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In Vitro Antioxidant Activity of 2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxychroman (α-CEHC), a Vitamin E Metabolite

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2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) has been identified as a major water-soluble metabolite of vitamin E, which circulates in the blood and is excreted with the urine. The aim of this study was to assess the antioxidant activity of α-CEHC using several methods with different prooxidant challenges. In the Oxygen Radical Absorbance Capacity assay, a fluorescent protein acts as a marker for oxidative damage induced by peroxyl radicals. In the Trolox Equivalent Antioxidant Capacity (TEAC) assay, a stable free radical, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) is reduced directly by antioxidants. Scavenging properties vs. reactive nitrogen species were studied measuring the effects on tyrosine nitration after reaction with peroxynitrite. Trolox, α-tocopherol, ascorbic acid, and (−)-epicatechin were simultaneously tested in order to compare their antioxidant activities. In all mentioned systems, α-CEHC exhibited antioxidant properties similar to those of Trolox. We conclude that α-CEHC is a molecule with good antioxidant activity, having the advantage over Trolox of being a naturally occurring compound. These properties might be useful for research or industrial purposes.

Keywords: α-CEHC; Vitamin E; Antioxidant; ORAC; TEAC; Peroxynitrite

INTRODUCTION

The term vitamin E comprises the whole family of lipid-soluble tocopherols and tocotrienols. Vitamin E is provided with the diet and RRR-α- and γ-tocopherol are the major forms in human plasma and tissues.[1] As an oxygen-based radical scavenger and a powerful chain-breaking antioxidant, vitamin E provides protection to lipid compartments, e.g. membranes and lipoproteins. High doses of oral vitamin E are suggested to prevent oxidative stress-related diseases. It has been shown that a high intake is negatively correlated with the incidence of cardiovascular death in patients suffering from coronary atherosclerosis[2,3] and that the use of vitamin E supplements among middle-aged women is associated with a lower risk for coronary heart disease.[4] It should be noted, however, that other recent studies showed that a daily moderate supplementation of vitamin E for 5–8 years had no effect on total mortality in male smokers—being instead associated with an increased risk of death from hemorragic stroke[5]—neither decreased the risk for large abdominal aortic aneurysm.[6] The major mechanism underlying the positive effects is likely the prevention of LDL oxidation. Other non-antioxidant cellular functions of α-tocopherol (α-TOH) might also play a role.[7–11]

The metabolism of α-TOH was thought to proceed via opening of the chroman ring after radical attack, yielding α-tocopherylquinone. The latter would be further metabolized to the water-soluble α-tocopheronic acid and yield α-tocopheronolactone.[12] In addition to these so-called Simon metabolites, other degradation products have been identified.

2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) was recently discovered as a metabolite of α-TOH detectable in human blood[13] and urine after tocopherol[14] and tocotrienol[15]

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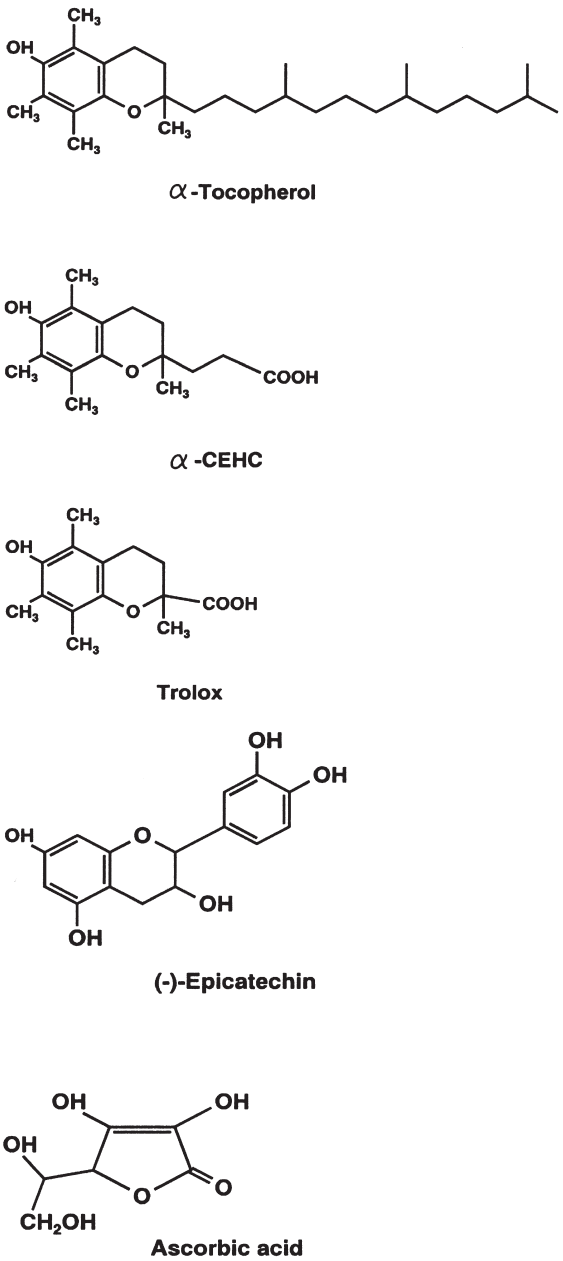

FIGURE 1 Chemical structures of the antioxidants tested and α-CEHC.

supplementation. Based on its structure, it was suggested that several oxidation reactions occur at the side chain of α-TOH, leaving the chroman ring, the redox-active moiety, intact. Detection and characterization of the α-carboxymethylbutyl-hydroxychroman (CMBHC), α-CEHC's intermediate precursor, in human urine[16,17] and in HepG2 cells[18] confirm this hypothesis. ω-Oxidation of α-TOH side chain has been attributed to the microsomal cytochrome P450-CYP3A monooxygenase using selective CYP3A inducers[16] and inhibitors[19] in model systems. A large amount of α-CEHC is excreted with urine as a conjugate to sulfate or to glucuronic acid; in plasma, free and conjugated α-CEHC are found.[13]

Baseline α-CEHC levels in human serum of healthy subjects have only been reported twice so far and were 7.1 ± 3.0 nmol/l using HPLC[13] and 12.6 ± 7.5 nmol/l applying GC/MS;[20] this is about 2500–6000 fold lower than the physiological levels for α-TOH. α-CEHC concentrations may increase up to 200 nmol/l upon RRR-α-TOH supplementation.[13]

In the present work, the antioxidant properties of α-CEHC were investigated.

MATERIALS AND METHODS

Materials

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*R*-Trolox), (−)-epicatechin, *R*-phycoerythrin from *Porphyra tenera*, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, L-tyrosine, 3-nitrotyrosine, 3-hydroxy-4-nitrobenzoic acid, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH) were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). L-Ascorbic acid was purchased from Merck (Darmstadt, Germany). 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was purchased from Polysciences (Warrington, USA). Racemic α-CEHC was kindly provided by BASF (Ludwigshafen, Germany) and RRR-α-tocopherol was obtained from Cognis (Düsseldorf, Germany). Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor, and H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. The final peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1700\ M^{-1}\ cm^{-1}$).

α-CEHC Antioxidant Activity Measured in the Oxygen Radical Absorption Capacity (ORAC) Assay

For comparison, RRR-α-TOH, *R*-Trolox, (−)-epicatechin and vitamin C (Fig. 1) were tested together with α-CEHC.

A highly fluorescent protein, *R*-phycoerythrin, was used as described by Cao *et al.*[21] to measure the ORAC. *R*-phycoerythrin loses rapidly its fluorescence when exposed to sources of free radicals. Thus, in the presence of radical scavengers loss of fluorescence is lowered. AAPH was used in the present study as a radical generator. Stock solutions of Trolox and ascorbic acid were prepared in 75 mM phosphate buffer (pH 7.0). α-TOH and α-CEHC were dissolved in ethanol and (−)-epicatechin in acetone prior to dilution with buffer. The final amount of

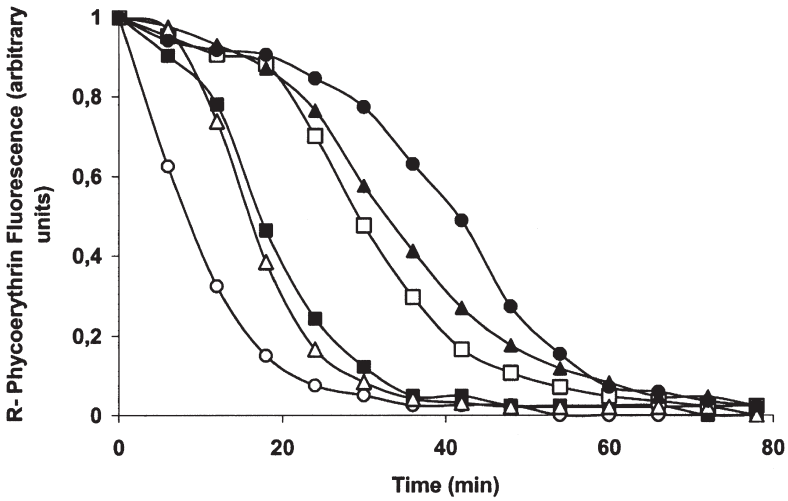

FIGURE 2 Decay of fluorescence of *R*-phycoerythrin upon addition of AAPH and extension of the lag phase with antioxidants. Different concentrations of test substance were tested in 3.4 mg/l *R*-phycoerythrin in 75 mM phosphate buffer (pH 7.0). The mixture was incubated for 10 min at 37°C and then its fluorescence measured. This value was set at fluorescence value equal to 1. AAPH (4 mM in phosphate buffer) was then added. Fluorescence was recorded at 37°C every 6 min until the fluorescence was less than 5% of the first reading. Control (only AAPH and solvents) (open circles); Trolox 1 μM (closed squares); ascorbic acid 2 μM (open triangles); Trolox 2 μM (open squares); α-CEHC 2 μM (closed triangles); (−)-epicatechin 2 μM (closed circles).

ethanol or acetone in the sample was below 0.1%, except for α-TOH (0.3%). Test substance (0.1 ml) was mixed in a cuvette with 0.1 ml 3.4 mg/l *R*-phycoerythrin (final concentration) and 1.75 ml 75 mM phosphate buffer (pH 7.0). The mixture was equilibrated for 10 min at 37°C and then its fluorescence measured at 540 nm (excitation) and 565 nm (emission) wavelengths in a Perkin–Elmer LS-5 Luminescence Photometer/Fluorimeter. This value was used as baseline and set to 100% fluorescence. After addition of 50 μl of 4 mM AAPH in phosphate buffer, the mixture was vortexed. Fluorescence was first recorded exactly 15 s after AAPH addition and then every 6 min until the fluorescence was less than 5% of the starting value. The rack containing the cuvettes was kept at 37°C in a water bath. Appropriate controls with ethanol and acetone and an antioxidant standard containing 0.1 ml of 1 μM Trolox were run in each assay. Each compound was tested in triplicate at four concentrations. Final results (ORAC values) were calculated using the formula proposed by Cao *et al.*[21]

$$\text{ORAC value } (\mu\text{M}) = 20\,k\frac{(S_{\text{sample}} - S_{\text{control}})}{(S_{\text{Trolox}} - S_{\text{control}})}$$

where k is the sample dilution factor and S is the area under the fluorescence decay curve of the sample, Trolox or control. ORAC values, thus, express the μM concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 μM solution of the test substance. To assess the inter-day variation of the method, the whole set of experiments was repeated on three different days.

α-CEHC Antioxidant Activity Measured in the Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The antioxidant capacity of α-CEHC and other antioxidants was also measured as described by Re *et al.*[22] This assay is based on the ability of different substances to scavenge the $ABTS^{\bullet+}$ radical cation in comparison to a standard (Trolox). The radical cation was prepared by adding 5 ml of a 4.9 mM potassium persulfate solution to 5 ml of a 14 mM ABTS solution. The mixture shows a maximal blue-green color after 6 h revealing ABTS oxidation. Fresh $ABTS^{\bullet+}$ solution was prepared each day. The solution was diluted in ethanol to yield an absorbance of 0.76 (±0.02) at 734 nm and preincubated at 30°C for equilibration prior to use. All compounds were dissolved in ethanol to a final concentration of 3 mM for stock solutions. The final reaction mixture contained 10 μl of standard or test compound in 1 ml $ABTS^{\bullet+}$ solution. The samples were vortexed for 10 s, and 1, 3 and 6 min after addition the absorbance at 734 nm was measured using a Beckman DU 530 spectrophotometer. Controls with ethanol were run in each series. Each compound was tested in triplicate at four concentrations. To assess inter-day variation the procedure was repeated at three different days. The slope obtained from Fig. 3 was divided by the slope of the reference compound Trolox, which shows a linear relationship. This is defined as the antioxidant activity expressed as TEAC values. TEAC values express the μmols of Trolox having the antioxidant capacity corresponding to 1.0 μmol of the test substance.

TABLE I Antioxidant capacity measured as ORAC units [calculated according to the formula proposed by Cao *et al.* (see text); values express the μM concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 μM solution of the test substance], as TEAC units [values express the μM concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 μM solution of the test substance], and as half-maximal inhibitory concentrations (IC_{50}) of peroxynitrite-mediated nitration of tyrosine. Results are expressed as mean values ± SD. *t- Student* test was applied for determining significant differences with Trolox; (*) $p \leq 0.05$; (**) $p \leq 0.001$

Test Compound	ORAC (μmol Trolox Equivalents)	TEAC (μmol Trolox Equivalents)	IC_{50} (μmol/L)
α-CEHC	1.42 ± 0.35	0.96 ± 0.02	46
Trolox	1.01 ± 0.34	1.00 ± 0.00	52
(-)-Epicatechin	2.64 ± 0.25*	1.45 ± 0.03**	12
Ascorbic Acid	0.58 ± 0.39*	1.26 ± 0.07*	-
α-Tocopherol	-	0.87 ± 0.01	-

α-CEHC Antioxidant Activity Measured as Inhibition of Peroxynitrite-mediated Nitration of Tyrosine

Protection against peroxynitrite-mediated nitration of tyrosine was performed as described by Pannala *et al.*[23] with minor modifications. A 200 μM tyrosine stock solution was prepared in 100 mM phosphate buffer/0.1 mM EDTA (pH 7.3). The concentration was confirmed spectrophotometrically at 274 nm ($\varepsilon = 1405\,M^{-1}\,cm^{-1}$). A 350 μM peroxynitrite solution was added as bolus to 100 μM tyrosine under vortexing. The protective effects of preincubating tyrosine for 10 min with α-CEHC, Trolox and (−)-epicatechin (0–40 μM) were examined. Fifty microliter of each sample containing 100 μM 3-hydroxy-4-nitrobenzoic acid as an internal standard was injected into a C-18 reverse-phase column (150 mm × 4.6 mm; Merck, Darmstadt, Germany) using a 655 A-40 autosampler (Merck–Hitachi). For separation a 50 mM potassium phosphate buffer (pH 7)/acetonitrile was used as mobile phase applying a step gradient (Merck–Hitachi L-7100 HPLC). The following gradient system was used (min/% acetonitrile): 0/5, 5/50, 13/5 and 26/5 at a flow rate of 1.0 ml/min. 3-Nitrotyrosine was detected with a Merck–Hitachi UV/Vis. detector at 430 nm. Ratios of peak areas of 3-nitrotyrosine standard vs. internal standard were used for calibration and quantification.

RESULTS

α-CEHC Antioxidant Activity Measured in the Oxygen Radical Absorption Capacity (ORAC) Assay

In Fig. 2, the decay in fluorescence of *R*-phycoerythrin when exposed to AAPH in the absence and presence of scavengers is shown. Each antioxidant induced a delay in the loss of fluorescence, a so-called lag phase, proportional to the initial scavenger concentration in the reaction mixture. With the antioxidants and concentrations applied, a decrease of 95% in the fluorescence readouts was achieved in a maximal time of 70 min, in agreement with previous results.[21] As shown in Table I, α-CEHC exhibited a slightly higher peroxyl quenching activity than Trolox, but the

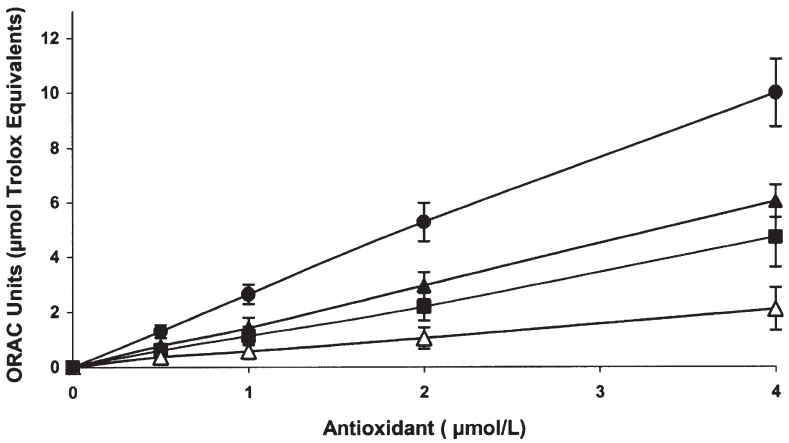

FIGURE 3 Antioxidant-mediated inhibition of oxidative damage to *R*-phycoerythrin (dose–response curves). Ascorbic acid (open triangles); Trolox (closed squares); α-CEHC (closed triangles); (−)-epicatechin (closed circles); $n = 9$. Results are mean values ± SD.

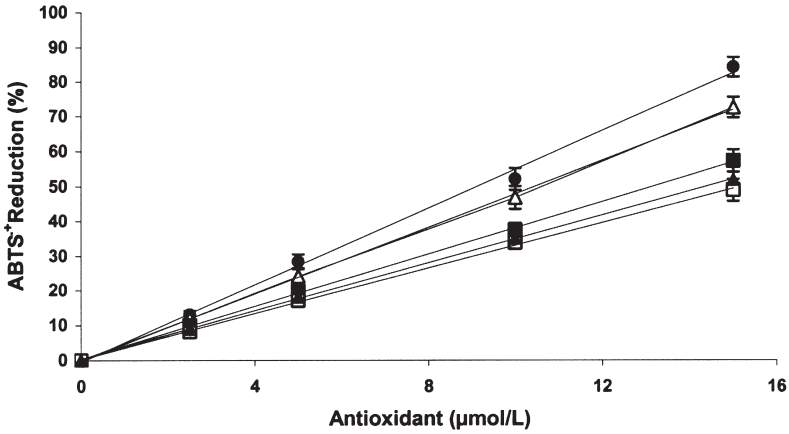

FIGURE 4 $ABTS^{+}$ reduction by antioxidants (dose–response curves). Ascorbic acid (open triangles); Trolox (closed squares); α-CEHC (closed triangles); (−)-epicatechin (closed circles); α-TOH (open squares). The radical cation solution was diluted in ethanol to obtain an absorbance of 0.76 (±0.02) at 734 nm. The final reaction mixture contained 10 μl standard or test compound (0–15 μM) in 1 ml $ABTS^{+}$ solution. Dose–response curves were plotted expressing absorbance at 6 min as a percentage of the absorbance of the original radical cation. $n = 9$; results are mean values ± SD.

difference was not statistically significant. (−)-Epicatechin showed the best protective effects. (−)-Epicatechin is a flavanol containing three hydroxyl substituents more than the flavonoid precursor chalcone, which makes its antioxidant capacity higher than that of other common nutrient antioxidants, such as vitamin C or E. Vitamin C was the least active compound. In the case of α-TOH, we assumed that the stability of the protein had been affected by the presence of higher amounts of the organic cosolvent. A linear correlation between ORAC values and concentrations (0–4 μM) for all the substances tested was found ($r^2 = 0.9996–0.9972$) as shown in Fig. 3. Coefficients of variation (SD of the mean expressed in percentage) were calculated for intra- and inter-day precision and ranged from 1.95 to 9.62% and from 4.29 to 16.67%, respectively.

α-CEHC Antioxidant Activity Measured in the Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The antioxidant response of α-CEHC resembled the antioxidant action exerted by Trolox, without significant differences between their scores (Table I). The most pronounced $ABTS^{\bullet+}$ reduction effect was seen after treatment with (−)-epicatechin, as occurred in the ORAC assay. Ascorbic acid was a slightly better reducing agent in comparison to Trolox whereas α-TOH showed a slightly less activity. These values are in agreement with already published data.[22] For all the compounds tested, reaction with the radical was complete after 1 min. A good correlation was found for all the tested antioxidants between the concentrations applied

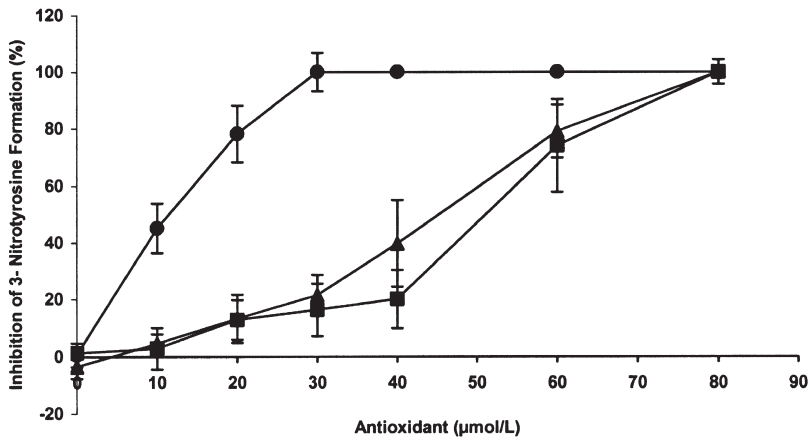

FIGURE 5 Inhibition of peroxynitrite-mediated formation of 3-nitrotyrosine. 350 μM peroxynitrite was added as bolus addition to 100 μM tyrosine. The protective effects of α-CEHC, Trolox and (−)-epicatechin (0–40 μM) were examined. Ratios of peak area of generated 3-nitrotyrosine vs. internal standard were used for plotting dose–response curves. Trolox (closed squares); α-CEHC (closed triangles); (−)-epicatechin (closed circles); $n = 3$. Results are expressed as mean values ± SD.

and percentage of $ABTS^{\bullet+}$ reduction to the neutral ABTS, as depicted in Fig. 4. The TEAC value of each test compound was calculated by dividing the slope of its concentration–response curve by the slope for the concentration–response curve corresponding to Trolox. Within-day variation coefficients for TEAC values ranged from 5.20 to 11.79% and inter-day variation ranged between 0.41 and 5.66%.

α-CEHC Antioxidant Activity Measured as Inhibition of Peroxynitrite-mediated Nitration of Tyrosine

As shown in Fig. 5, α-CEHC and Trolox showed also similar reactivities against reactive nitrogen species. (−)-Epicatechin was the most efficient inhibitor of tyrosine nitration, which is in agreement with previous results.[24] In the absence of test compound, but in the presence of vehicle (0.4% methanol), the concentration of generated nitrotyrosine was $21.6 \pm 1.9\,\mu mol/l$; this value was considered 0% inhibition of nitration. Half-maximal inhibitory concentrations for (−)-epicatechin, α-CEHC and Trolox were 12, 46 and 52 μmol antioxidant/l, respectively (Table I). (−)-Epicatechin is unsubstituted in the 2′ and 5′ positions of its B ring, which enables the formation of stable nitro adducts, thereby competing with tyrosine in peroxynitrite scavenging. Trolox and α-CEHC, as structural analogues of α-TOH, are completely substituted and a pure antioxidant action is thought to be responsible for their protective effect. Recent data, however, suggest that the reaction is likely to occur via a two-electron oxidation process yielding stable quinones that would not able to become *re*-reduced to chromanol-based structures by ascorbic acid.[25]

DISCUSSION

The α-TOH metabolite, α-CEHC yields a protective antioxidant action *in vitro* when stressed with different free radical sources that resembles the properties of Trolox very closely.

In vivo, the relative efficacy of α-CEHC will depend on both its bioavailability and reactivity. Nothing is known about the bioavailability of α-CEHC itself, but of its parent compounds, α-TOH and α-tocotrienol. Under sustained supplementation with 500 IU (335 mg) RRR-α-TOH/day over a period of 7 weeks, plasma α-CEHC levels were reported to reach 200 nmol/l.[13] At such low levels, Trolox exhibits antioxidant activity in the ORAC and TEAC assays (data not shown). Based on the similarities in the antioxidant activity of Trolox and α-CEHC, it may be suggested that α-CEHC might contribute, at least to some extent, to the plasma antioxidative status. Complementary information about percentage of α-CEHC conjugates in human plasma after vitamin E liver metabolism as well as their own antioxidant activity would also be necessary to clarify this issue.

An increasing number of synthetic water-soluble vitamin E analogues, including Trolox,[26] are assessed in different laboratories for potential advantages over tocopherols or tocotrienols in the acute treatment of diseases related to oxidative impairment, such as ischemia- reperfusion and coronary heart disease in general,[27] vascular complications in diabetes,[28] or hepatotoxicity triggered by radicals.[29] Some are rendering very promising results. Considering the structural similarity of α-CEHC with these analogues, we think that it should be tested in this set of experiments. Due to its well-proved antioxidant action *in vitro*, Trolox has been gradually included as a control in several standard methodologies to assess the total antioxidant capacities of a large variety of compounds and mixtures,[21,22,30–32] as well as in experiments aimed to find protective agents against lipid oxidation, either using liposome[33] or lipoprotein[34] suspensions. Since α-CEHC is more liposoluble than Trolox, it will likely exhibit a better performance in biphasic systems, including cell membranes. Besides, α-CEHC has the advantage over Trolox of being a natural-occurring compound.

In addition to vitamin E analogues, also (−)-epicatechin and vitamin C have been investigated in the present study, as positive controls. Our data regarding (−)-epicatechin are consistent with the literature and confirm the compound as an excellent antioxidant,[35,36] although most of present research in the prevention of cardiovascular disease with green tea polyphenols is focused on their non-antioxidant actions.[37–39]

In conclusion, we suggest that the properties of α-CEHC should be taken into account and be used to explore possible pharmacological, industrial or research applications.

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