The Coantioxidative Effects of Carboxyethyl-6-Hydroxycromans and α-Tocopherol

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Summary  α-Tocopherol (α-Toc) is abundant in LDL and thought to prevent the oxidation of LDL together with various water-soluble antioxidants. Recently, it was reported that α-Toc and γ-Toc metabolites, α-carboxyethyl-6-hydroxycromans (CEHC) and γ-CEHC, are water-soluble antioxidants. In this study, we investigated the interaction between α-Toc and CEHC against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals and LDL oxidation. We administered 600 mg of α-Toc to healthy male volunteers to obtain LDL including high levels of α-Toc before antioxidant administration. The α-Toc content of their LDL was increased after consumption at 24 h (18.3 μg/mL) above the level before consumption (6.6 μg/mL). The lag time of LDL at 24 h after α-Toc consumption (α-Toc rich LDL) with α-CEHC (98.5 ± 8.2 min) or γ-CEHC (101.3 ± 9.0 min) was longer than that of only α-Toc-rich LDL (78.1 ± 9.0 min). Furthermore, we examined the interaction of LDL with CEHC and α-Toc in vitro (5–20 μg/mL). The lag times of 5 and 10 μg/mL α-Toc were 65.5 ± 18.9 min and 69.5 ± 15.5 min, and that of 20 μg/mL α-Toc (83.5 ± 20.2 min) was longer than the control value (55.7 ± 14.1 min). The lag time of 20 μg/mL α-Toc with α-CEHC (98.7 ± 25.7 min) or γ-CEHC (100.6 ± 25.3 min) was longer than that of only α-Toc (83.5 ± 20.2 min). These results suggest that CEHC has the potential to delay the oxidation of LDL, while enhancing the antioxidative activity of α-Toc both in vitro and ex vivo.

Key Words  antioxidant, LDL oxidation, α-tocopherol, γ-tocopherol, CEHC

Dietary supplementation with antioxidants has been shown to reduce the number of cardiac events in patients with documented coronary heart diseases (CHD) in prospective studies (1–3). The best known of these trials, the Cambridge heart antioxidant study (CHAOS), found a reduction in fatal and nonfatal myocardial infarction in patients with proven coronary atherosclerosis who were given 400 IU or 800 IU (equivalent to 400 mg or 800 mg) of vitamin E daily (3). The oxidative modification of low-density lipoproteins (LDL) has been linked to increased risks for the development of atherosclerosis, as well as in patients with CHD. Vitamin E, known to be a fat-soluble antioxidant, has eight forms: α-, β-, γ- and δ-tocopherols (Toc) and α-, β-, γ- and δ-tocotrienols. α-Toc and γ-Toc are especially abundant in foods such as nuts and vegetable oils. α-Toc and γ-Toc are absorbed equally well from the small intestine (4, 5). After the uptake into intestinal cells, they are secreted into chylomicrons. Chylomicrons are subsequently catabolized in the circulation by lipoprotein lipase. After the uptake of chylomicron remnants by the liver, α-Toc and γ-Toc are secreted in association with very low density lipoprotein and they circulate in plasma and tissue. However, biodiscrimination by α-Toc transfer protein (α-TTP) in the liver is related to the bioavailability of each Toc. The relative affinity for α-TTP is as follows: α-Toc 100%, β-Toc 38%, γ-Toc 9%, and δ-Toc 2% (6).

Several studies have indicated that the interaction between α-Toc and various water-soluble antioxidants might be important in maintaining the resistance of LDL to oxidation (7–10). The α-Toc metabolite, α-CEHC and γ-CEHC exist in blood and act as water-soluble antioxidants in vitro (11–13). Galli et al. (12) showed that γ-CEHC had a slightly but significantly greater inhibitory effect on macrophage-induced LDL oxidation than α-CEHC. Furthermore, Yoshida and Niki (13) indicated that CEHC scavenged aqueous radicals but inhibited lipid peroxidation within membranes.

In this study, we aimed to determine whether α-Toc and CEHC act as coantioxidants using the LDL oxidation system both in vitro and ex vivo.
MATERIALS AND METHODS

Materials. α-Toc, α-CEHC, and γ-CEHC were donated by the Eisai Co. (Tokyo, Japan). 2,2'-azobisis(4-methoxy-2,4-dimethyl valeronitrile) (AMVN-CH$_3$O) and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All agarose gel reagents were purchased from Helena Laboratories Corp. (TX, USA). All reagents used in this study were of reagent grade.

DPPH radical scavenging activity. DPPH traps the hydrogen of a phenol compound, and the DPPH radical disappears. The radical scavenger activity of α-CEHC, γ-CEHC, and α-Toc was measured spectrophotometrically using the DPPH radical. Various volumes of α-CEHC, γ-CEHC (60 ng/mL), and α-Toc (5, 10, 20 μg/mL) were mixed with 2 mL of 0.1 mM DPPH in ethanol. The solution was shaken and incubated at 37°C for 20 min in the dark. The decrease in absorbance of DPPH was measured at 516 nm using a DU 650 spectrophotometer (Beckman Coulter, Inc., CA, USA). The volume of each sample required to cause a 50% decrease in absorbance at 516 nm relative to the control (ethanol) was then calculated.

Measurement of the LDL oxidative modification. Plasma samples were separated from blood collected in EDTA (1 mg/mL) by centrifugation at 2,000 × g for 15 min at 4°C. The LDL (d=1.019–1.063 g/mL) was isolated from 1.1 mL of plasma by a single-spin density gradient ultracentrifugation (417,000 × g. 40 min, 4°C) (14). The protein content of LDL was then determined using a BCA protein assay (Pierce Laboratories Inc., IL, USA) (15). α-CEHC, γ-CEHC, and α-Toc were dissolved in ethanol. LDL samples were diluted with 0.01 M PBS to give a final concentration of 70 μg/mL LDL protein for the assay of the LDL oxidation lag time. The LDL solution containing α-CEHC, γ-CEHC (60 ng/mL), α-Toc (5, 10, 20 μg/mL) or ethanol (control) was prepared using a BCA protein assay (Pierce Laboratories Inc., IL, USA). The mobile phase was hexane/isopropyl alcohol (98.4/1.6, v/v) delivered at a flow rate of 1.0 mL/min. The detection wavelength was 268 nm.

Statistical analysis. Statistical analyses were performed using the Stat View Version 5.0 software package (SAS Institute Inc., NC, USA). All results were expressed as the means ± SD. Any significant differences among 3 or more groups were evaluated using ANOVA and the post hoc test (Scheffe test).

RESULTS

In vitro assay of the antioxidative activities of CEHC

For α-CEHC and γ-CEHC, it took 306 μL and 247 μL to scavenge 50% of the DPPH radicals, respectively, and

| α-CEHC | γ-CEHC |
|--------|--------|
| 306±36 | 247±29 |

CEHC indicates carboxyethyl-6-hydroxychromans. These results are expressed as the mean±SD of n=8 for the DPPH test. The volume and the amount of CEHC needed to decrease by 50% the level of DPPH radical relative to the control (ethanol).

| CEHCs (ng/mL) | LDL oxidizability |
|---------------|-------------------|
|               | Lag time (min)    |
| Control       | 47.5±16.0         |
| α-CEHC 30     | 55.4±10.4         |
|              | 60.5±10.7         |
|              | 62.6±10.9         |
| 120           | 67.7±12.3         |
| 240           | 74.9±16.8         |
| 480           | 74.9±16.8         |
| γ-CEHC 30     | 57.2±9.5          |
|              | 63.2±12.6         |
| 60            | 65.2±12.6         |
| 120           | 70.1±14.9         |
| 240           | 85.3±20.1         |

CEHC indicates carboxyethyl-6-hydroxychromans. CEHCs (0–480 ng/mL) were added to the LDL solution (70 μg protein/mL). The results are expressed as the mean±SD of n=13 for LDL oxidizability. Different superscripts indicate a significant difference according to the Scheffe post hoc test (p<0.05).
CEHC is likely to have scavenging activity (Table 1). The LDL oxidation lag time of α-CEHC at 60 ng/mL (p < 0.05), 120 ng/mL (p < 0.01), 240 ng/mL (p < 0.001), 480 ng/mL (p < 0.001) was significantly longer than that of the control (Table 2). On the other hand, the LDL oxidation lag time of γ-CEHC at 60 ng/mL (p < 0.01), 120 ng/mL (p < 0.001), 240 ng/mL (p < 0.001), 480 ng/mL (p < 0.001) was also significantly longer than that of the control (Table 2). The LDL charge changed toward the anode side after Cu2+ oxidation (OX-LDL line) although the charges in LDL particles in the presence of α-CEHC and γ-CEHC at a final concentration of 60 ng/mL. Agarose gel electrophoresis was performed at 400 V for 15 min.

The α-Toc content of the LDL solution after the administration of 600 mg significantly increased by 2.7 fold to 18.3 μg/mL at 24 h from the basal value (6.6 μg/mL; p < 0.001) (data not shown).

**In vitro assay in antioxidative activities between α-Toc and CEHC**

As shown in Table 3, the lag time of 20 μg/mL α-Toc was longer than that of the control (p < 0.001). The lag times of 20 μg/mL α-Toc and α-CEHC (p < 0.05) or γ-CEHC (p < 0.05) were longer than that of only 20 μg/mL α-Toc. In addition, the 50% scavenging activity of 5 μg/mL α-Toc with α-CEHC (p < 0.001) or γ-CEHC (p < 0.001) was stronger than that of 5 μg/mL α-Toc. The 50% scavenging activity of 10 μg/mL α-Toc with α-CEHC (p < 0.01) or γ-CEHC (p < 0.01) was stronger than that of 10 μg/mL α-Toc.

**Ex vivo assay in antioxidative activities between α-Toc and CEHC**

The lag time of the LDL at 24 h after the consumption of α-Toc (α-Toc-rich LDL) (p < 0.001) significantly increased in comparison to that of the control (Table 4). The lag times of α-Toc-rich LDL added to 60 ng/mL α-CEHC (p < 0.01) and γ-CEHC (p < 0.01) were significantly longer than that of α-Toc-rich LDL.

**DISCUSSION**

Yoshida and Niki (13) indicated that CEHC scavenged aqueous radicals but inhibited lipid peroxidation within membranes less efficiently than the corresponding α-Toc and γ-Toc. Furthermore, the present study showed that α-Toc and CEHC might act as coantioxidants with the oxidation of LDL.

Since Stahl et al. (11) reported that α-CEHC content was about 60 ng/mL when humans took 500 IU (equivalent to 500 mg) of RRR-α-Toc, we investigated...
the antioxidative activity of 60 ng/mL of α-CEHC and γ-CEHC in vitro. Our results showed that α-CEHC and γ-CEHC had antioxidative activity in vitro (Table 2). Galli et al. (12) reported that α-CEHC and γ-CEHC show similar concentration-dependent inhibition of plasma and LDL lipid oxidation. Our findings were similar to those of Galli et al. (Table 2). These results might thus indicate that not only γ-Toc but γ-CEHC had antioxidative activity and inhibitory effects against LDL oxidation. On the other hand, it has been reported that γ-CEHC acts as a natriuretic hormone (20, 21). Therefore, γ-CEHC is expected to exhibit important pharmacological activities both of the antioxidant and the natriuresis.

It is not yet clear how CEHC circulates inside the body; but Stahl et al. (11) reported that serum levels of both α-CEHC and γ-CEHC are higher following supplementation with α-Toc. However, α-Toc and the water soluble antioxidant CEHC coexist in the blood. Alternatively, several studies have indicated the interaction between α-Toc and various water soluble antioxidants such as vitamin C (7), uric acid (8), sesame lignans (9), and tea catechin (10) to be important in maintaining the resistance of LDL to oxidation. Therefore, we examined the antioxidative interaction between α-Toc and either α-CEHC or γ-CEHC. In this experiment, both α-CEHC and γ-CEHC were added to LDL in vitro at 24 h after 600 mg of α-Toc was consumed. Since we wanted to obtain LDL containing a lot of α-Toc before the administration, we administered 600 mg of α-Toc to 4 healthy male volunteers. The amount of α-Toc in LDL was 5–20 μg/mL at physiological levels. The α-Toc content and the oxidation-lag time in LDL increased the most, namely by about 2.7-fold and 2.0-fold at 24 h, respectively, in comparison to that before the consumption of α-Toc (Table 4). The oxidation-lag time of α-Toc-rich LDL with both CEHCs was significantly enhanced in comparison to that of α-Toc rich LDL (Table 3). Recently, Yoshikawa et al. reported that the plasma γ-CEHC concentration increased but α-Toc concentration decreased during γ-Toc administration (22). Therefore, further studies will be needed to elucidate the coantioxidative effects of CEHC and α-Toc after the variable ratio of α-Toc and γ-Toc administration.

On the other hand, Bowry et al. (23–25) reported that α-Toc caught lipid radicals and itself changed the α-Toc radical when LDL was oxidized. Vitamin C can also prevent the pro-oxidative activity of α-Toc by reducing the α-tocopheroyl radical to α-Toc, thereby acting as a coantioxidant and regenerate α-Toc (7). Yoshida and Niki (13) indicated that CEHC inhibited lipid peroxidation within the membranes less efficiently than the corresponding α-Toc and γ-Toc. However, the present study showed that CEHC might have the potential to delay the oxidation of LDL as a water soluble antioxidant (such as vitamin C, uric acid, sesame lignans, or tea catechin). These results suggest that CEHC may act as an antioxidant in vivo especially in those who take a tocopherol supplement. In addition, it is necessary to further investigate the regenerative effect of CEHC in the future. Therefore, the quality not quantity of a vitamin E supplement may be important to obtain the greatest physiological responses to vitamin E.

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