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Antioxidant effects of 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran and α-tocopherol in hyperlipidemic mice as evaluated by hydroxyoctadecadienoic acid and 7-hydroxycholesterol

Yasukazu Yoshida [a,*], *Mieko Hayakawa* [a], *Nanako Itoh* [a], *Yoko Habuchi* [a], *Ruriko Inoue* [a], *Zhi-Hua Chen* [a], *Jiaofei Cao* [a], *Osamu Cynshi* [b], *Kou-Ichi Jishage* [b], *Etsuo Niki* [a]

[a] *Human Stress Signal Research Center (HSSRC), National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan*

[b] *Fujigotemba Research Laboratories, Chugai Pharmaceutical Co. Ltd., Komakado, Gotemba, Shizuoka 412-8513, Japan*

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ABSTRACT

It has been hypothesized that oxidative modification of low density lipoprotein plays a key role in the pathogenesis of atherosclerosis. In order to elucidate the role of lipid oxidation and its inhibition in vivo, apolipoprotein E and α-tocopherol (αT) transfer protein double knockout ($ApoE^{-/-}\alpha\text{-}TTP^{-/-}$) and apolipoprotein E knockout ($ApoE^{-/-}$) mice fed with a vitamin E-depleted diet and a diet containing 0.002 wt.% αT, respectively, were used with or without the treatment of a synthetic antioxidant 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran (BO-653, 0.2 wt.%). The lipid oxidation markers of total hydroxylinoleic acid (tHODE), 8-*iso*-prostaglandin $F_{2\alpha}$, and 7-hydroxycholesterol (t7-OHCh) in the blood, liver, and brain were inclusively measured with or without an excessive cholesterol-feeding (Ch-diet). The tHODE levels were elevated by Ch-diet in the plasma and brain of $ApoE^{-/-}\alpha\text{-}TTP^{-/-}$ mice and in the liver of $ApoE^{-/-}$ mice without BO-653. The levels of t7-OHCh in the liver were also increased due to the Ch-diet, and the ratio of t7-OHCh to the parent compound cholesterol was reduced to the control levels by BO-653.

In summary, it was demonstrated by biomarkers, tHODE and t7-OHCh, that the added BO-653 in their diets exerted antioxidative effects in vivo under the condition of reduced vitamin E.

1. Introduction

Lipid oxidation may cause direct damage to biological molecules and membranes and may also induce the generation of toxic and signaling molecules [1,2]. Accordingly, lipid oxidation products have attracted considerable attention as indices for oxidative stress [3–5]. The oxidative modification of lipoprotein has been hypothesized to play a key role in the

* *Corresponding author*. Tel.: +81 72 751 8183; fax: +81 72 751 9964.
E-mail address: yoshida-ya@aist.go.jp (Y. Yoshida).

Abbreviations: $ApoE^{-/-}$, apolipoprotein E knockout mice; BHT, 2,6-di-*tert*-butyl-4-methylphenol; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; BO-653, 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran; Ch, cholesterol; CoQ9, coenzyme Q_9 (ubiquinol-9 + ubiquinone-9); 7-OHCh, 7-hydroxycholesterol; GPT, glutamate pyruvate transaminase; tHODE, total hydroxyoctadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid; 8-*iso*-$PGF_{2\alpha}$, 8-*iso*-prostaglandin $F_{2\alpha}$; LDL, low density lipoprotein; PBS, phosphate-buffered saline; T, tocopherol; $\alpha\text{-}TTP^{-/-}$, α-tocopherol transfer protein knockout

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pathogenesis of atherosclerosis. In order to elucidate the in vivo relationship between lipid oxidation and atherosclerosis and the role of antioxidants such as vitamin E, gene disrupted mice have been proposed recently. Among them, apolipoprotein E knockout (ApoE$^{-/-}$) mice have been used frequently as models of atherosclerosis [6–8]. Furthermore, apolipoprotein and α-tocopherol (αT) transfer protein double knockout (ApoE$^{-/-}$α-TTP$^{-/-}$) mice were used to assess the efficacy of α-tocopherol as antioxidant in vivo [9]. 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 [10]. Therefore, the amount of lipid hydroperoxides measured does not always reflect the extent of in vivo lipid peroxidation. We have recently developed a method for the in vivo measurement of lipid oxidation. In this method, the total hydroxyoctadecadienoic acid (tHODE) and hydroxycholesterol (t7-OHCh) are determined from physiological samples after reduction with sodium borohydride and saponification by potassium hydroxide [11], and the biomarkers were evaluated as indices of the in vivo lipid oxidation [12–16]. In this method, hydroperoxides and ketones as well as hydroxides of both the free and ester forms of linoleic acid and cholesterol are measured as tHODE and t7-OHCh, respectively.

BO-653, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran, is a synthetic antioxidant that has been designed as a potent radical-scavenging antioxidant [17]. BO-653 scavenges free radicals as rapidly as αT and the aryloxyl radical derived from BO-653 is much more stable than the α-tocopheroxyl radical [18]. Further, BO-653 inhibits the oxidation of low density lipoprotein [19] and plasma lipids [20] more efficiently than αT. In the present study, to investigate the involvement of the lipid oxidation in the onset of the dysfunction induced by hyperlipidemia with an excessive cholesterol-feeding and the protective effects of vitamin E and BO-653 against oxidative stress, hyperlipidemic ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$ mice were used, and the tHODE and t7-OHCh in their blood and tissues were assessed. The ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$ mice were fed a diet that was deficient and rich in vitamin E, respectively; this enabled us to clarify the role of vitamin E on the lipid oxidation in hyperlipidemic mice. Further, BO-653 was fortified in the diet, when necessary, to clarify whether the BO-653 can compensate for the vitamin E deficiency.

2. Materials and methods

2.1. Chemicals

BO-653 was prepared as described previously [21]. 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 Cayman Chemical Company (MI, 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 commercially available grade.

2.2. Experimental animal

Male mice (specific pathogen-free, C57BL/6J (α-TTP$^{+/+}$ mice), weighing 19–24 g) were purchased from Nippon Clea Co. (Tokyo, Japan). Male apolipoprotein E knockout (ApoE$^{-/-}$) mice and apolipoprotein E and α-tocopherol transfer protein double knockout (ApoE$^{-/-}$ α-TTP$^{-/-}$) mice, both of which were on the C57BL/6J background, from in-house colony were used (weighing 19–24 g). Mice were fed a vitamin E free diet (Funabashi Nojyo, Chiba, Japan, the composition was shown in the ref.[16]) or a controlled diet (0.002 wt.% vitamin E (more than 99.7 wt.% natural D-α-tocopherol), Funabashi Nojyo, Chiba, Japan) containing 5 wt.% stripped corn oil (Funabashi Nojyo, Chiba, Japan) for 10 weeks. When necessary, 0.2 wt.% BO-653 and/or 2 wt.% cholesterol were mixed with the diets. As previously reported [22,23], cholesterol was added, as a stripped corn oil (5 wt.%) solution with 0.5 wt.% cholic acid, to the diets, which was prepared by the manufacturer (Funabashi Nojyo, Chiba, Japan). BO-653 was added to the diet which was mixed with water beforehand, followed by drying on an air-conditioned bench over night. The diets used in this study were stored under 4 °C and it was confirmed that there was no detectable oxidized lipids in the diets during experimental period. The diets on the mice cages were replaced by stored ones day by day to avoid the artificial oxidation and deterioration. Mice were divided into eight groups depending on the genotype and types of diets (Table 1). Mice were maintained under standardized conditions of light (7 a.m. to 7 p.m.), temperature 22 °C, and humidity (70%). They were sacrificed under anesthesia with diethyl ether. The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Institute of Advanced Industrial Science and Technology.

2.3. Analyses of tHODE and 8-iso-PGF$_{2\alpha}$ in plasma, liver, and brain

Total HODE and 8-*iso*-PGF$_{2\alpha}$ were measured as follows by the slightly modified method reported previously [11]. 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 with a fourfold volume of saline to remove plasma and white blood cells and adjusted to hematocrit value (HV) around 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 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 subjected to the analyses of tHODE, t7-OHCh, and antioxidants immediately. Liver and brain were also collected after perfusion with saline (1.0 ml) and stored under −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

Table 1 – The study design

	ApoE$^{-/-}$α-TTP$^{-/-}$[a]				ApoE$^{-/-}$[a]			
	A[b] (4[c])	B[b] (9[c])	C[b] (4[c])	D[b] (6[c])	E[b] (4[c])	F[b] (11[c])	G[b] (4[c])	H[b] (10[c])
Diet								
Cholesterol (Ch)	–	+	–	+	–	+	–	+
Antioxidants								
Vitamin E (E)	–	–	–	–	+	+	+	+
BO-653 (BO)	–	–	+	+	–	–	+	+

Mice were separated into eight groups according to the with (+) and without (–) cholesterol and antioxidants. Ch, BO, and E were fortified into the diet as 2.0, 0.2, 0.002 wt.% of the diet, respectively.
[a] Mouse genotype.
[b] Group.
[c] Number of animals studied.

were added to the plasma and 1 ml extracts from the erythrocytes 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) in saline (liver or brain:saline = 1:3, w/w), and the aliquot (300 μl) was diluted with saline (1700 μl). The internal standards and 1 ml of methanol were added to this solution followed by the reduction as described above. Then the reduced sample was 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*, for 10 min, at 4 °C) and the supernatant was diluted with fourfold volume of water (pH 3) and acidified (pH 3) using 2N HCl. The acidified sample was centrifuged (3000 × *g*, for 10 min, at 4 °C) and the supernatant was subjected to the solid phase extraction [11]. The solution was evaporated under nitrogen and 30 μl of the silylating agent, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), was added to the dried residue. The solution was vigorously mixed by a vortex mixer for 1 min and incubated for 60 min at 60 °C 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 the gas chromatograph (GC 6890N, Agilent Technologies Co. Ltd.) equipped with a quadrupole mass spectrometer (5973 Network, Agilent Technologies Co. Ltd.). Fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30 m × 0.25 mm, Agilent Technologies Co. Ltd.) was used. Helium was used as the carrier gas at a flow rate of 1.2 ml/min. Temperature programming was performed from 60 to 280 °C at 10 °C/min. The injector temperature was set at 250 °C and temperatures of transfer line to mass detector and ion source were 250 and 230 °C, respectively. Electron energy was set at 70 eV. The identification of HODE and 8-*iso*-$PGF_{2\alpha}$ was conducted by their retention times and mass patterns (*m*/*z* = 440, 369, 225 for HODE and 571, 481 for 8-*iso*-$PGF_{2\alpha}$) and precursor ions at 440 and 481 were selected for quantification for 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 most sensitive among detected ions. In this method, 9-(*E*, *Z*)-HODE and 13-(*Z*, *E*)-HODE, 9-(*E*, *E*)-HODE, 13-(*E*, *E*)-HODE, and 8-*iso*-$PGF_{2\alpha}$ were measured at a time and isolated on the GC-MS chromatograph. By using these methods, artificial oxidation of lipids during sample work-up was kept minimal and it was confirmed that the experimental errors were within ±10% [11].

2.4. Analysis of cholesterol and t7-OHCh in erythrocytes, liver, and brain

t7-OHCh in erythrocytes, liver, and brain were also measured as follows. The erythrocyte sample was extracted with fourfold volume of methanol containing 100 μM BHT. The internal standard, 16-hydroxyhexadecanoic acid, was added to the methanol solution of lipid extracts. An aliquot of liver and brain homogenates (50 μl) described above was mixed with 400 μl of methanol containing 100 μM BHT followed by the addition of the internal standard. Reduction of lipid hydroperoxides was carried out by the addition of excessive amount of sodium borohydride to the methanol solution followed by the saponification. The reduced sample was suspended in 1 M KOH in methanol and pure ether and mixed for 30 min in the dark at 40 °C in a shaker. The mixture was acidified with 20% acetic acid in water and extracted with hexane. The sample was evaporated to dryness under nitrogen and derivatized with BSTFA for 1 h at 60 °C. An aliquot of this solution was injected into the same gas chromatograph equipped with a quadrupole mass spectrometer as mentioned above. The identification of 7-OHCh was conducted by their retention time and mass pattern (*m*/*z* = 546, 456) and ions at 456 was selected for the quantification. 7α-OHCh and 7β-OHCh were identified by retention times of 28.5 and 30.9 min, respectively. Total cholesterol (tCh) (*m*/*z* = 458) was also measured by this method.

2.5. HPLC analysis

Plasma antioxidants were extracted by chloroform/methanol (2/1, by volume). Chloroform/methanol (100 μl, 2/1, by volume) was added to the homogenized suspension (25, w/w%, 50 μl) of liver and brain prepared as mentioned above. Then, lipids and vitamin E were extracted from plasma, liver, and brain by centrifugation (20,400 × *g*, at 4 °C for 10 min) after mixing vigorously with a vortex mixer. α- and γ-Tocopherols and BO-653 were measured using an HPLC by an amperometric electrochemical detector (NANOSPACE SI-1, Shiseido, Tokyo, Japan) set at 800 mV, with an ODS column (LC-18, 5 μm, 250 mm × 4.6 mm, Supelco, Japan) and methanol/*tert*-butyl alcohol (95/5 by volume) containing 50 mM sodium perchlorate as eluent at 1 ml/min. Plasma, liver, brain levels of ascorbic acid were measured by an HPLC with an UV detector

(SPD-10AV, Shimadzu, Japan, 263 nm). NH_2 column (Wakosil $5NH_2$, 5 μm, 250 mm × 4.6 mm, Wako, Japan) was used and 40 mM PBS/methanol (1/9, by volume) was delivered as eluent at 1 ml/min. Plasma and homogenates (25 wt.%) of liver and brain were diluted with methanol (1/4, by volume) and mixed vigorously by a vortex mixer for 1 min followed by centrifugation (20,400 × *g*, 10 min) and immediately an aliquot of the upper layer was injected to the HPLC. Ubiquinols and ubiquinones were also measured by using HPLC by an amperometric electrochemical detector (NANOSPACE SI-1, Shiseido, Tokyo, Japan) set at 700 mV, with a reverse phase column (LC-8, 5 μm, 250 mm × 4.6 mm, Supelco, Japan) followed by reducing column (RC-10, 30 mm × 4 mm, Irika, Japan) and methanol/*tert*-butyl alcohol (85/15, by volume) containing 50 mM sodium perchlorate as eluent at 1 ml/min. Plasma and homogenates of liver and brain (25 wt.%) were diluted with methanol and hexane (1/5/10, by volume) and mixed vigorously by a vortex mixer for 1 min followed by centrifugation (20,400 × *g*, 10 min) and immediately an aliquot of the upper layer was injected to the HPLC.

2.6. Analysis of TBARS in liver

The amount of lipid oxidation in liver was also assayed by measuring thiobarbituric acid reactive substances (TBARS) as an additional parameter for comparison with tHODE and t8-*iso*-$PGF_{2\alpha}$. Thiobarbituric acid reaction was carried out by mixing 0.2 ml sodium dodecyl sulfate solution (8.1%, w/v), 1.5 ml acetic acid buffer (20%, v/v, pH 3.5), 1.5 ml thiobarbituric acid (1%, v/v), 0.7 ml water, and 0.05 ml ethanol containing BHT (0.8 wt.%, w/v) with 0.1 ml of liver homogenate (25 wt.%) prepared as mentioned above. The reaction mixture was incubated at 100 °C for 60 min and then cooled in ice followed by mixing vigorously with 1 ml water and 5 ml of *n*-butyl alcohol and pyridine (15/1, by volume). Then the mixture was centrifuged (4 °C, 1580 × *g*) for 10 min and the supernatant was measured spectrophotometrically at 535 nm. Tetramethoxypropane was used as a standard to estimate TBARS formation as nanomoles of malondialdehyde (MDA) equivalents per milligram of liver.

2.7. Analysis of fatty acids

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

2.8. Analysis of glutamate pyruvate transaminase in plasma

Glutamate pyruvate transaminase (GPT) was measured by spectrophotometric assay kits (Wako Pure Chemical Industries (Osaka, Japan)).

2.9. Statistical analysis

Statistical analyses were performed on a Microsoft personal computer by using non-parametric Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered significant. The number of animals used in this study is shown in Table 1. Data are expressed as mean values ± standard deviation (S.D.).

3. Results

The levels of tHODE and its stereoisomer ratio (*ZE*/*EE*), t7-OHCh, t8-*iso*-$PGF_{2\alpha}$, antioxidant, and lipid in the blood and tissues of hyperlipidemic mice were inclusively characterized. The ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$ mice were compared from the viewpoint of their vitamin E deficiency with and without a cholesterol-fortified diet. Additionally, the effects of BO-653 were investigated in each group. The summary of the effects of Ch, vitamin E, and BO-653 in the blood and tissues is shown in Table 2 as a ratio of either the tHODE or the t7-OHCh level with (+) to without (−) Ch, vitamin E, or BO-653 in each dietary group. The individual results observed in blood and tissues are described below.

3.1. Plasma analyses

As shown in Fig. 1(A), the plasma level of tHODE increased significantly with the excessive amount of cholesterol (2 wt.% in the diet) when the ApoE$^{-/-}$α-TTP$^{-/-}$ mice were fed diets without BO-653. However, the level of t8-*iso*-$PGF_{2\alpha}$ was not influenced remarkably by the cholesterol (Fig. 1(C)). Interestingly, both the tHODE and 8-iso$PGF_{2\alpha}$ levels tended to be lowered by the addition of 0.2 wt.% BO-653 to the diet both in the cases of the presence and absence of cholesterol, suggesting the involvement of oxidative stress and its inhibition by BO-653 in the hyperlipidemic mice. Inversely, the concentration of ascorbic acid in the plasma of ApoE$^{-/-}$α-TTP$^{-/-}$ mice decreased significantly due to the cholesterol-fortified diet (data not shown). These observations may imply that lipid peroxidation occurred in the plasma, particularly in LDL, in the case of mice fed with a Ch-fortified diet to result in the decrease in ascorbic acid. It should be noted that the plasma level of tHODE in the ApoE$^{-/-}$ mice that were fed a 0.002 wt.% vitamin E diet was significantly lower than that in ApoE$^{-/-}$α-TTP$^{-/-}$ mice that were fed a vitamin E-deficient diet when all the mice were fed a cholesterol-fortified diet without BO-653. In this case, inversely, the stereoisomer ratio of HODE (*ZE*/*EE*), which is a measure of the antioxidant capacity, in the ApoE$^{-/-}$ mice group was significantly higher than that in the

Table 2 – Summary of the effects of cholesterol, vitamin E (E), and BO-653 (BO) on tHODE and t7-OHCh in blood and tissues of mice

	Diet	Diet	Compared groups[a]	Plasma	Erythrocytes		Liver		Brain	
				tHODE	tHODE	t7-OHCh	tHODE	t7-OHCh	tHODE	t7-OHCh
+Ch/−Ch	−E	−BO	B/A	1.84*	0.95	1.80	1.91	70.0*	5.73*	1.50
		+BO	D/C	1.02	0.51	1.43	2.04	7.32*	15.4*	1.51
	+E	−BO	F/E	1.91	0.42	1.43	3.29*	9.04*	1.62	1.62
		+BO	H/G	0.89	0.32*	1.13	2.41	14.18*	1.82	1.82
+E/−E	−Ch	−BO	E/A	0.53	0.95	0.37	0.90	0.30	0.60	0.93
		+BO	G/C	0.78	0.55*	1.43	1.15	0.71	0.51	1.51
	+Ch	−BO	F/B	0.56*	0.43	0.29*	1.55	0.04*	0.17*	1.01
		+BO	H/D	0.72	0.34	1.14	1.36	1.38	0.06*	1.82
+BO/−BO	−Ch	−E	C/A	0.63	0.90	0.28	0.61	0.07*	1.23	0.77
		+E	G/E	0.91	0.52	1.09	0.78	0.17	1.10	1.25
	+Ch	−E	D/B	0.35*	0.49	0.22*	0.65	0.01*	3.46	0.78
		+E	H/F	0.45*	0.40	0.87	0.57	0.27*	1.31	1.41

The ratio of tHODE and t7-OHCh in the presence (+) of additives to that in the absence (−) are shown. ApoE$^{-/-}$ and ApoE$^{-/-}$ α-TTP$^{-/-}$ mice were fed diet with and without vitamin E (0.002 wt.%), respectively, for 10 weeks. Cholesterol (Ch, 2.0 wt.%) and BO-653 (0.2 wt.%) were added to the diet as shown in Table 1. The marks '*' indicates the significance $P < 0.05$. Statistical analysis was carried out by Mann–Whitney *U*-test.
[a] The compared groups are shown by using the group symbol shown in Table 1.

ApoE$^{-/-}$α-TTP$^{-/-}$ mice (Fig. 1(B)). These results suggest that vitamin E plays a pivotal role in the inhibition of lipid oxidation in the plasma of hyperlipidemic mice fed with a Ch-fortified diet. Further, BO-653 significantly decreased the tHODE levels in plasma when mice were fed Ch-fortified diets in the absence and presence of vitamin E, suggesting the strong efficacy of BO-653 in the suppression of oxidative stress induced by hyperlipidemia. On the other hand, a minor change was observed in the plasma levels of αT and total coenzyme Q_9 including its ratio of reduced to oxidized form (data not shown). The concentration of BO-653 in the plasma of mice fed with 0.2 wt.% BO-653-contained diet was about 20 times higher than that of αT. Needless to say, the level of αT in the plasma of the ApoE$^{-/-}$α-TTP$^{-/-}$ mice, which were fed with a vitamin E-free diet, was around the detection limit or below it. The liver damage marker – GPT – did not change significantly with the types of mice genotype, diet, and antioxidant (data not shown).

3.2. *Erythrocyte analyses*

Next, in order to clarify lipid oxidation in whole blood, the erythrocytes were analyzed for tHODE and t7-OHCh. The level of tHODE in the erythrocytes was not influenced by the Ch-fortified diet (Fig. 2(A)). On the contrary, the levels of t7-OHCh tended to increase, although not significant, due to the Ch-fortified diet and decreased significantly due to BO-653 (Fig. 2(B)) in ApoE$^{-/-}$α-TTP$^{-/-}$ mice, while the level of total cholesterol (tCh) did not increase significantly due to the diet (data not shown). In this case again, when all the mice were fed a Ch-fortified diet, the erythrocyte level of t7-OHCh in the erythrocytes of ApoE$^{-/-}$ mice that were fed a 0.002 wt.% vitamin E diet was significantly lower than that observed in the ApoE$^{-/-}$α-TTP$^{-/-}$ mice that were fed a vitamin E-free diet without BO-653. This suggests the inhibitory effect of vitamin E against lipid oxidation in erythrocytes. Interestingly, the ratio of 7β-OHCh to 7α-OHCh decreased due to BO-653 in the ApoE$^{-/-}$α-TTP$^{-/-}$ mice fed with Ch-fortified diet thereby

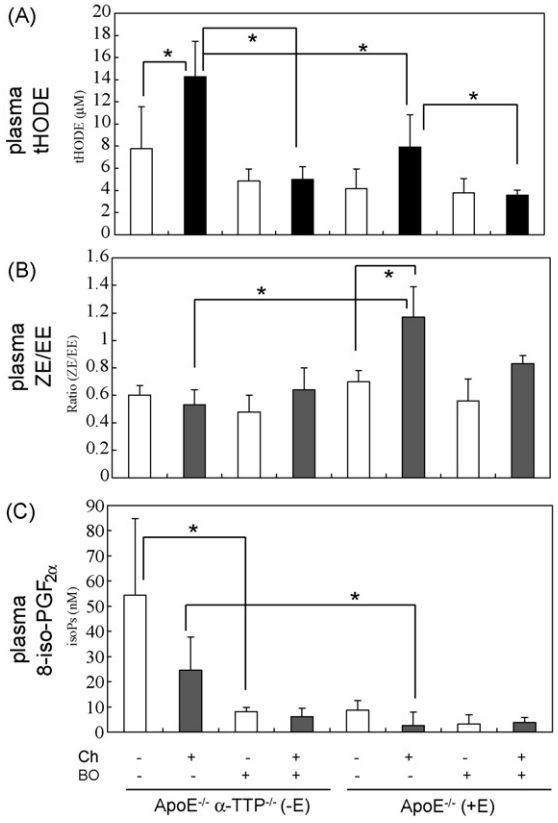

Fig. 1 – Levels of tHODE in plasma (A), stereo-isomer ratio of HODE (*ZE*/*EE*) (B), and t8-iso-PGF$_{2\alpha}$ (C) of ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$ mice that were fed a vitamin-E free and control (0.002 wt.% vitamin E) diet, respectively, for 10 weeks. Diets contained 2.0 wt.% cholesterol and 0.2 wt.% BO-653, when necessary. Open bar, without cholesterol; solid bar, with cholesterol. Mann–Whitney *U*-test was carried out and statistic significance is shown as an asterisk '*' ($P < 0.05$) in the figure.

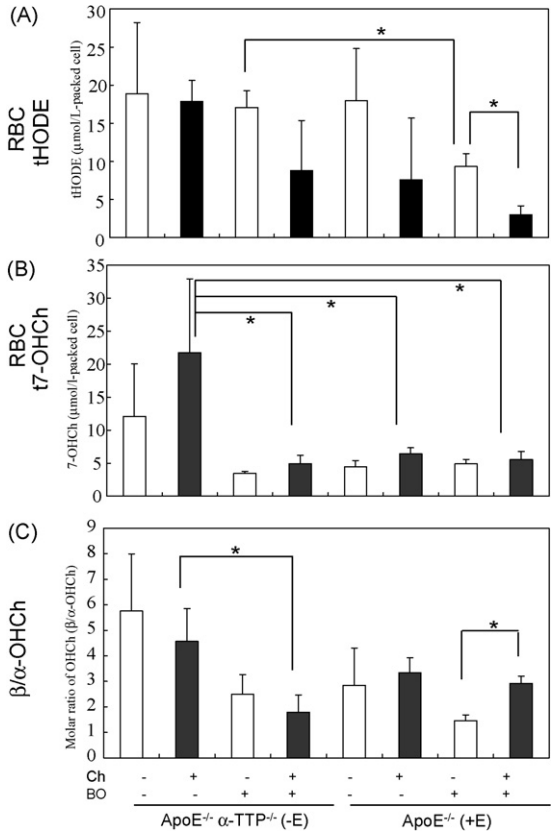

Fig. 2 – Levels of tHODE in erythrocytes (A), t7-OHCh (B), and the ratio of 7β-OHCh to 7α-OHCh (C). Experimental conditions are similar to those in Fig. 1.

implying that free radical-mediated oxidation was suppressed by the BO-653 and that lipid peroxidation was observed in the erythrocytes (Fig. 2(C)).

3.3. *Liver analyses*

As shown in Fig. 3(A) and (B), the level of tHODE in the liver tended to increase and its stereo-isomer ratio (*ZE*/*EE*) tended to decrease due to the Ch-fortified diet, irrespective of the mice type. This observation was in good agreement with the TBARS assessment (data not shown). As the level of t8-iso-$PGF_{2\alpha}$ was two to three orders of magnitude lower than that of tHODE, the sensitive influence on the level was not observed (data not shown). It was clearly demonstrated that the ratio of t7-OHCh to its parent compound tCh, which was elevated by the Ch-fortified diet in ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$ mice, decreased to the control levels by a BO-653 diet (Fig. 3(C)). It should be noted that this t7-OHCh observation in the liver is consistent with that observed in the erythrocytes mentioned above. The influence of the Ch-fortified diet on the tCh in the liver was apparent, as shown in Fig. 3(D). The fatty acid analysis clearly revealed that the total fatty acids (tFA) decreased due to a Ch-fortified diet (Fig. 3(E)). Furthermore, linoleic acid and arachidonic acid, which are susceptible to peroxidation to yield HODE and 8-iso-$PGF_{2\alpha}$, respectively, also tended to decrease due to the diet (data not shown). Cholesteryl linoleates and arachidonates in the liver were measured by an HPLC method and it was found that their levels increased drastically due to the cholesterol-fortified diet (data not shown). The reason for the decrease in the tFA in the liver due to the Ch-fortified diet may be attributed to the acceleration of cholesterol ester formation and its subsequent secretion from the liver to the blood. The concentration of αT in the liver was significantly decreased by BO-653 (Fig. 3(F)), which is consistent with the observation in aortas of ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$α-TTP$^{-/-}$ mice reported previously [24]. It may be reasonable because the concentration of BO-653 in the livers of mice fed with the 0.2 wt.% BO-653 diet was more than one order of magnitude higher than that of αT in the ApoE$^{-/-}$ mice (Fig. 3(F), inseted panel). The level of total coenzyme Q_9 in the liver tended to increase by the Ch-fortified diets (data not shown), which may be due to the influence on biosynthesis of coenzyme Q_9 by an excessive amount of cholesterol. There was little significant change in ascorbic acid in the liver by types of diets (data not shown).

3.4. *Brain analyses*

The enhancement of lipid oxidation in the brain due to the Ch-fortified diet and the pivotal role of vitamin E in the suppression of lipid oxidation were observed in tHODE but not in t7-OHCh. As shown in Fig. 4(A) and (B), the level of the tHODE in the brains of the ApoE$^{-/-}$α-TTP$^{-/-}$ mice increased and its stereo-isomer ratio (*ZE*/*EE*) decreased due to cholesterol where BO-653 exerted little effect on the level in the brains. On the other hand, the level of tHODE in the brains of the ApoE$^{-/-}$ mice that were fed with a 0.002 wt.% vitamin E diet was dramatically lower than that of the ApoE$^{-/-}$α-TTP$^{-/-}$ mice that were fed with a vitamin E-free diet when all the mice were fed with a Ch-fortified diet. Further, the ratio of t7-OHCh to tCh which was increased due to the Ch-fortified diet in ApoE$^{-/-}$α-TTP$^{-/-}$ mice was decreased significantly by vitamin E but not BO-653 (Fig. 4(C)). The level of αT in the brains of ApoE$^{-/-}$ mice and that of total coenzyme Q_9 but not its ratio of reduced to oxidized form in the ApoE$^{-/-}$α-TTP$^{-/-}$ mice increased significantly due to the cholesterol-fortified diet (Figs. 4(D) and (E)).

4. Discussion

The present study was carried out to elucidate the involvement of lipid oxidation in dysfunction induced by hyperlipidemia and to clarify the antioxidative effects of vitamin E and BO-653. For the former objective, it is important to assess well-grounded and quantitative measures. The acceleration of lipid peroxidation by reactive oxygen species and inhibitory effects by antioxidants are apparently dependent on the class of the lipid, sites of radical formation, and antioxidant localization. With regard to this, oxidative stress markers, antioxidant levels, and lipid concentrations in blood and tissues, as far as possible, were inclusively characterized in this study. Many biomarkers have been proposed and among them, F2-isoprostanes have been reported as the gold standard for the assessment of in vivo oxidative injury [25,26]. Numerous papers have shown that F2-isoprostanes are indeed good biomarkers (for example Ref. [27]). There are several observations that reported the relationship between isoprostanes and

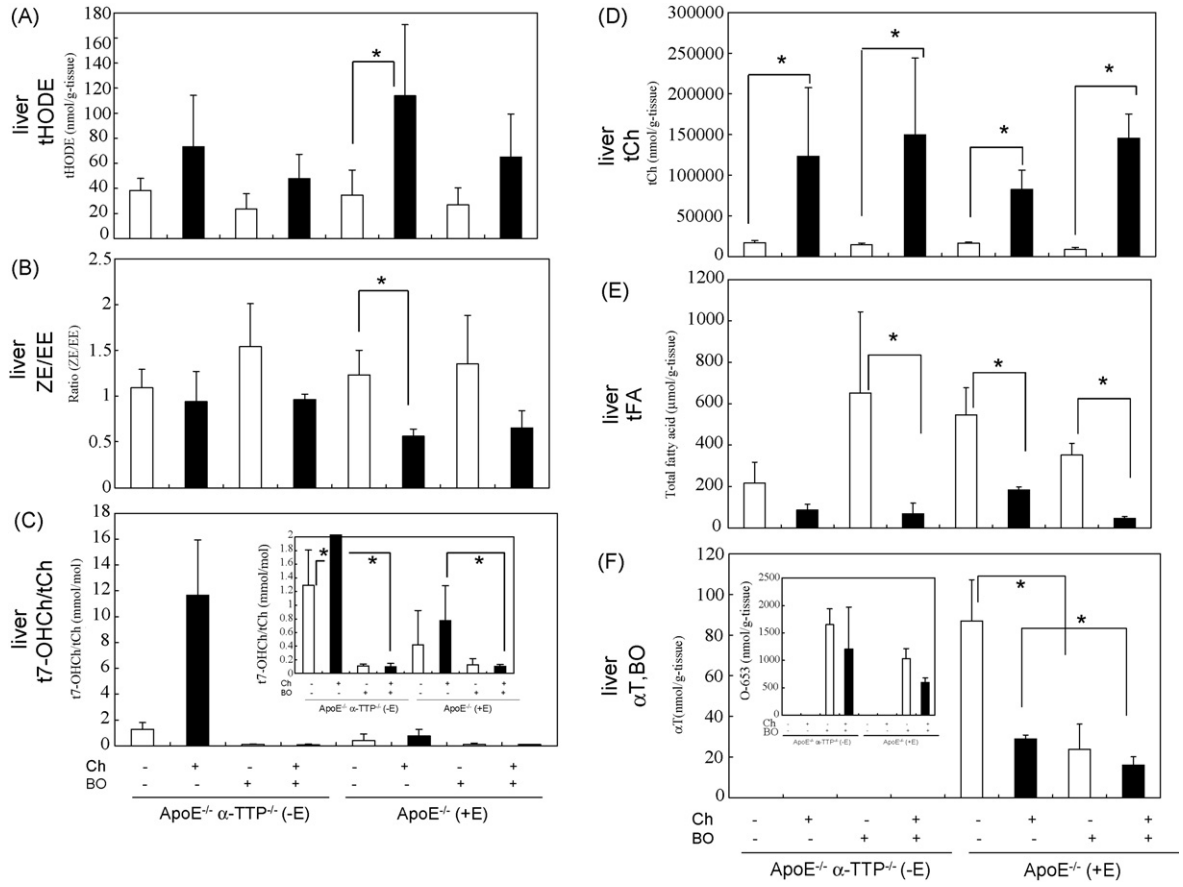

Fig. 3 – Levels of tHODE in liver (A), stereo-isomer ratio of HODE (*ZE/EE*) (B), the ratio of t7-OHCh to tCh (C), tCh (D), total fatty acids (tFA) (E), and αT (F) and BO-653 (F, inseted panel). The inseted panel in (C) is the *y*-axis focused figure of (C). Experimental conditions are similar to those in Fig. 1.

the progression of atherosclerotic lesions [24,28–30]. It has also been reported that cholesteryl ester hydroperoxides and hydroxides were directly detected in the aorta of apoE-knockout mice [31]. Linoleates are major in vivo polyunsaturated fatty acids and their oxidation proceeds by a straight forward mechanism to yield 9- and 13-hydroperoxyoctadecadienotes (HPODE) as the major products [32]. Therefore, the tHODE thus measured may account for much of the in vivo lipid peroxidation. On the other hand, arachidonates, although more reactive toward oxygen radicals than linoleates, are contained in a lesser amount than linoleates, and their oxidation proceeds by complicated mechanisms to yield versatile products, making F2-isoprostanes only minor products. The advantage of tHODE over 8-*iso*-$PGF_{2\alpha}$ as a biomarker is its higher concentration [11]. 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 t8-*iso*-$PGF_{2\alpha}$. Another advantage of HODE over 8-*iso*-$PGF_{2\alpha}$ is that the regional and stereoisomers can be measured separately [11]. The ratio of *cis*-, *trans*-to-*trans*, *trans*-HODE is a measure of the capacity of H-atom donation at the site of oxidation. It is known that the lipid peroxidation proceeds by different mechanisms depending on the oxidants to subsequently yield different isomers. Further, more importantly, the efficacy of the antioxidants depends on the oxidants [33]. A plasma analysis revealed that the level of tHODE decreased due to vitamin E and BO-653, and its stereo-isomer ratio (*ZE/EE*) increased due to vitamin E when the mice were fed with a cholesterol-fortified diet. However, this result was not observed in the tissues. The levels of tHODE in the liver and their isomer ratio clearly increased and decreased, respectively, due to cholesterol; however, neither measure indicated significant antioxidative effects in either vitamin E or BO-653. The reasons for this are not clear at present; however, the excretion of fatty acids from liver can be considered to be one of the reasons. Furthermore, the levels of tHODE in the brain and its isomer ratio remarkably increased and decreased, respectively, due to cholesterol, particularly in the $ApoE^{-/-}\alpha\text{-}TTP^{-/-}$ mice, and vitamin E; however, BO-653 exerted a profound antioxidative effect.

The oxidation products of cholesterol, often called as oxysterols, have received considerable attention because of their regulatory role in lipid metabolism [34] and proatherogenic properties [35]. They may be generated either enzymatically or nonenzymatically and may also originate from dietary sources. The oxysterols at 7-position such as 7β-hydroxy-, 7α- and 7β-hydroperoxy-, and 7-ketocholesterol are formed by nonenzymatic oxidation, while 7α-hydroxycholesterol is generated by both enzymatic and nonenzymatic oxidation [35]. The allylic hydrogens of

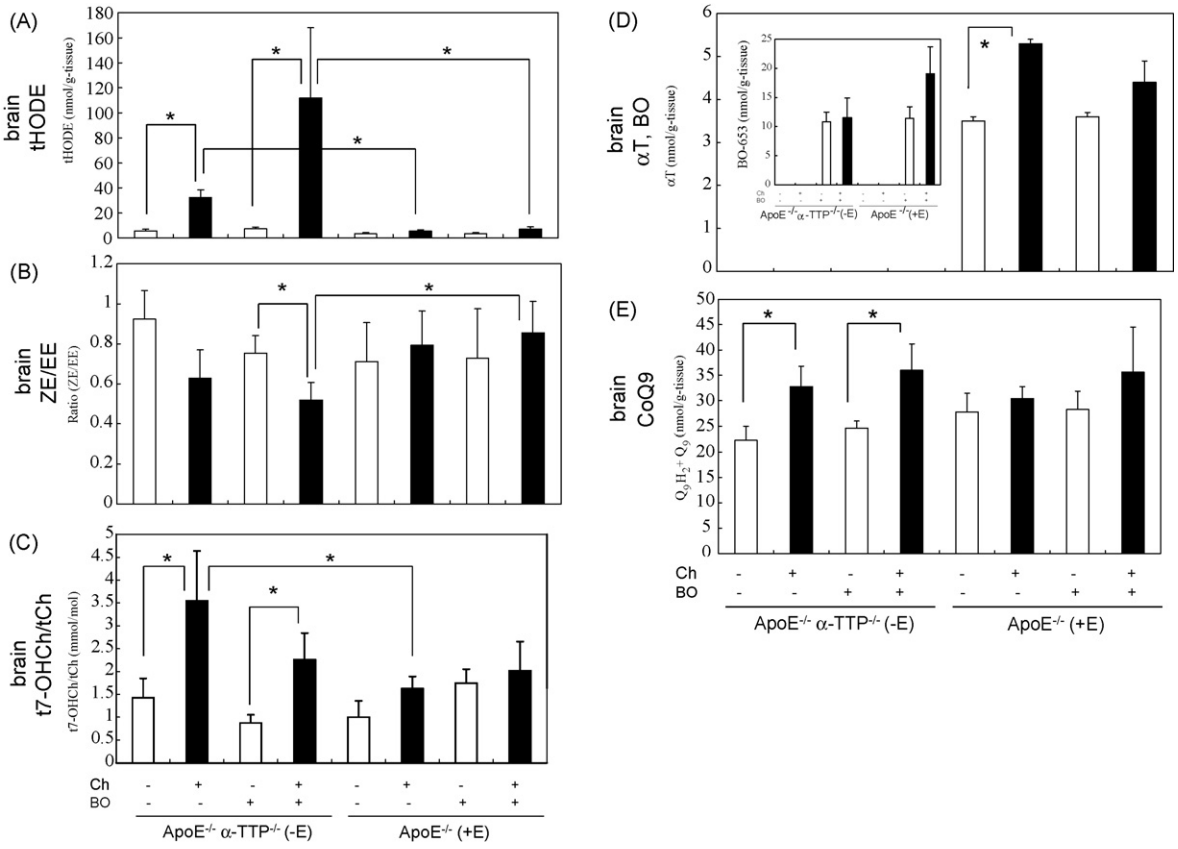

Fig. 4 – Levels of tHODE in brain (A), stereo-isomer ratio of HODE (*ZE/EE*) (B), the ratio of t7-OHCh to tCh (C), αT (D) and BO-653 (D, inseted panel), and coenzyme Q_9 (E). Experimental conditions are similar to those in Fig. 1.

cholesterol are considerably less reactive toward oxygen radicals than the bisallylic hydrogens of polyunsaturated lipids such as linoleates. However, oxysterols have been detected in physiological samples [36], and this study shows that t7-OHCh measured by the present method may serve as another good marker for in vivo oxidative stress. In this study, the levels of t7-OHCh in erythrocytes and the liver levels but not the brain of the ApoE$^{-/-}$α-TTP$^{-/-}$ mice decreased dramatically due to vitamin E and BO-653 when the mice were fed with a cholesterol-fortified diet. However, neither tHODE nor t8-*iso*-$PGF_{2\alpha}$ in the erythrocytes exhibited a significant change even when the mice were fed a cholesterol- or antioxidant-fortified diet. Needless to say, no sole gold standard exists for the evaluation of in vivo lipid peroxidation and antioxidant efficacy. Further, the level of any marker depends on the oxidants and the rates of formation, metabolism, and clearance. This may be the reason why tHODE, its stereo-isomer (ZE/EE), t7-OHCh, and 8-*iso*-$PGF_{2\alpha}$ exhibited different changes depending on the blood and tissues. Thus, it is important that an appropriate marker be selected for the evaluation of oxidative dysfunction in the specific sites. In the present study, a remarkable accumulation of cholesterol and decline in fatty acids were observed in the liver but not in the brain when the mice were fed with a cholesterol-fortified diet. This may result in that the liver dysfunction due to cholesterol is evaluated by t7-OHCh, being more reliable than by tHODE.

Terasawa et al. [9] reported that ApoE$^{-/-}$α-TTP$^{-/-}$ mice caused the increase in the severity of atherosclerotic lesion and its increase was associated with increased levels of isoprostanes. On the contrary, the dissociation between atherosclerosis and lipid oxidation based on the F2-isoprostane levels in aortas was reported by Suarna et al. although they also observed the increase in lesion area of both aortic sinus and abdominal aorta in ApoE$^{-/-}$α-TTP$^{-/-}$ mice [24]. They pointed out that F2-isoprostane was significantly increased in the abdominal aorta where lesion sizes did not differ between ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$mice in the Terasawa's work. Although we have not measured the sizes of atherosclerotic lesions in aorta as reported previously [9,31], we compared inclusively both ApoE$^{-/-}$α-TTP$^{-/-}$ and ApoE$^{-/-}$mice with and without excessive amount of cholesterol which should increase severity of atherosclerosis. In the present study, all data but t8-*iso*-$PGF_{2\alpha}$ showed the increase in oxidized lipids including tHODE and t7-OHCh by cholesterol without antioxidants, suggesting the increase in oxidative stress. Needless to say, because of the different experimental conditions such as types of diets and feeding period, and kinds of biomarkers assessed as oxidative stress, we are not able to compare directly the present results with previous ones. Our biomarkers, tHODE and t7-OHCh were present at the concentrations more than 100-fold higher than F2-isoprostanes in vivo. Further, we have recently reported that the concentrations of tHODE and t7-OHCh in oxidized LDL were much higher than

intact one, which was fractionated from healthy human [37]. Thus, we believe that tHODE and t7-OHCh are indeed good markers for the detection of progression of oxidative stress.

In conclusion, it was demonstrated by biomarkers, tHODE and t7-OHCh, that the added BO-653 in their diets exerted antioxidative effects in vivo under the condition of reduced vitamin E.

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