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The role of α-tocopherol in motor hypofunction with aging in α-tocopherol transfer protein knockout mice as assessed by oxidative stress biomarkers

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Abstract

It has been hypothesized that oxidative stress plays a key role in aging. In order to elucidate the role of the antioxidant network — including α-tocopherol (αT) and αT transfer protein — in aging in vivo, α-tocopherol transfer protein knockout ($\alpha TTP^{-/-}$) mice were fed a vitamin-E-depleted diet, and wild-type (WT) mice were fed a diet containing 0.002 wt.% αT from the age of 3 months to 1 1/2 years. The lipid oxidation markers total hydroxyoctadecadienoic acid (tHODE) and 8-*iso*-prostaglandin $F_2\alpha$, and antioxidant levels in the blood, liver and brain were measured at 3, 6, 12 and 18 months. tHODE levels in the plasma of $\alpha TTP^{-/-}$ mice were elevated at 6 months compared to 3 months, and were significantly higher those in WT mice, although they decreased thereafter. On the other hand, tHODE levels in the liver and brain were constantly higher in $\alpha TTP^{-/-}$ mice than in WT mice. Motor activities decreased with aging in both mouse types; however, those in the $\alpha TTP^{-/-}$ mice were lower than those in the WT mice. It is intriguing to note that motor activities were significantly correlated with the stereoisomer ratio (*Z*,*E*/*E*,*E*) of HODE, which is a measure of antioxidant capacity in vivo, in the plasma, in the liver and even in the brain, but not with other factors such as antioxidant levels. In summary, using the biomarker tHODE and its stereoisomer ratio, we demonstrated that αT depletion was associated with a decrease in motor function, and that this may be primarily attributable to a decrease in the total antioxidant capacity in vivo.
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Keywords: Aging; Motor activity; Lipid peroxidation; Total hydroxyoctadecadienoic acid (tHODE); Stereoisomer ratio

1. Introduction

It has been suggested that biological molecules and membranes are directly damaged by reactive oxygen and nitrogen species, as well as by toxic and signaling molecules generated [1,2]. Indeed, lipid oxidation products, which may be yielded initially, have attracted considerable attention as indices of oxidative stress [3–5]. On the other hand, the antioxidant effects of vitamin E have been studied extensively in both in vivo and in vitro systems. However, there still remains a fundamental and controversial question as to whether vitamin E acts as an antioxidant to prevent

Abbreviations: αT, α-tocopherol; $\alpha TTP^{-/-}$, α-tocopherol transfer protein knockout; WT, wild type; tHODE, total hydroxyoctadecadienoic acid; t7-OHCh, hydroxycholesterol; 8-*iso*-$PGF_2\alpha$, 8-*iso*-prostaglandin $F_2\alpha$; 8-*iso*-$PGF_2\alpha$-d_4, 8-*iso*-prostaglandin $F_2\alpha$-d_4; 9-(*E*,*Z*)-HODE, 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-(*Z*,*E*)-HODE, 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 9-HODE-d_4, 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic-9,10,12,13-d_4 acid; 9-(*E*,*E*)-HODE, 9-hydroxy-10(*E*),12(*E*)-octadecadienoic acid; 13-(*E*,*E*)-HODE, 13-hydroxy-9(*E*),11(*E*)-octadecadienoic acid; HV, hematocrit value; BHT, 2,6-di-*tert*-butyl-4-methylphenol; HPLC, high-performance liquid chromatography; TR, thioredoxin reductase; TBARS, thiobarbituric-acid-reactive substances; GPT, glutamate pyruvate transaminase; ANOVA, analysis of variance.

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cardiovascular disease and whether the major role of this vitamin is as an antioxidant [6,7]. Recently, Roberts et al. [8] reported that vitamin E decreases systemic oxidant stress in vivo in humans at a high dose (>1600 IU/day) by measuring F_2-isoprostane. Needless to say, there are abundant in vivo animal studies demonstrating the antioxidant and inhibitory effects of vitamin E on atherosclerosis. For example, apolipoprotein and α-tocopherol transfer protein double knockout ($ApoE^{-/-}\alpha TTP^{-/-}$) mice were used to assess the efficacy of αT as an antioxidant in vivo [9,10]. However, it should be kept in mind that many animal studies are terminated before the onset of atherosclerosis.

The first targets in biological fluids attacked by reactive oxygen species are polyunsaturated fatty acids. Lipid hydroperoxides are the major primary products in the oxidation of polyunsaturated fatty acids and their esters; however, hydroperoxides are the substrates of many enzymes such as glutathione peroxidases and phospholipases, and they also undergo nonenzymatic secondary reactions [11]. Therefore, the amount of lipid hydroperoxides measured does not necessarily reflect the extent of in vivo lipid peroxidation. We have proposed a method for the in vivo measurement of lipid oxidation. In this method, total hydroxyoctadecadienoic acid (tHODE) and hydroxycholesterol (t7-OHCh) are determined from physiological samples after reduction with sodium borohydride and saponification by potassium hydroxide [12], and biomarkers are evaluated as indices of in vivo lipid oxidation [13–17]. Using this method, hydroperoxides and ketones, as well as hydroxides of both the free form and the ester form of linoleic acid and cholesterol, are measured as tHODE and t7-OHCh, respectively.

We performed the present study in order to elucidate whether vitamin E can act as an antioxidant not only during the early stages but also during the later stages of aging in animals, and, additionally, to establish whether it can suppress motor hypofunction at an advanced stage of aging. α-Tocopherol transfer protein knockout ($\alpha TTP^{-/-}$) mice were used in a long-term study during which they were fed a vitamin-E-depleted diet. By administering this diet, it is expected that the concentration of αT in the blood and tissues will fall to zero. Wild-type (WT) mice that were fed a diet containing 0.002 wt.% vitamin E were used as controls. Blood and tissue levels, as well as activity performance, of biomarkers and antioxidants were followed for 1 1/2 years.

2. Materials and methods

2.1. Chemicals

8-*iso*-Prostaglandin $F_2\alpha$ (8-*iso*-$PGF_2\alpha$), 8-*iso*-prostaglandin $F_2\alpha$-d_4 (8-*iso*-$PGF_2\alpha$-d_4), 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid (9-(*E*,*Z*)-HODE), 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13-(*Z*,*E*)-HODE) and 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic-9,10,12,13-d_4 acid (9-HODE-d_4) were obtained from the Cayman Chemical Co. (Michigan, USA). 9-Hydroxy-10(*E*),12(*E*)-octadecadienoic acid (9-(*E*,*E*)-HODE) and 13-hydroxy-9(*E*),11(*E*)-octadecadienoic acid (13-(*E*,*E*)-HODE) were obtained from Larodan Fine Chemicals AB (Malmo, Sweden). Other materials were of the highest grade available commercially.

2.2. Experimental animals

Male and female mice [specific pathogen-free C57BL/6J ($\alpha TTP^{+/+}$ mice), weighing 19–24 g] were purchased from Nippon Clea Co. (Tokyo, Japan). We also used male and female $\alpha TTP^{-/-}$ mice (weighing 19–24 g) of C57BL/6J background from an in-house colony. Mice were fed a vitamin-E-free diet (the composition of which is given in Yoshida et al. [17]; Funabashi Nojyo, Chiba, Japan) or a control diet [0.002 wt.% vitamin E (>99.7 wt.% natural D-αT); Funabashi Nojyo]. The diets used in this study were stored at 4°C, and it was confirmed that there were no detectable oxidized lipids in the diets during the experimental period. The diets in the mouse cages were replaced daily with stored feeds in order to avoid artificial oxidation and deterioration. The mice were maintained under standardized conditions of light (0700–1900 h), temperature (22°C) and humidity (70%). They were sacrificed under diethyl ether anesthesia. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology. The number of mice used in this study is shown in Table 1.

Table 1
The number of mice used in this study and statistical conditions

	Age (month)	3 (months)	6 (months)	12 (months)	18 (months)
αTTP	♂	8	12	12	16
	♀	7	10	8	5
WT	♂	6	5	7	5
	♀	6	5	8	4

Unpaired *t* test for two groups shown

ANOVA–Tukey's test was carried out in four groups of male and female α-$TTP^{-/-}$ and WT mice. (Significance is shown as the * in the figure ($P < 0.05$)).

2.3. Motor performance test

The motor function of $\alpha TTP^{-/-}$ and WT mice was investigated using a rotating rod apparatus (Muromachi Kikai Co., Ltd., Tokyo, Japan). The mice were tested at 3, 6, 12 and 18 months of age. The mice were placed on the rod (30 mm in diameter) for five consecutive trials. The data of the final three trials were used to derive an average score, which expresses the latency to fall. During each trial, the rotating rod underwent linear acceleration from 0 to 40 rpm over a period of 5 min and thereafter remained at the maximum speed.

2.4. Analyses of tHODE and 8-iso-PGF$_2\alpha$ in the plasma, liver and brain

tHODE and 8-*iso*-PGF$_2\alpha$ were measured as follows using a slightly modified version of a previously reported method [12]. Animal blood was collected from the inferior vena cava using a heparinized syringe, and blood cells and plasma were separated by centrifugation (1580×*g* at 4°C for 10 min). Plasma (0.3 ml) was used for the analyses of tHODE and antioxidants immediately after collection. The blood cells were washed twice using a fourfold volume of saline in order to remove plasma and white blood cells, and then adjusted to a hematocrit value (HV) of approximately 40% with saline. Accurate HV was later determined by a hematocrit capillary (Cosmo-bio Ltd., Tokyo, Japan). The erythrocyte sample (HV ca. 40%) was extracted with a fourfold volume of methanol containing 100 μM 2,6-di-*tert*-butyl-4-methylphenol (BHT) by vortexing and centrifugation (20,400×*g* at 4°C for 10 min), and then immediately subjected to tHODE, t7-OHCh and antioxidant analyses. The livers and brains of mice were also collected after perfusion with saline (1.0 ml) and stored at −80°C until analysis. Internal standards — 8-*iso*-PGF$_2\alpha$-d$_4$ (100 ng) and 9-HODE-d$_4$ (100 ng) — and 1 ml of methanol were added to the plasma and to 1-ml extracts from the erythrocyte sample, followed by reduction with an excessive amount of sodium borohydride at room temperature for 5 min under nitrogen. The liver and brain were homogenized (Polytron PT-3100; Kinematica AG, Lucerne, Switzerland) in saline (liver:saline or brain: saline=1:3, wt/wt), and 300-μl aliquots were diluted with saline (1700 μl). The internal standards and 1 ml of methanol were added to this solution, followed by reduction as described above. The reduced sample was then mixed with 1 M KOH in methanol (1 ml) under nitrogen and incubated for 30 min in the dark at 40°C in a shaker. The sample was centrifuged (3000×*g* at 4°C for 10 min), and the supernatant was diluted with a fourfold volume of water (pH 3) and acidified (pH 3) using 2 N HCl. The acidified sample was centrifuged (3000×*g* at 4°C for 10 min), and the supernatant was subjected to solid-phase extraction [12]. The solution was evaporated under nitrogen, and 30 μl of the silylating agent *N*,*O*-bis(trimethylsilyl)trifluoroacetamide was added to the dried residue. The solution was vigorously mixed using a vortex mixer for 1 min and incubated for 60 min at 60°C in order to obtain the trimethylsilyl esters and ethers. The eluent obtained was diluted with 70 μl of acetone, and then an aliquot of this sample was injected into a gas chromatograph (GC 6890 N; Agilent Technologies Co., Ltd.) equipped with a quadrupole mass spectrometer (5973 Network; Agilent Technologies Co., Ltd.) and a fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30 m×0.25 mm; Agilent Technologies Co., Ltd.). Helium was used as carrier gas at a flow rate of 1.2 ml/min. The temperature of the column was programmed from 60 to 280°C at a rate of 10°C/min. The injector temperature was set at 250°C, and the temperatures of transfer lines to the mass detector and ion source were 250 and 230°C, respectively. Electron impact mass spectrometry was carried out; electron energy was set at 70 eV. HODE and 8-*iso*-PGF$_2\alpha$ were identified on the basis of their retention times and mass patterns (m/z=440, 369 and 225 for HODE; m/z=571 and 481 for 8-*iso*-PGF$_2\alpha$), and precursor ions at m/z=440 and 481 were selected for quantification of HODE and 8-*iso*-PGF$_2\alpha$, respectively, using the internal standards 9-HODE-d$_4$ (m/z=444) and 8-*iso*-PGF$_2\alpha$-d$_4$ (m/z=485). These precursor ions were indeed the most sensitive among the detected ions. With this method, 9-(*E*,*Z*)-HODE, 13-(*Z*,*E*)-HODE, 9-(*E*,*E*)-HODE, 13-(*E*,*E*)-HODE and 8-*iso*-PGF$_2\alpha$ were measured simultaneously and isolated on the gas chromatography/mass spectrometry chromatograph. By using these methods, artificial oxidation of lipids during the sample workup was kept to a minimum, and it was confirmed that the experimental errors were within ±10% [12].

2.5. High-performance liquid chromatography analysis

Plasma antioxidants were extracted by chloroform/methanol (2/1, vol/vol). Chloroform/methanol (100 μl, 2/1, vol/vol) was added to the homogenized suspension (25 wt/wt.%, 50 μl) of the liver and brain, prepared as described above. Lipids and vitamin E were then extracted from the plasma, liver and brain by centrifugation (20,400×*g* at 4°C for 10 min) after mixing vigorously using a vortex mixer. αT and γ-tocopherol were measured using a high-performance liquid chromatography (HPLC) system equipped with an amperometric electrochemical detector (NANOSPACE SI-1; Shiseido, Tokyo, Japan) set at 800 mV and an ODS column (LC-18, 5 μm, 250×4.6 mm; Supelco Co., Ltd., Japan); methanol/*tert*-butyl alcohol (95/5, vol/vol) containing 50 mM sodium perchlorate as eluent, was used at a flow rate of 1 ml/min. Ascorbic acid levels in the plasma, liver and brain were measured using an HPLC system equipped with a UV detector (SPD-10AV, 263 nm; Shimadzu, Japan) and an NH_2 column (Wakosil 5NH$_2$, 5 μm, 250×4.6 mm; Wako Pure Chemical Industries, Osaka, Japan); 40 mM phosphate-buffered saline/methanol (1/9, vol/vol) delivered at a flow rate of 1 ml/min was used as eluent. Plasma and homogenates (25 wt.%) of the liver and brain were diluted with methanol (1/4, vol/vol) and mixed vigorously for 1 min

using a vortex mixer, followed by centrifugation (20,400×*g*, 10 min). An aliquot of the resulting upper layer was injected into the HPLC system. Ubiquinols and ubiquinones were also measured by using an HPLC system equipped with an amperometric electrochemical detector (NANOSPACE SI-1; Shiseido) set at 700 mV and a reverse-phase column (LC-8, 5 μm, 250 mm×4.6 mm; Supelco Co., Ltd.), followed by a reducing column (RC-10, 30 mm×4 mm; Irika, Japan). Methanol/*tert*-butyl alcohol (85/15, vol/vol) containing 50 mM sodium perchlorate delivered at a flow rate of 1 ml/min was used as eluent. Plasma and homogenates of the liver and brain (25 wt.%) were diluted with methanol and hexane (1/5/10, vol/vol/vol) and mixed vigorously for 1 min using a vortex mixer, followed by centrifugation (20,400×*g*, 10 min). An aliquot of the resulting upper layer was injected into the HPLC.

2.6. Thioredoxin reductase activity

Human recombinant thioredoxin [thioredoxin reductase (TR) 0.8 μM] in phosphate buffer (50 mM) containing 1 mM EDTA, 0.2 mM NADPH and 80 μM insulin was used. The oxidation of NADPH was recorded at 340 nm. TR reactivity was calculated from oxidized NADPH.

2.7. Analysis of thiobarbituric-acid-reactive substances in the liver

The amount of lipid oxidation in the liver was also assayed by measuring thiobarbituric-acid-reactive substances (TBARS) as an additional parameter for comparison with tHODE and t8-*iso*-$PGF_2\alpha$. The thiobarbituric acid reaction was carried out by mixing 0.2 ml of sodium dodecyl sulfate solution (8.1%, wt/vol), 1.5 ml of acetic acid buffer (20%, vol/vol, pH 3.5), 1.5 ml of thiobarbituric acid (1%, vol/vol), 0.7 ml of water and 0.05 ml of ethanol containing BHT (0.8 wt.%, wt/vol) with 0.1 ml of liver homogenate (25 wt.%), prepared as mentioned above. The reaction mixture was incubated at 100°C for 60 min, cooled in ice and then mixed vigorously with 1 ml of water and 5 ml of *n*-butyl alcohol and pyridine (15/1, vol/vol). The mixture was then centrifuged (1580×*g* at 4°C for 10 min), and the supernatant was measured spectrophotometrically at 535 nm. Tetramethoxypropane was used as standard to estimate TBARS formation as nanomoles of malondialdehyde equivalents per milligram of liver.

2.8. Analysis of fatty acids

Chloroform/methanol (100 μl, 2/1, vol/vol) containing an internal standard, tridecanoic acid (600 μg), was added to the homogenized suspension (25 wt.%, 50 μl) of the liver, and lipids were extracted by centrifugation (20,400×*g* at 4°C for 10 min) after mixing vigorously using a vortex mixer. An aliquot of this solution was evaporated, and 2 ml of methanol/benzene (4/1, vol/vol) was added. Acetyl chloride (200 μl) was added slowly to this solution, and the mixture was subjected to methanolysis at 100°C for 1 h. After the sample had been cooled in water, 5 ml of potassium carbonate (6% in water) was added slowly to stop the reaction and to neutralize the mixture. The solution was mixed vigorously using a vortex mixer and centrifuged (1580×*g*, 10 min), and an aliquot of the resulting benzene layer was injected into a gas chromatograph (GC 6890 N; Agilent Technologies Co., Ltd.) equipped with a flame ionization detector in conjunction with a fused-silica capillary column (SP-2560, 100 m×0.25 mm; Supelco Co., Ltd.). Helium was used as carrier gas at a flow rate of 1.2 ml/min. The temperature of the column was programmed from 140 to 240°C at a rate of 4°C/min, and the injector temperature was set at 250°C.

2.9. Analysis of glutamate pyruvate transaminase in plasma

Glutamate pyruvate transaminase (GPT) was measured using spectrophotometric assay kits (Wako Pure Chemical Industries).

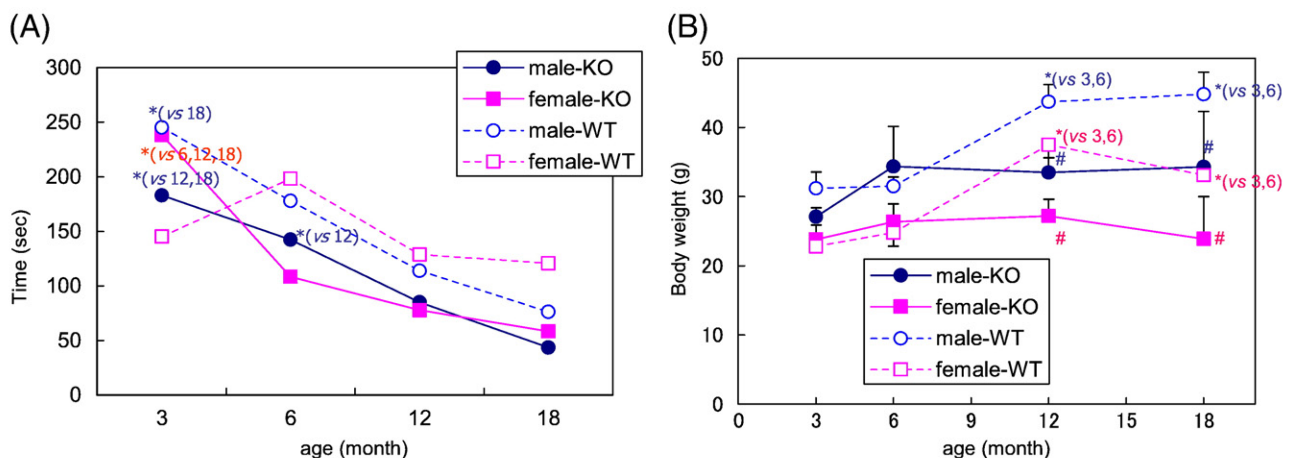

Fig. 1. (A) Performance of α-$TTP^{-/-}$ and WT mice on an accelerating rotating rod apparatus and (B) their body weights. Statistical analysis was carried out as described in Materials and Methods and as shown in Table 1. The symbols (*) and (#) indicate significance ($P<.05$). α$TTP^{-/-}$ mice were fed a vitamin-E-depleted diet, whereas WT mice were fed a control (0.002 wt.% vitamin E) diet from birth until the age indicated.

2.10. Statistical analysis

For statistical analysis of aging within sex and genotype groups, analysis of variance (ANOVA) was performed, followed by Tukey's test for multiple comparisons. Analysis of genotype and sex differences at the same age was performed using *t* test. The analytical method is summarized in Table 1. Data are expressed as mean±standard deviation (S.D.). Correlations were analyzed using Pearson correlation coefficient and are presented as scatter plots. $P<.05$ was considered significant.

3. Results

Male and female $\alpha TTP^{-/-}$ mice fed a vitamin-E-deficient diet and WT mice fed a diet containing 0.002 wt.% vitamin E performed a rotating rod test for acute motor activity at 3, 6, 12 and 18 months of age. As shown in Fig. 1A, performance on the rotating rod test deteriorated with aging in the four groups (male/female $\alpha TTP^{-/-}$ and WT) tested. As expected, the performance of $\alpha TTP^{-/-}$ mice was poorer than that of the WT mice when animals of the same sex were compared. Interestingly, the body weights of the $\alpha TTP^{-/-}$ mice were significantly lower than those of the WT mice at the ages of 12 and 18 months (Fig. 1B). Furthermore, the body weight of both male and female WT mice was greater at the age of 12 months than at 6 months; however, this trend was not observed in $\alpha TTP^{-/-}$ mice. Additionally, males were heavier than females, irrespective of the type of mouse.

The levels of tHODE and its stereoisomer ratio (*Z*,*E*/*E*,*E*), t8-*iso*-$PGF_2\alpha$, vitamin E, vitamin C and coenzyme Q_9 (ubiquinol-9+ubiquinone-9) in the blood and tissues of $\alpha TTP^{-/-}$ and WT mice were characterized inclusively. The individual results observed in the blood and tissues are described below.

3.1. Plasma analyses

As shown in Fig. 2A, the plasma levels of tHODE in $\alpha TTP^{-/-}$ mice were significantly higher but had a large S.D. at the age of 6 months compared to the same-sex WT mice. It is interesting to note that the tHODE levels decreased with aging, and that there was no difference observed in the four groups at ages older than 6 months; the reason for this observation is not known at present. Apparently, the concentration of αT in the plasma of $\alpha TTP^{-/-}$ mice was zero even at the early age of 3 months (Fig. 2D). The concentration of vitamin C and the ubiquinol-9 (Q_9H_2/total coenzyme Q_9) content in male $\alpha TTP^{-/-}$ mice at the age of 6 months were significantly lower than those of male WT mice (Fig. 2E and G). This result is in good agreement with the increase in tHODE. The stereoisomer ratio of HODE (*Z*,*E*/*E*,*E*), which is a measure of antioxidant capacity in vivo, tended to decrease with age; however, there was no obvious difference among the four groups (Fig. 2B). The level of t8-*iso*-$PGF_2\alpha$ was not influenced remarkably by aging; however, the level in $\alpha TTP^{-/-}$ mice tended to be higher than that in WT mice when animals of the same sex were compared (Fig. 2C). Another interesting feature is that the Q_9H_2 levels in female mice were higher than those in male mice irrespective of the type of mouse (Fig. 2F). The liver damage marker GPT did not change significantly with mouse genotype, diet or antioxidant (data not shown).

3.2. Liver analyses

As shown in Fig. 3A and C, the levels of tHODE and t8-*iso*-$PGF_2\alpha$ in the liver of $\alpha TTP^{-/-}$ mice tended to be higher than those in the corresponding WT mice of the same sex over the same period of study. Among these, the tHODE level in male $\alpha TTP^{-/-}$ mice was significantly higher than that in male WT mice (Fig. 3A). The stereoisomer ratio of HODE decreased with age, which is the same trend observed in plasma (Fig. 3B). It was revealed using the traditional lipid peroxidation marker TBARS that the levels in male $\alpha TTP^{-/-}$ mice at the age of 18 months were significantly higher than those in WT mice (Fig. 3D) — the same as the results obtained for tHODE. The absolute level of TBARS was, however, five times higher than that of tHODE, suggesting nonspecific detection of TBARS. As expected, αT was not detected in the liver of $\alpha TTP^{-/-}$ mice (Fig. 3E). It should be added that the plasma level of αT in male WT mice was higher than that in female WT mice; however, the inverse result was observed in the liver, of which the reason is not known at present. The results for antioxidants observed in the liver were almost the same as those observed in plasma (Fig. 3F–H).

3.3. Brain analyses

Enhancement of lipid oxidation in the brain due to vitamin E depletion was observed in t8-*iso*-$PGF_2\alpha$ (Fig. 4C) rather than in tHODE (Fig. 4A). This result may be due to the fatty acid composition in the brain. The arachidonic acid content in the brain is higher than that in the blood and liver. It was clear that the content of Q_9H_2 in $\alpha TTP^{-/-}$ mice was lower than that in WT mice (Fig. 4G). Interestingly, in contrast to plasma and liver, there was no marked difference between the sexes in terms of brain Q_9H_2 content (Fig. 4F). This observation should be further investigated.

3.4. Correlation study

As the phenotype of $\alpha TTP^{-/-}$ mice was clearly shown by motor function (Fig. 1A), the correlation between motor function and the biomarkers assessed was investigated individually. As a result, motor function was found to correlate significantly with the stereoisomer ratio of HODE (*Z*,*E*/*E*,*E*) in the plasma, liver and brain (Fig. 5A–C). Furthermore, it is interesting to note that TR activity in the brain was significantly correlated with motor function (r=.243, $P<.05$). Unfortunately, it is difficult to specify the mechanism of motor hypofunction, since the whole brain was used to measure the biomarkers. However, it is

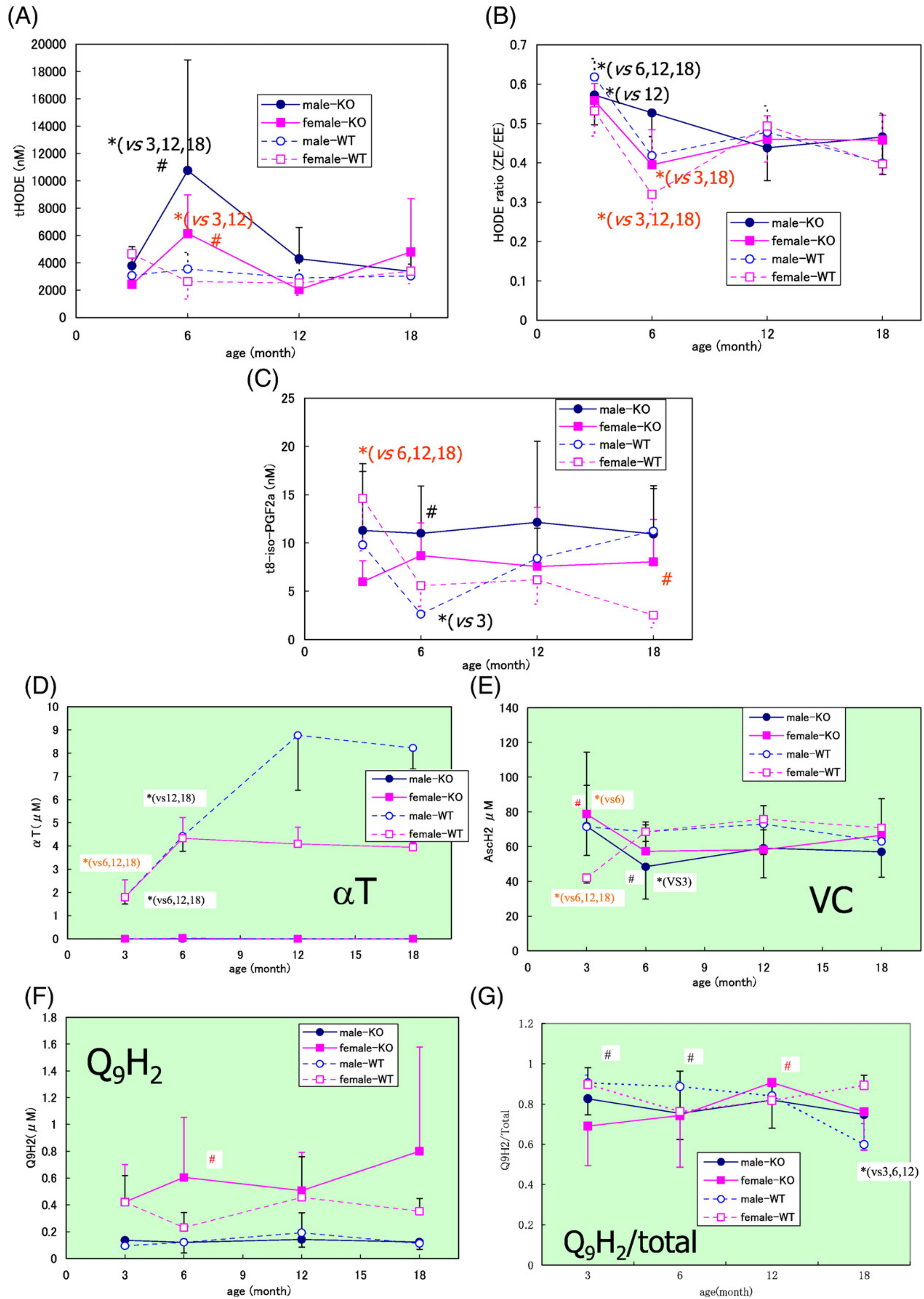

Fig. 2. Levels of tHODE in plasma (A); the stereoisomer ratio of HODE (*Z,E*/*E,E*) (B); t8-*iso*-PGF$_2\alpha$ (C); αT (D); vitamin C (E); and the reduced form of coenzyme Q_9 (F and G) in $\alpha TTP^{-/-}$ and WT mice. Experimental conditions are similar to those described in Fig. 1.

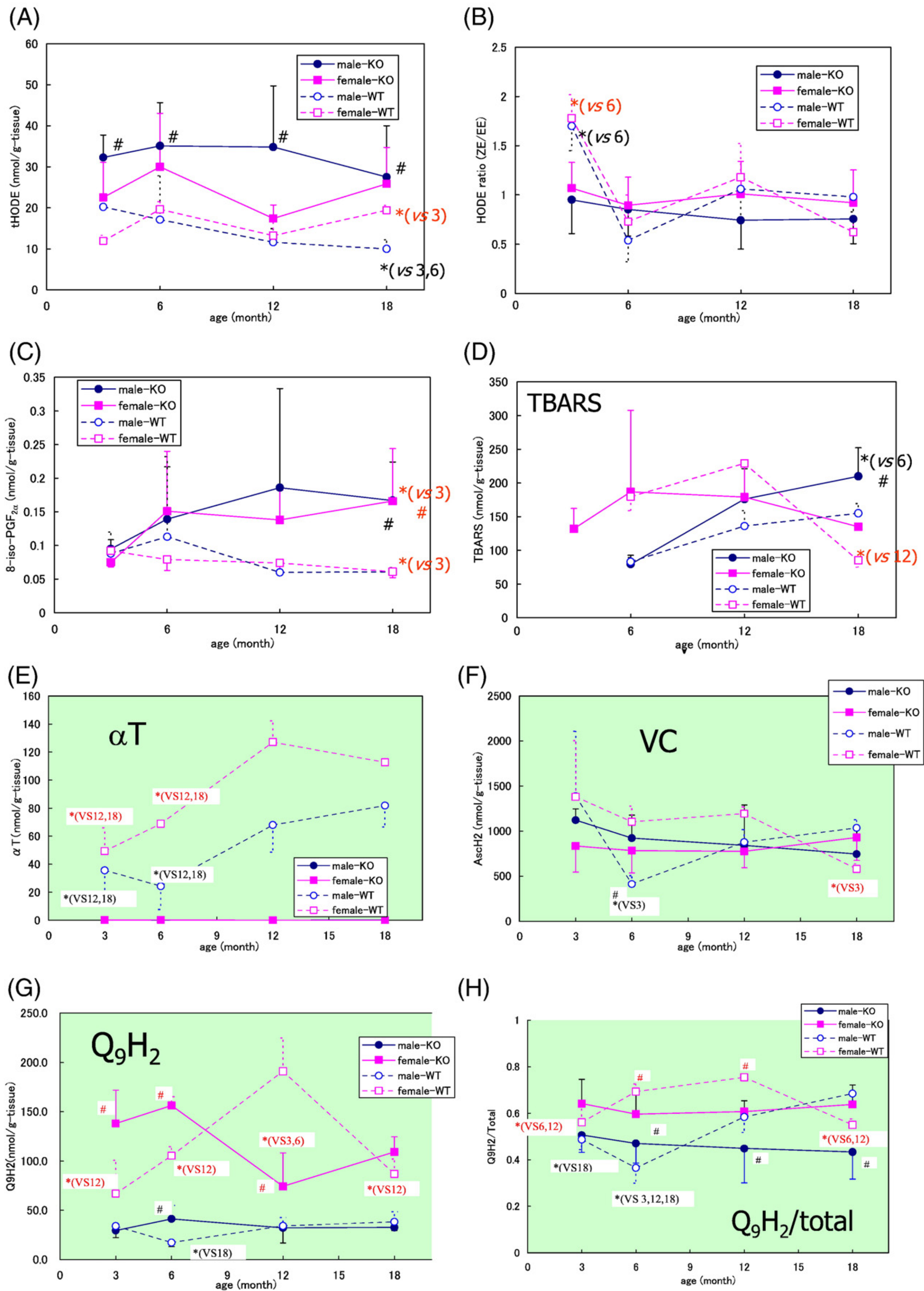

Fig. 3. Levels of tHODE in the liver (A); the stereoisomer ratio of HODE (*Z,E*/*E,E*) (B); t8-*iso*-PGF$_2$α (C); TBARS (D); αT (E); vitamin C (F); and the reduced form of coenzyme Q$_9$ (G and H) in αTTP$^{-/-}$ and WT mice. Experimental conditions are similar to those described in Fig. 1.

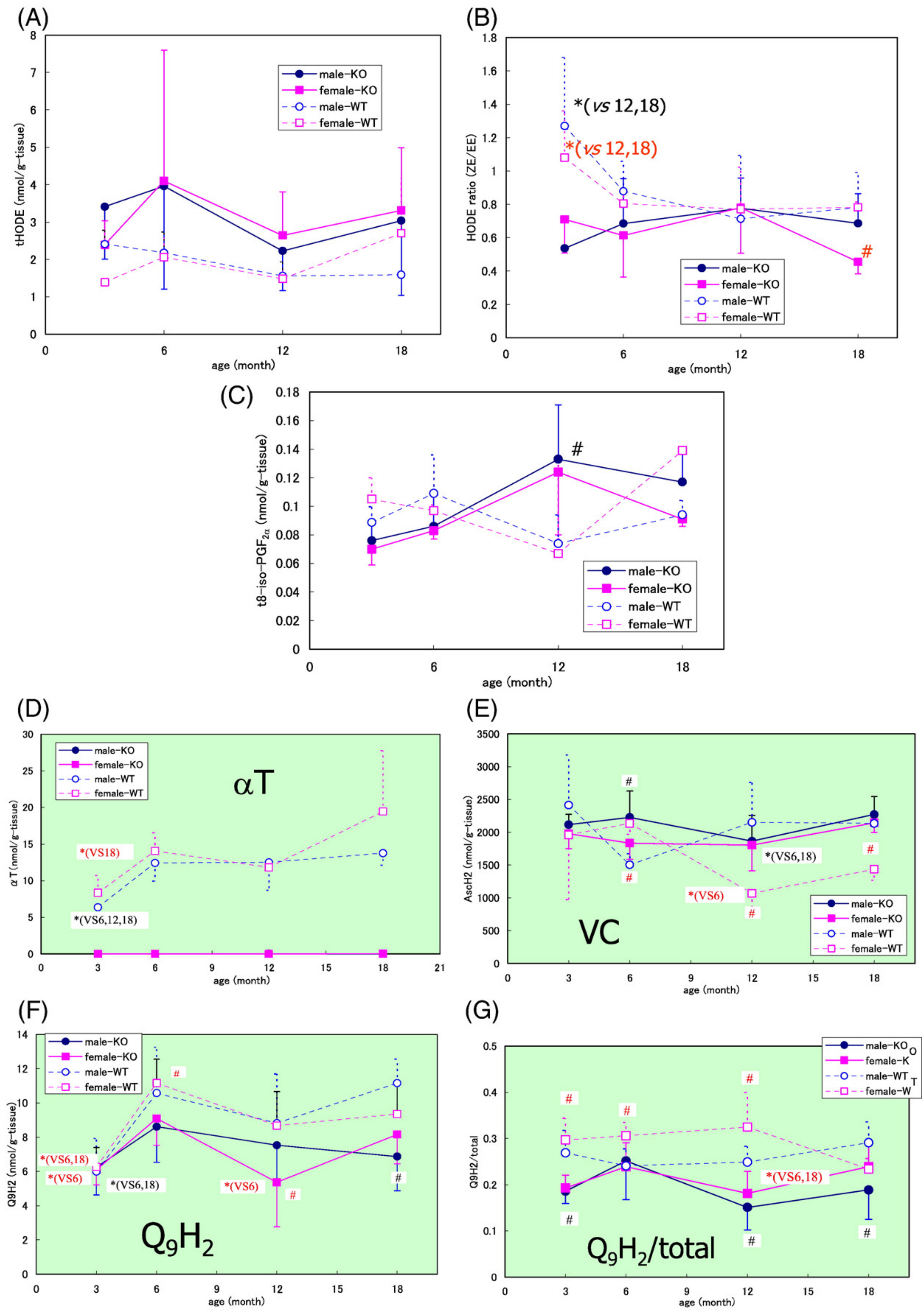

Fig. 4. Levels of tHODE in the brain (A); the stereoisomer ratio of HODE (*Z,E*/*E,E*) (B); t8-*iso*-PGF$_2α$ (C); aT (D); vitamin C (E); and the reduced form of coenzyme Q_9 (F and G) in $αTTP^{-/-}$ and WT mice. Experimental conditions are similar to those described in Fig. 1.

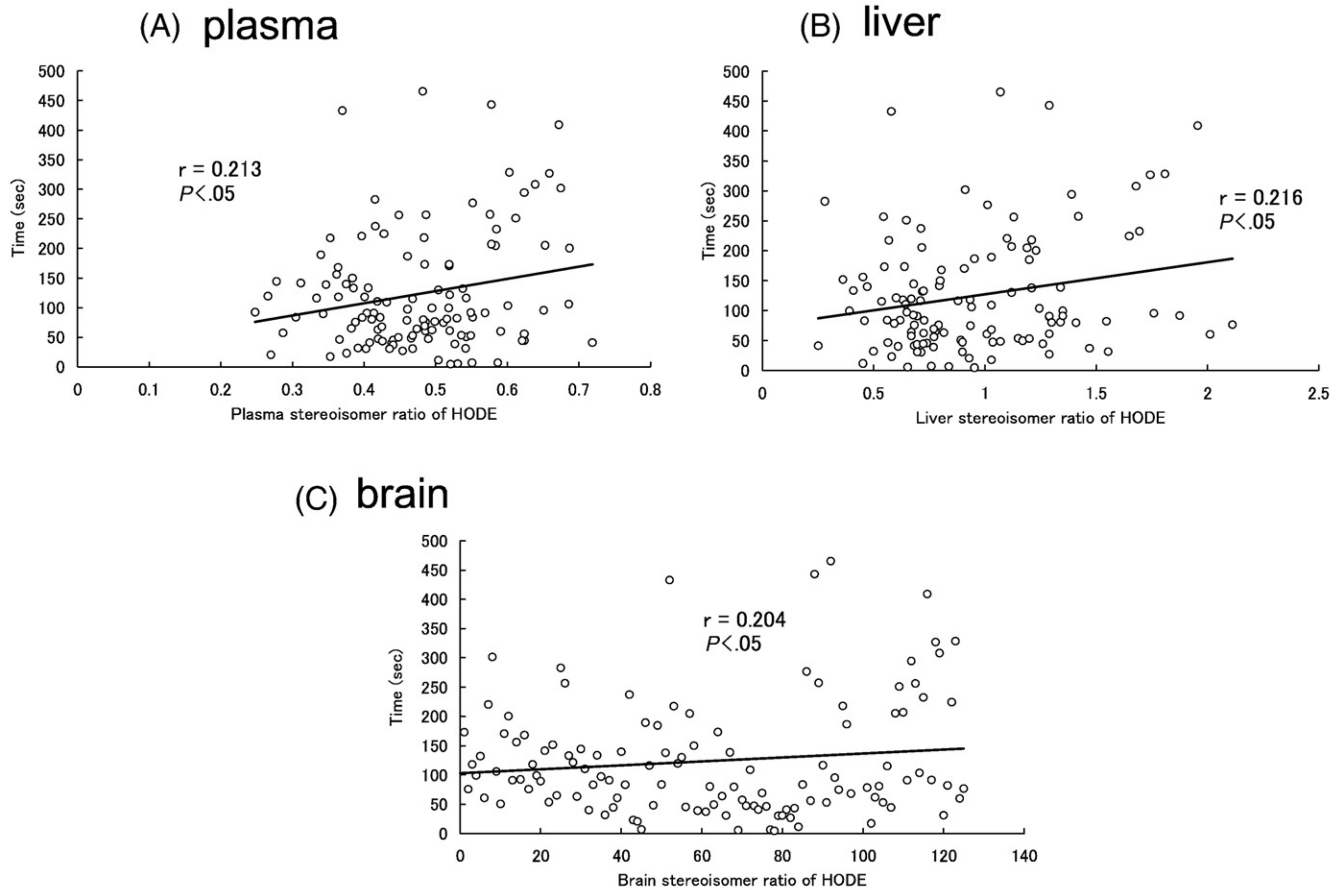

Fig. 5. The relationship between performance of motor activity and the stereoisomer ratio of HODE in the plasma (A), liver (B), and brain (C).

important to note that the measure of total antioxidant capacity in vivo (the stereoisomer ratio of HODE) revealed a significant correlation with the decrease in motor activity with aging. Furthermore, the stereoisomer ratio in plasma and liver exhibited a significant inverse correlation with age (data not shown), which has also been observed in a human plasma study [18]. Additionally, the plasma levels of tHODE were significantly correlated with liver and brain levels of tHODE (data not shown), consistent with our previous observations [19].

4. Discussion

The present study was carried out in order to elucidate the involvement of vitamin E deficiency in motor hypofunction and to clarify the causative role of lipid peroxidation by using $\alpha TTP^{-/-}$ mice. It has previously been reported that $\alpha TTP^{-/-}$ mice reared on a vitamin-E-depleted diet exhibit neuronal degeneration, leading to a decrease in motor activity [20]. It was emphasized in this literature that $\alpha TTP^{-/-}$ mice were good models for late-onset slowly progressive neuronal degeneration caused by chronic oxidative stress. Furthermore, Gohil et al. [21] reported that the behavior of young $\alpha TTP^{-/-}$ mice was essentially normal, but older mice showed inactivity, ataxia and memory dysfunction. We further extended this work to encompass a detailed study in which we demonstrated, using lipid peroxidation biomarkers, a decrease in motor activity with aging by focusing on the brain, as well as on the blood and liver. For this purpose, it is important to assess well-established and quantitative measures. In this respect, we have evaluated a promising biomarker, tHODE, in both plasma and tissues. The most striking finding was that motor activity is significantly correlated with the stereoisomer ratio of HODE (*Z*,*E*/*E*,*E*) (Fig. 5), suggesting that total antioxidant capacity affects motor activity. Complete depletion of αT in tissues apparently influenced other antioxidant levels such as vitamin C and coenzyme Q. Among these, the levels and contents of the reduced form of coenzyme Q_9 in the brain, but not in the plasma or liver, were decreased by the depletion of αT compared to the levels in WT mice. We have previously reported that coenzyme Q levels are significantly correlated with the stereoisomer ratio of HODE in healthy humans [18] and experimental animals fed diets including several types of supplement [17]. Another important observation is that the activity of TR is significantly correlated with motor activity. TR, thioredoxin and

NADPH comprise a highly conserved and ubiquitous network, which plays an important role in the redox regulation of multiple intracellular processes. Furthermore, it has been suggested that induction of TR might serve as an adaptive response to the oxidative damage induced by hydrogen peroxide [22].

The acceleration of lipid peroxidation by reactive oxygen species and the inhibitory effects of antioxidants are apparently dependent on the class of lipid, sites of radical formation and antioxidant localization. Consequently, no single gold standard exists for the evaluation of in vivo lipid peroxidation and antioxidant efficacy. Furthermore, the level of any marker depends on the oxidants and their rates of formation, metabolism and clearance. Many biomarkers have been proposed; among these, F_2-isoprostanes have been reported as the gold standard for the assessment of in vivo oxidative injury [23,24]. Several observations have revealed a relationship between isoprostanes and progression of atherosclerotic lesions [25,26]. However, to our knowledge, there is no available report indicating a relationship between isoprostane and ataxia with isolated vitamin E deficiency (AVED). As suggested, $\alpha TTP^{-/-}$ mice are a good model for AVED. Thus, we attempted to measure as many biomarkers as possible, including total 8-*iso*-$PGF_2\alpha$ and our novel tHODE, in order to clarify the involvement of lipid peroxidation in AVED.

Linoleates are a major class of in vivo polyunsaturated fatty acids, and their oxidation proceeds by a straightforward mechanism to yield 9-hydroperoxyoctadecadienote and 13-hydroperoxyoctadecadienote (hydroperoxyoctadecadienoic acids) as the major products [27]. Therefore, the tHODE thus measured may account for much of the in vivo lipid peroxidation. On the other hand, arachidonates — although more reactive to oxygen radicals than linoleates — are present in smaller amounts than linoleates, and their oxidation proceeds by complicated mechanisms to yield versatile products, of which F_2-isoprostanes comprise only a small fraction. Consequently, the advantage of tHODE over 8-*iso*-$PGF_2\alpha$ as a biomarker is its higher concentration [12]. Indeed, the present study demonstrated that the levels of tHODE in the plasma and liver were two to three orders of magnitude higher than the corresponding levels of t8-*iso*-$PGF_2\alpha$. It is well known that lipid peroxidation proceeds by different mechanisms, depending on the oxidants, to subsequently yield different isomers. More importantly, the efficacy of the antioxidants depends on the oxidants [28]. Thus, it is very important to assess the total antioxidant efficacy by using a solid measure that accurately reflects the in vivo phenotype. Another advantage of HODE over 8-*iso*-$PGF_2\alpha$ is that regional isomers and stereoisomers can be measured separately [12]. The ratio of *cis–trans* to *trans–trans* HODE is a measure of the capacity of H-atom donation at the site of oxidation.

In conclusion, this study demonstrates that lipid oxidation is indeed involved in the progression of motor hypofunction with aging in vitamin-E-depleted $\alpha TTP^{-/-}$ mice. Vitamin E can play a pivotal role in the enhancement of antioxidant function, leading to the suppression of lipid peroxidation, particularly in the brain. Needless to say, we need more solid data demonstrating the beneficial effects of vitamin E on the suppression of motor hypofunction with aging in vivo.

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