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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

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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

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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