fig. 3, α-TTP protein levels increased from 3 to 12 months of age and then decreased until 24 months of age. Protein levels of α-TTP in livers of mice at 12 months of age were 122 and 161 % higher than that of mice at 3 and 24 months of age, respectively (Fig. 3b). However, there was no significant difference in α-TTP mRNA expression level in the liver during aging (Fig. 4a).

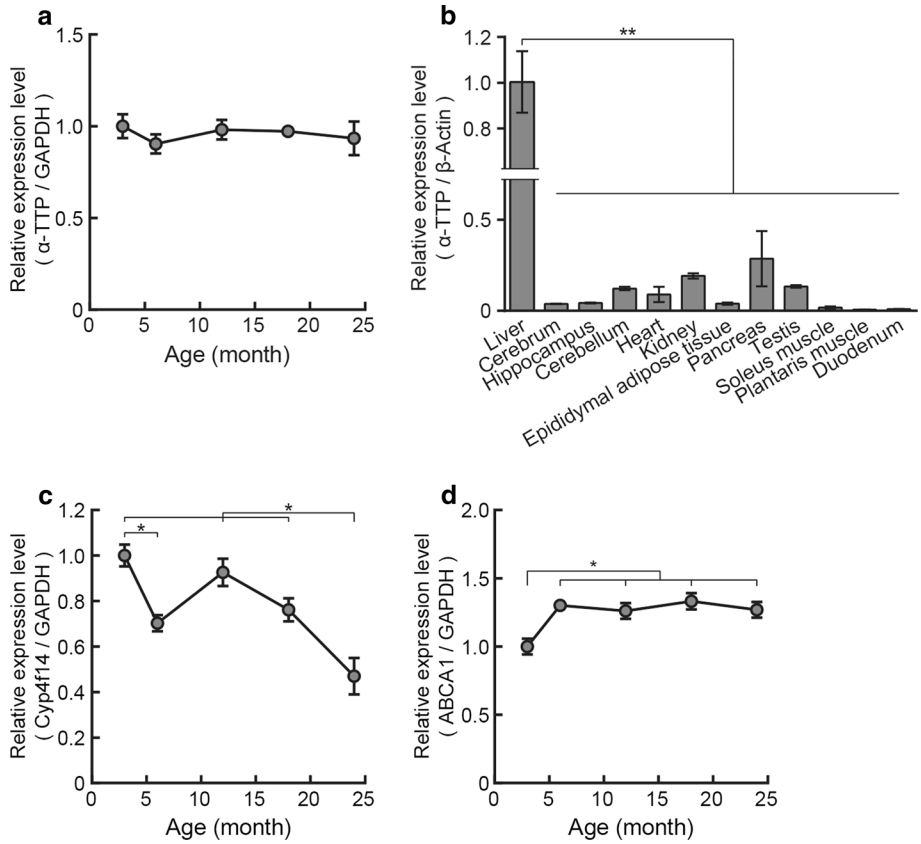

Fig. 4 Age-related changes of α-TTP, Cyp4f14, and ABCA1 mRNA expression levels in the liver and α-TTP mRNA expression levels in multiple tissues. (**a**, **c**, **d**) The α-TTP (**a**), Cyp4f14 (**c**), and ABCA1 (**d**) mRNA expression levels in livers of mice at 3, 6, 12, 18, and 24 months of age were determined by qPCR analysis as described in "Materials and methods" section. The α-TTP, Cyp4f14, and ABCA1 mRNA expression levels in livers from mice of increasing ages were evaluated relative to the GAPDH mRNA expression levels and expressed as the ratio when compared to 3-month-old mice. Values are expressed as mean ± SEM of five animals. $*P < 0.01$. **b** The α-TTP mRNA expression levels in liver, cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum of male mice at 12 months of age were determined by qPCR analysis. The α-TTP mRNA expression levels in tissues were evaluated relative to the β-actin mRNA expression levels and expressed as the ratio when compared to livers from 12-month-old mice. Values are expressed as mean ± SEM of three animals. $*P < 0.05$, $**P < 0.01$

α-TTP mRNA expression in various tissues

To confirm the α-TTP mRNA expression levels in various tissues, we performed qPCR analysis. All tissues examined contained α-TTP mRNA, i.e., cerebrum, hippocampus, cerebellum, heart, kidney, epididymal adipose tissue, testis, pancreas, soleus muscle, plantaris muscle, and duodenum, although the levels in these tissues were much lower than that in the liver (Fig. 4b).

Age-related changes of Cyp4f14 and ABCA1 mRNA expression levels in the liver

As well-documented, α-tocopherol is transported to the plasma membrane by α-TTP in liver cells, transferred to plasma lipoproteins such as very low density lipoprotein (VLDL) by ABCA1, and then distributed to peripheral tissues [4, 5]. Moreover, α-tocopherol is catabolized by Cyp4f14 in mouse livers [25, 26]. Thus, hepatic Cyp4f14 and ABCA1 are also key factors for determining α-tocopherol levels in plasma and tissues [27]. Therefore, we examined Cyp4f14 and ABCA1 mRNA expression in livers of mice at 3, 6, 12, 18, and 24 months of age (Fig. 4c, d). Cyp4f14 mRNA expression decreased from 3 to 6 months of age, increased from 6 to 12 months of age, then gradually decreased for the next 12–24 months (Fig. 4c). Cyp4f14 mRNA expression was 30 % lower in the liver at 6 months than at 3 months of age and, by 24 months of age, was 49 % lower than that at 12 months of age. On the other hand, ABCA1 mRNA expression increased from 3 to 6 months of age and then remained at almost the same levels during 6–24 months of age (Fig. 4d). ABCA1 mRNA expression levels in the livers of 6-month-old mice was 30 % higher than that at 3 months of age.

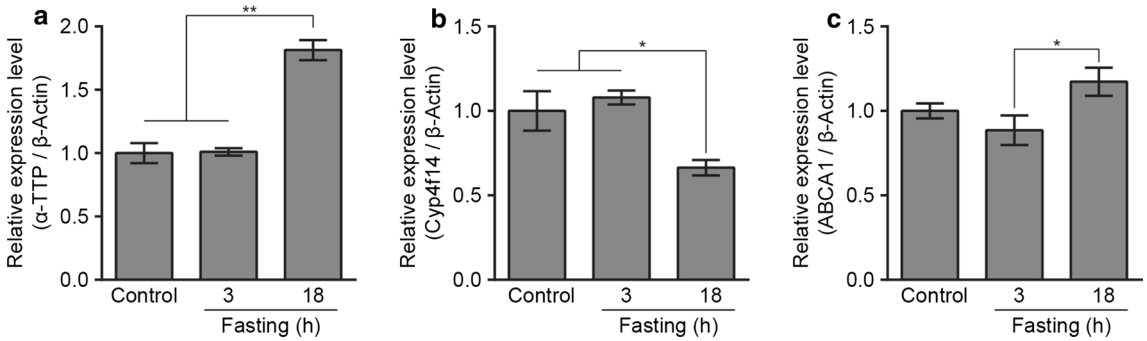

Fig. 5 Effects of fasting on α-TTP, Cyp4f14, and ABCA1 mRNA expression levels in the liver. The **a** α-TTP, **b** Cyp4f14, and **c** ABCA1 mRNA expression levels in livers from mice fasted for 3 and 18 h and non-fasted mice as a control were determined by qPCR analysis. The mRNA expression levels were evaluated relative to the β-actin mRNA expression levels and are presented as ratios compared to those in livers from non-fasted mice. Values are expressed as mean ± SEM of five to six animals. $^{*}P < 0.05$, $^{**}P < 0.01$

Effect of fasting on α-TTP, Cyp4f14, and ABCA1 mRNA expression levels in liver

To elucidate the effect of fasting on the α-TTP, Cyp4f14, and ABCA1 mRNA, we examined these mRNA expression levels in livers from 3- to 18-h-fasted mice and non-fasted, matched controls. The α-TTP mRNA expression levels in livers from 18-h-fasted mice were 1.8-fold higher than that from 3-h-fasted and non-fasted mice (Fig. 5a). However, no significant difference was evident in α-TTP mRNA expression levels between 3-h-fasted and non-fasted mice. Cyp4f14 mRNA expression in livers from 18-h-fasted mice was 30 % lower than in 3-h-fasted mice and non-fasted mice (Fig. 5b), whereas the 3-h-fast and non-fasted state produced no significant difference in Cyp4f14 mRNA expression. The ABCA1 mRNA expression levels in livers from 18-h-fasted mice were 1.3-fold higher than that from 3-h-fasted mice (Fig. 5c). However, there was no significant difference of ABCA1 mRNA expression between mice fasted for 3 or 18-h and non-fasted mice.

Discussion

In the years-long debate about the efficacy of VE as an antioxidant that benefits human health, this is the first report to verify the content of α-tocopherol, a major component of VE, in plasma and key tissues of mice during aging. We documented significant differences during aging, most notably in the liver and epididymal adipose tissue. Moreover, hepatic α-TTP protein levels showed age-related changes.

In this study, we first clarified the α-tocopherol levels in plasma and 12 tissues of male mice as they advanced from 3 to 24 months of age. We also examined TG content in plasma, liver, and epididymal adipose tissue of mice during aging and normalized α-tocopherol levels by TG, because α-tocopherol is a lipid-soluble antioxidant and α-tocopherol level in tissues might be influenced by the amount of lipid. Interestingly, the hepatic TG content increased dramatically from 3 to 6 months of age; in contrast, hepatic α-tocopherol levels normalized with TG decreased from 3 to 6 months of age. These results indicate that hepatic α-tocopherol level is not directly proportional to the hepatic TG contents during aging. Moreover, the hepatic α-tocopherol level normalized according to tissue weight decreased during the period from 6 to 24 months, but no such differences occurred in hepatic α-tocopherol level normalized with TG for the same 6–24 month span. Thus, age-related changes of hepatic α-tocopherol levels varied depending on the normalization agent.

We found that hepatic α-TTP protein levels increased from 3 to 12 months of age and then decreased until 24 months of age, although age-related changes of hepatic α-tocopherol levels did coincide with hepatic α-TTP protein levels, which were normalized with tissue weight or TG. Since α-TTP is responsible for releasing α-tocopherol from the liver [27], reduction of hepatic α-TTP protein levels late in life (in mice from 12 to 24 months of age) might result in the accumulation of hepatic α-tocopherol. However, hepatic α-tocopherol levels decreased when normalized to tissue weight or maintained almost same levels when normalized to TG from 12 to 24 months. This discrepancy suggests that hepatic α-tocopherol content must be controlled not only by hepatic α-TTP protein but also other factors that may participate in the transport and/or metabolism of α-tocopherol in the liver. As mentioned earlier, ABCA1, which secrets α-tocopherol out of liver cells, and Cyp4f14 that oxidatively catabolizes α-tocopherol are possible candidates to control the hepatic α-tocopherol level as well as α-TTP. We examined Cyp4f14 and ABCA1 mRNA expression in livers of mice during aging and found that Cyp4f14 mRNA expression levels decreased from 12 to 24 months of age, yet ABCA1 remained at almost static

from 6 to 24 months of age. Thus, age-related changes of ABCA1 mRNA expression levels in the liver were coincident with hepatic α-tocopherol levels normalized with TG. However, age-related changes of Cyp4f14 mRNA expression levels in the liver did not correlate with hepatic α-tocopherol levels normalized with both tissue weight and TG, because reductions in Cyp4F14 should promote the accumulation of α-tocopherol in the liver at progressive ages. Grebenstein et al. [28] reported that hepatic α-tocopherol levels reduced in α-TTP knockout mice and sesamin which is a Cyp4F14 inhibitor did not affect the α-tocopherol catabolism. Accordingly, age-related changes of hepatic α-tocopherol levels may not be due to the α-tocopherol catabolism involved in Cyp4F14.

Hepatic α-TTP protein levels showed age-related changes; however, there were no significant differences of α-TTP mRNA expression levels in the liver during aging. To account for this discrepancy, we examined the effect of fasting on the α-TTP mRNA expression. In mice fasted to avoid the influence of food intake, we noted that α-TTP mRNA expression in the liver from 18-h-fasted mice was 1.8-fold higher than that from 3-h-fasted and non-fasted mice. Thus, fasting clearly increased α-TTP mRNA expression. Thakur et al. [29] reported that intracellular α-tocopherol inhibited α-TTP protein degradation of HepG2-TetOn-α-TTP cells, which stably expressed HA-tagged α-TTP protein under the inducible TetOn promoter. Therefore, α-TTP protein stability is presumably influenced by intracellular α-tocopherol levels at progressive ages, thereby providing a possible reason for the discrepancy in age-related changes between α-TTP protein and mRNA expression. On the other hand, Cyp4f14 mRNA expression levels decreased from 12 to 24 months of age and also decreased after 18 h of fasting. The catabolic pathway of α-tocopherol might be suppressed to avoid the reduction of hepatic α-tocopherol levels and also to avoid a reduction of α-tocopherol levels in the liver at progressive ages. Moreover, ABCA1 mRNA expression remained at virtually the same level throughout 6–24 months of aging and increased slightly after 18 h of fasting when compared to the 3-h-fasting; however, there was no significant difference in ABCA1 mRNA expression levels between both 3- and 18-h-fasted and non-fasted mice. Consequently, ABCA1 might be expressed constitutively to maintain lipid metabolism and hardly be influenced by α-tocopherol levels and aging.

In this study, epididymal adipose tissue weights increased from 3 to 12 months of age and then decreased until 24 months of age. These age-related changes of epididymal adipose tissue weights were in good agreement with previous reports [30, 31]. This phenomenon must be due to reduced differentiation capacity of preadipocytes and fat deposit during aging caused by decreased adipogenic transcription factor such as CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ), and increased anti-adipogenic regulators such as tumor necrosis factor α (TNFα) as reviewed by Kirkland et al. [32].

Moreover, in epididymal adipose tissue, α-tocopherol levels increased from 6 to 24 months of age irrespective of normalization. Interestingly, α-tocopherol levels normalized with tissue weight and TG at 24 months of age were 25- and 18-fold higher than that at 6 months of age, respectively. On the other hand, TG contents in epididymal tissue increased only slightly from 18 to 24 months of age, strongly suggesting that aged animals must accumulate high concentrations of α-tocopherol in the epididymis. If so, high concentrations of α-tocopherol may play an important role in preventing the oxidation of stored lipids. Furthermore, epidermal tissue may be the place to store α-tocopherol and to release it for maintenance of physiological functions in the brain. Also, specific machinery may exist to incorporate and accumulate α-tocopherol into adipose tissue. Further analyses are needed to clarify that hypothesis.

In the case of plasma, no significant differences were apparent in α-tocopherol levels during aging even when α-tocopherol levels were normalized with TG. Plasma α-tocopherol might be strictly controlled at constant levels during aging.

Fukui et al. [33] reported that α-tocopherol-deficient rats displayed cognitive dysfunction. Others noted that α-tocopherol was essential for Purkinje neuron integrity and prevented high-fat, high-carbohydrate diet-induced memory impairment [34, 35]. In the present study, α-tocopherol levels in the cerebrum, hippocampus, and cerebrum normalized with tissue weight increased for animals from 3 to 18 months of age and maintained α-tocopherol levels at those progressive ages. Therefore, α-tocopherol may be essential to prevent memory impairment and to maintain neuronal cells during aging.

To summarize, α-tocopherol levels in the liver showed significant differences during aging when normalized with tissue weights. Moreover, hepatic α-TTP protein levels underwent age-related changes, although α-TTP mRNA expression measured after overnight fasting was not altered during aging. Interestingly, the α-tocopherol level in epididymal adipose tissue, when normalized with tissue weight and with TG, increased profoundly from 6 to 24 months of age; however, no such age-related changes were observed in plasma. This detailed investigation of bodily changes in content of the VE component, α-tocopherol, during the aging process provides a new foundation for pursuing the usefulness of VE supplementation to human health.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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