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A comparison between the antioxidant and peroxynitrite-scavenging functions of the vitamin E metabolites α- and γ-carboxyethyl-6-hydroxychromans

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Abstract: Carboxyethyl-6-hydroxychromans (CEHC) are vitamin E metabolites with proposed *in vitro* antioxidant function. In this study we compared the antioxidant potency of the two main CEHC metabolites found in biological fluids (i.e., α-CEHC and γ-CEHC) using two different experimental models of lipid oxidation: 1) plasma diluted 1/50 vol/vol in phosphate buffered saline (PBS) exposed to 50 μM Cu^{2+} ions, and 2) LDL (100 μg of proteins) exposed to different pro-oxidants as 2.5 μM Cu^{2+}, 1 mM of the water soluble peroxyl radical generator 2,2'-Azobis(2-amidinopropane) hydrochloride (AAPH) and human macrophages (4×10^5 cells). Moreover, the two CEHC homologues were assessed for the inhibitory effect on the peroxynitrite ($ONOO^-$)-induced nitration of tyrosine (Tyr).

The results showed that in the concentration range 0.015–5 μM the CEHC metabolites and the hydrosoluble analogue Trolox exert similar concentration-dependent inhibition of the Cu^{2+}-induced lipid oxidation of plasma. After *in vitro* exposure to *tert*-butyl hydroperoxide/Fe^{2+}, CEHC formed chromanoxyl radicals with electron spin resonance spectra matching exactly those of their parent tocopherols. The LDL oxidation induced by AAPH or Cu^{2+} was significantly and similarly inhibited by 1 μM of both the CEHC homologues and Trolox. γ-CEHC showed a slight but significantly higher inhibition of the macrophage-induced low-density lipoprotein (LDL) oxidation than α-CEHC. Both the CEHC homologues inhibit Tyr nitration induced by $ONOO^-$. However, γ-CEHC produced a slightly greater inhibitory effect than α-CEHC through the formation of the nitrated congener 5-nitro-γ-CEHC. In all the systems under investigation, low nanomolar concentrations of CEHC (i.e., the concentration range in the blood of subjects with normal dietary intake of vitamin E) produced feeble antioxidant effects.

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In conclusion, γ-CEHC and α-CEHC show similar concentration-dependent inhibition of plasma and LDL lipid oxidation. γ-CEHC has a fairly higher potency than α-CEHC as $ONOO^-$ scavenger through the formation of 5-nitro-γ-CEHC. CEHC metabolites show the same *in vitro* antioxidant chemistry of their parent tocopherols, but the characteristic hydrophilicity of these metabolites could result in different biopotency and roles. Further studies are needed to clarify whether CEHC could contribute to the antioxidant network in biological fluids and tissues.

Key words: Vitamin E, tocopherol, vitamin E metabolites, carboxyethyl-hydroxychromans, CEHC, antioxidants, chromanoxyl radical, peroxynitrite, 3-nitrothyrosine, lipid oxidation, LDL

Introduction

A still growing body of evidence proves that one of the most important biological roles of tocopherols and tocotrienols (vitamin E) is that of preventing lipid peroxidation in low-density lipoprotein (LDL) and cell membranes through its chain-breaking antioxidant activity [1, 2]. Together with other biological properties [reviewed in refs. 3–5], the antioxidant function of these natural compounds put forward a role in the prevention of some major chronic degenerative states in which inflammation – and thus oxidant stress – can play a pathogenic role such as the atherosclerotic cardiovascular disease and neuroinflammatory syndromes.

Due to tissue concentrations and biochemical properties, α- and γ-tocopherol (α-T and γ-T) are considered the two most biologically relevant forms in mammals, the biological activity of which appears to be different and complementary. In fact, α-T was described as a more efficient antioxidant [6], while γ-T could be a specific scavenger of reactive congeners of the gaseous free radical nitric oxide (NOx) that can be produced during either the normal cell metabolism or inflammatory events [7, 8].

The 2,5,7,8-tetramethyl- and 2,7,8-trimethyl-2(2'-carboxyethyl)-6-hydroxychromans (α-CEHC and γ-CEHC, respectively) are preferential metabolic products of the tocopherols and tocotrienols found in biological fluids [9–11]. These form in hepatic cells by the shortening of the pythyl chain by cytochrome P450-mediated ω-oxidation and subsequent β-oxidation, without modification of the chroman ring [12, 13].

Recent investigation suggested that these metabolites show biological functions resembling those of their parent vitamers. In one[14], γ-CEHC was described to protect against the metal-induced nephrotoxicity in the rat model through an antioxidant mechanism and its potency has been described to be higher than γ-T and α-T. Betancor-Fernandez *et al* [15] showed that α-CEHC functions as a peroxyl radical scavenger and inhibits the peroxynitrite-induced nitration of the amino acid tyrosine (Tyr). In this study the potency of α-CEHC was observed to be comparable to that of the water-soluble vitamin E analogue Trolox.

However, these studies did not investigate the antioxidant function of CEHC metabolites in the concentration range in which they have been found in biological fluids. Typical CEHC concentrations in the blood and urine are usually in the low nanomolar and low micromolar range, respectively [11]. This makes it difficult to imagine a real contribution of CEHC to the antioxidant defense in the blood. In vitamin E-supplemented individuals these concentrations increase substantially and this could be particularly true in the case of γ-CEHC as γ-T, contrarily to α-T, is largely processed through the catabolic pathway [9, 16]. In fact, peak levels of plasma γ-CEHC reach low micromolar levels even after taking relatively small amounts (100 mg) of γ-T acetate [11, 16] while subjects supplemented with larger amounts (300 mg) of RRR-α-T showed peak concentrations of plasma α-CEHC more than twofold lower [17, 18]. These findings suggest that specific dietary regimens or vitamin E supplements can lead to a steady sustained production of CEHC (particularly as γ-CEHC) that in turn might justify a role for these metabolites as components of the antioxidant network in the blood.

Moreover, γ-CEHC, originally identified as endogenous natriuretic factor [19], has been described to be more potent than γ-T as inhibitor of the cycloxygenase-2 (COX-2) activity [20], a well-known pro-oxidant enzyme expressed in macrophages and epithelial cells, while α-T was not an effective inhibitor. Although the underlying mechanism of this effect remains unknown, the findings in this study further suggest close functional similarity between the vitamin precursors and CEHC metabolites. In the case of gamma vitamers and CEHC metabolite, it could be particularly important to verify functional homology as regards their activity as NOx scavengers that could be important to protect biomolecule targets as protein Tyr and lipid unsaturations [7, 8, 21].

Altogether this evidence suggests that α-CEHC and γ-

CEHC, as main vitamin E metabolites found in biological fluids, may show different biological properties that could contribute to vitamin E functions, the antioxidant one included. However, as observed for their vitamer precursors, differences in the structure and *in vivo* concentrations of these two CEHC homologues could strikingly influence their role as antioxidants.

Therefore, we compared *in vitro* the capability of submicromolar and low micromolar concentrations of α-CEHC and γ-CEHC to inhibit the lipid oxidation of plasma and LDL lipids induced by different agents as copper ions (Cu^{2+}), peroxyl radicals and macrophages, and the Tyr nitration reaction induced by peroxynitrite ($ONOO^-$). The antioxidant activity of these vitamin E metabolites was also compared with that of the water-soluble analogue Trolox and in some experiments with the parent vitamers α-T and γ-T.

Experimental Procedures

Chemicals

All compounds were of the highest quality available and unless otherwise specified were purchased from Sigma Chemical Co., Fluka or Carlo Erba (Milan, Italy).

α- and γ-CEHC (purity of 98.0% and 99.6% by HPLC analysis) and tocopherols were a kind gift of Eisai Co., Japan. Stock solutions (10 mM) of tocopherols and CEHC were made in absolute ethanol and kept at –20°C. Stock solutions were diluted in phosphate-buffered saline (PBS)-ethanol 1:1 vol/vol (working solutions) at a final concentration of 100 μM.

2,2'-Azobis(2-amidinopropane) hydrochloride (AAPH) was from Plysciences (Warrington, PA). Stock solutions of this compound were prepared fresh in 10 mM phosphate buffer pH 7.2. AAPH is a hydrophilic compound that thermally decomposes to generate peroxyl radicals ($ROO^{\bullet}$) at a constant rate.

Peroxynitrite was prepared fresh before each experiment and used accordingly with [22]. Its concentration was checked at 302 nm ($\varepsilon = 1670\ M^{-1} \times cm^{-1}$).

The 5'-nitro derivatives of γ-T and γ-CEHC (5N-γ-T and 5N-γ-CEHC, respectively) were prepared by the reaction of 1 mg of the two compounds with an equimolar solution of $NaNO_2$ in 4% (vol/vol) aqueous solution of glacial acetic acid [8]. Alternatively, $ONOO^-$ was used employing the procedure described by Hoglen *et al* [23] with some minor changes. Briefly, a bolus of $ONOO^-$ in 0.1 N NaOH (final concentration in the reaction mixture 400 μM) was rapidly mixed with γ-T and γ-CEHC (final concentration range 0.1–100 μM) suspended in 100 mM phosphate buffer (pH 7.4). Nitrated forms were obtained as major reaction products and were purified from unreacted substrates and other products by high-performance liquid chromatography (HPLC) (see below). Their purity and concentrations were checked by spectroscopic and HPLC analysis [23].

For the preparation of 3'-nitro-Tyr (3N-Tyr), a bolus (25 μL) of $ONOO^-$ in 0.1 N NaOH was rapidly mixed with a suspension of Tyr in PBS. The reaction was carried out at room temperature (22°C) in a final volume of 1 mL and with a final molar ratio $ONOO^-$/Tyr of 4/1. The product showed a characteristic spectroscopic profile with absorbance maximum at 287 and 430 nm and purity was > 98% as tested by HPLC analysis.

Assay of the Cu^{2+}-induced oxidation of plasma lipids

The antioxidant activity of tocopherols and CEHC metabolites was preliminarily assessed as the ability to inhibit the Cu^{2+}-induced lipid oxidation in unfractionated plasma according to the assay procedure described by Schnitzer *et al* [24], with some changes. Briefly, in a 1 mL quartz cuvette, 20 μL of citrate-containing plasma were diluted in PBS containing tocopherols and/or their metabolites suspended to the desired final concentrations. The mixtures were allowed to equilibrate at room temperature for 5 minutes and then $CuCl_2$ (final concentration 50 μM in PBS) and PBS up to 1 mL of final volume were added to start the reaction. Ethanol in the reaction mixture was < 0.1% of the final volume. The rate of lipid oxidation was measured at a temperature of 37°C as conjugate dienes (CD) formation in the time unit by continuous spectroscopic analysis at 245 nm. Appropriate blanks containing all the components except than LDL, $CuCl_2$, or antioxidants were evaluated.

The data of absorbance were plotted using the software Microcal Origin 6.0 and the sigmoidal curves produced by the oxidation reactions were analyzed according with [25] to calculate: 1) *lag phase,* as the time (minutes) preceding the oxidation reaction, 2) *propagation rate* (in Abs units/minute) that is the slope of the curve in the tract with maximal rate of increase of the optical density (OD) [$(\Delta OD/t)_{max}$], and 3) the *maximal absorbance* (in Abs units) or OD_{max}.

Preliminary experiments showed that in samples without supplemental antioxidants the oxidation reactions reached their V_{max} (0.32–0.37 OD_{245} units) within 150 minutes from copper addition. The antioxidants added up to a concentration of 5 μM for CEHC and Trolox, and 30 μM for tocopherols that (?) mainly influenced the lag phase and $(\Delta OD/t)_{max}$ values. The extent of inhibition of the oxidation reaction by the test compounds as either increase of the lag phase or decrease of the $(\Delta OD/t)_{max}$ and OD_{max}-

at 150 minutes were comparable as magnitude and correlated with the concentration of the antioxidant tested. Therefore, in routine experiments we arbitrarily decided to monitor the reactions for 150 minutes and only lag-phase and propagation rate values were calculated and used to compare the antioxidant potency of test compounds.

LDL oxidation experiments

LDL was prepared by density gradient ultracentrifugation. 20 ml of venous blood was obtained from two different normolipidemic healthy volunteers by venipuncture of the antecubital vein of the arm using vacutainer tubes containing citrate as anticoagulant. After centrifugation, plasma was collected and processed immediately as described in [24, 26]. Immediately before the incubation with the different pro-oxidant substrates, the LDL suspension was passed through a PD-10 column equilibrated with Chelex 100-treated PBS to remove ethylenediaminetetraacetic acid (EDTA) and sterilized by filtration through 0.45-μm filters. The final protein concentration in LDL preparations was ≈0.55 mg/mL.

Human macrophages (MØ) were prepared from the mononuclear leukocyte layer obtained by density gradient fractionation using the Histopaque-1077 separation media according to the manufacturer's procedure (Sigma Diagnostics, Milan, Italy). Fifty mL of heparinized blood, kindly provided by the local blood bank, were used in each experiment. Purified monocytes (≥ 85% pure as judged by non-specific esterase staining) were adhered in 22-mm cell culture wells (Falcon) at 2.5×10^6 cells per well and cultured in RPMI 1640 containing 10% (v/v) human serum, 20 mM glutamine, 100 units/mL penicillin G, and 100 μg/mL streptomycin for a time sufficient to let them differentiate to MØ. The cells were cultured in a humidified incubator at 37°C in 5% CO_2 in air. Cell viability was assessed by the trypan blue exclusion test. Prior to experiments, cells were washed twice with warm PBS.

LDL oxidation by MØ (4×10^5 cells/well) was performed by the incubation with 100 μg of LDL proteins resuspended in the culture media without phenol red or PBS-glucose in the presence of the antioxidants in PBS or PBS alone (blank). After gentle mixing, the plates were incubated at 37°C in 5% CO_2/air for 3 hrs under gentle shaking. At different time points, LDL was recovered and resuspended to a volume suitable to perform the spectrophotometric assays (O.D. range was between 0.2 and 0.4 after blank correction). Optical density values were corrected for LDL protein concentration.

Alternatively, LDL at the same protein and antioxidant concentration of above were oxidized using 2.5 μM Cu^{2+} or 1 mM AAPH in PBS at 37°C. In some experiments copper ions were also used at different concentrations in the range 0.1–10 μM.

Spectrophotometric analysis and lag phase and $(\Delta OD/t)_{max}$ calculation were performed as described above.

Peroxynitrite-induced Tyr nitration

A bolus (25 μL) of concentrated $ONOO^-$ (40 mM) in 0.1 N NaOH was added in a final volume of 1 mL to a solution of 0.1 mM Tyr in 100 mM PBS (pH 7.4) in the presence or absence of α-CEHC, γ-CEHC or Trolox in the final concentration range of 0.01–100 μM. The final Tyr/$ONOO^-$ molar ratio of this experiment was ¼, but in some experiments it was reduced to 1/10 by decreasing the concentration of Tyr in the reaction mixture. After rapid vortexing, the rate of generation of 3N-Tyr at 37°C was immediately recorded by continuous spectrophotometric analysis at 430 nm. The formation of 3N-Tyr was confirmed also by HPLC analysis as described in the section below.

HPLC analysis of tocopherols and CEHC metabolites, their nitrated congeners and 3N-T

The absolute concentration of CEHC, tocopherols, and Trolox in the reaction mixtures was measured by HPLC analysis using the assay procedure described in [18], with some changes. Briefly, after addition of the internal standards 1-naphthol and tocol, a single-step liquid-liquid extraction protocol was carried out on the samples after acidification with 20 μL of 100% acetic acid as described in detail in [11]. The samples were mixed with 10 mL of hexane/dichloromethane (2/1 vol/vol) containing 1% (wt/vol) butylated hydroxytoluene (BHT). The samples were vortexed for 1 minute at room temperature. After centrifugation (3200 rpm for 15 minutes at 10°C), the organic layer was collected and dried in the dark under a stream of nitrogen at 50°C and resuspended in 100 μL of mobile phase for the analysis (see below).

Alternatively, the HPLC assay of CEHC metabolites and tocopherols was performed using a simple procedure that allows avoiding the extraction step with solvents. In fact, after this step the sample (from 100 μL to 0.5 mL) was directly injected into an enrichment column (mm 15×4.6 mm i.d.) packed with C18 resin (5 μm particle size) and mounted instead of the injection loop. With the injector in position load, the enrichment column was washed twice with 250 μL of 20% methanol in ultrapure water, then the analysis was performed by switching the injector to the position inject. This procedure allows to measure in the same run CEHC metabolites [electrochemical (EC) detection] and tocopherols [ultraviolet

(UV) detection]. For this purpose, a discontinuous gradient using two different mobile phases was used. The phase A (35% vol/vol acetonitrile/water containing 5 mM tetrabutylammonium-bromide and 0.19 mg/mL EDTA, pH 4.5) was maintained for 10 minutes (i.e., the time required for CEHC elution) and then changed in 1 minute to 100% phase B (phase A with acetonitrile 95% vol/vol) and maintained in this condition for 8 minutes. Then in 1 minute, the mobile phase returned to 100% of A the column was left to equilibrate for at least 5 minutes.

For the analysis of 5N-γ-CEHC and 5N-γ-T (EC and UV/visible detection) the samples were assessed by direct injection (see the procedure described above) and compound elution was obtained respectively with 45% and 95% (vol/vol) acetonitrile in water. This procedure was also used to purify nitrated forms of γ-CEHC and γ-T by collection of the elution fractions and extraction in hexane.

The separation of the different compounds was carried out using a C8 Kromasil 100, 5 μ (mm 250 × 4.6 i.d.) (Eka Nobel, Sweden) under isocratic conditions (flow rate = 1 mL/minute). Compounds were detected using a LC 90 UV spectrophotometric detector (Perkin Elmer) mounted in series with a model 400 EC detector (EG&G, Princeton Applied Research). The EC detector was set in the oxidation mode with a potential of 550 mV and record scale 0.5 nA for CEHC metabolites and 50 nA for tocopherol detection. The UV detector was set at 290 nm. Correction factors for the different homologues and internal standards were determined. The intra-assay coefficient of variation (CV) for the CEHC and Trolox was < 2.5%. Peak identity was confirmed by spiking external standards to samples. In preliminary experiments the spectroscopic analysis of the peaks corresponding to CEHC metabolites and tocopherols was performed using a diode array detector model 9060 Polychrom (Varian).

The analysis of 3N-Tyr was carried out using an ODS2 5 μ column. The mobile phase used was 0.1% vol/vol heptafluorobutyric acid in 30% vol/vol acetonitrile. The separation was achieved under isocratic conditions at a flow rate of 1 mL/minute. The UV/vis detector was set at 287 nm for the first 7 minutes and at 430 nm for the remaining time of the analysis. The external standard prepared as above was used for peak identification and assay calibration. The intra-assay CV was 4.8%.

Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectroscopy was used to investigate the formation of chromanoxyl radicals in CEHC and tocopherols while reacting with *tert*-butyl hydroperoxide (TBH) and ferrous ions [27]. TBH 98% was diluted in dichloromethane; ferrous sulfate was dissolved in deionized water and used within one hour of the preparation. The solutions of α-T or α-CEHC were also freshly prepared the same day of the experiment. They were dissolved in methanol, protected from light, and stored at 4°C until used. The reaction mixture contained always 1mol/L TBH, 0.2 mM ferrous sulfate, and 0.2 mM of α-T or α-CEHC; it was incubated at room temperature for 1 minute and then transferred in capillary tube, sealed with Critoseal, and then drawn in a quartz tube for ESR analysis. ESR spectra were recorded on a Bruker ESP 300E (Bruker Spectrospin, Karlsruhe, Germany). Instrumental conditions were: microwave power, 20 mW; modulation amplitude, 1 G; scan width, 50 G; time constant 82 ms.; sweep time, 168 s. Acquisition was performed by a signal averaging two scans, in order to obtain a low signal to noise ratio.

Data and statistical analysis

Data were expressed as means ± SD. The effect of different concentrations of antioxidants compared to control samples and the difference between different antioxidants were corrected for the degree of purity. Differences were evaluated for statistical significance by Student's *t*-test for repeated measures or one-way ANOVA test. Values of $p < 0.05$ were considered significant.

Results

Inhibition of the Cu^{2+}-mediated oxidation in plasma

Figure 1 shows a typical experiment of Cu^{2+}-induced oxidation carried out in 1:50 (vol/vol) diluted plasma in the presence of α-CEHC, γ-CEHC, Trolox, and α-T at different concentrations. These antioxidants caused a concentration-dependent inhibition of the oxidation reaction as shown by the increase in the lag phase and propagation rate [as $(\Delta OD/t)_{max}$] of the reaction.

As described in detail in the comparison of lag phase values shown in Figure 2, both the CEHC homologues and Trolox produced a significant inhibition of the oxidation reaction when used at concentrations ≥ 0.75 μM, while a weak inhibitory effect was found at concentrations ≤ 0.15 μM. At the same time, the data show that CEHC and Trolox were much effective than α-T as inhibitors of the Cu^{2+}-induced oxidation of plasma lipids (Fig. 2). γ-T gave almost the same dose-dependent response than α-T.

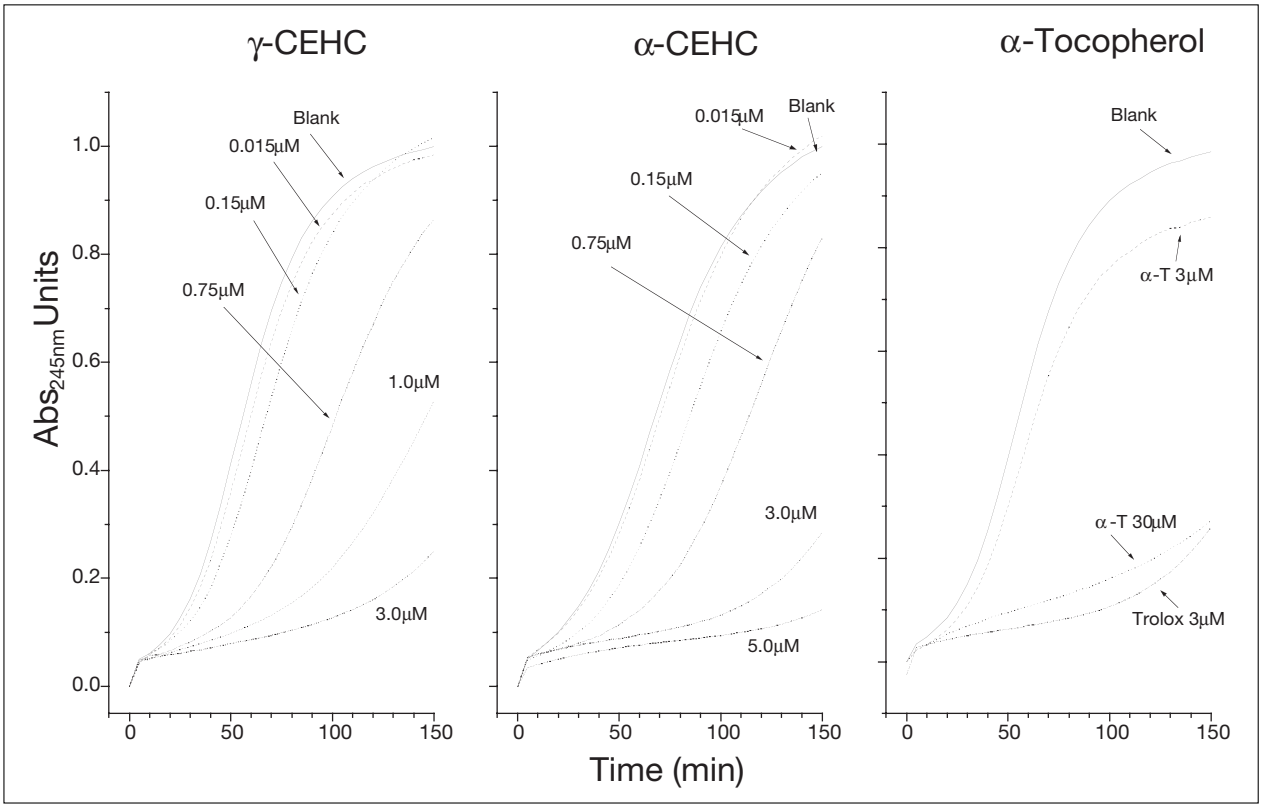

Figure 1: Copper-induced oxidation of lipids plasma in the presence of different concentrations of α- and γ-CEHC, α-tocopherol and Trolox. The graph shows data from an experiment representative of all the assays carried out. The oxidation experiments were carried out on plasma diluted 1/50 vol/vol in PBS to which different amounts of CEHC, Trolox, or tocopherols working stock solutions were added to reach the concentrations given in the figure. The reaction was started when Cu^{2+} ions were added to the final concentration of 50 μM. Lipid oxidation was assessed by spectrophotometric analysis at 245 nm (recorder gain 3 X). The curves were analyzed to calculate lag-phase values (see Fig. 2).

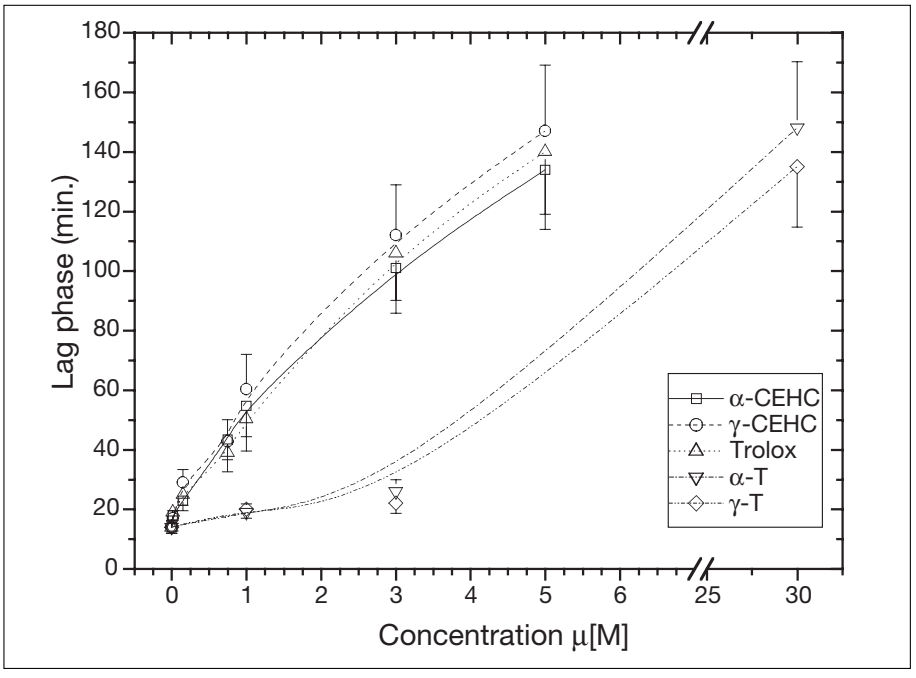

Figure 2: Concentration-dependent effect of CEHC, tocopherols, and Trolox on the lag phase of the Cu^{2+}-induced oxidation of plasma lipids. Lag-phase values of the copper-induced oxidation of plasma lipid was calculated according to [24, 25] and was plotted against the concentrations of the test substances. The oxidation reactions were carried out as described in the text and Figure 1. The data were expressed as means ± SD of four separate experiments carried out in duplicate.

Inhibition of LDL oxidation

To investigate more in detail the antioxidant properties of CEHC, we used purified LDL as lipid substrate for the oxidation reaction catalyzed by different systems such as $CuCl_2$, AAPH, or MØ (Fig. 3). The Cu^{2+} used at the final concentrations of 2.5 μM provided a comparable degree of LDL oxidation as did 1 mM AAPH, while MØ were a less effective pro-oxidant system. Independent of the oxidation system used, the concentration of 1 μM of either the CEHC homologues or Trolox lead to a significant increase of the lag phase and decrease of the propagation rate of LDL oxidation ($p < 0.05$ or greater in both cases). The two CEHC homologues and Trolox showed the same antioxidant potency when tested in the experiments in which copper ions and AAPH were used as oxidation catalysts, but γ-CEHC showed slightly higher potency as compared with α-CEHC or Trolox when MØ were used ($p < 0.05$).

In the concentration range between 0.01 and 1 μM γ-CEHC showed a dose-dependent inhibitory effect of the LDL oxidation reaction induced by either 0.1 or 1 μM Cu2+, but not that induced by 10 μM Cu^{2+} (Fig. 4).

Inhibition of $ONOO^-$-induced formation of 3N-Tyr by CEHC

The formation of 3N-Tyr in the presence of a Tyr/$ONOO^-$ molar ratio of ¼ was inhibited in a concentration-dependent way by α-CEHC, γ-CEHC, and Trolox (Fig. 5, left

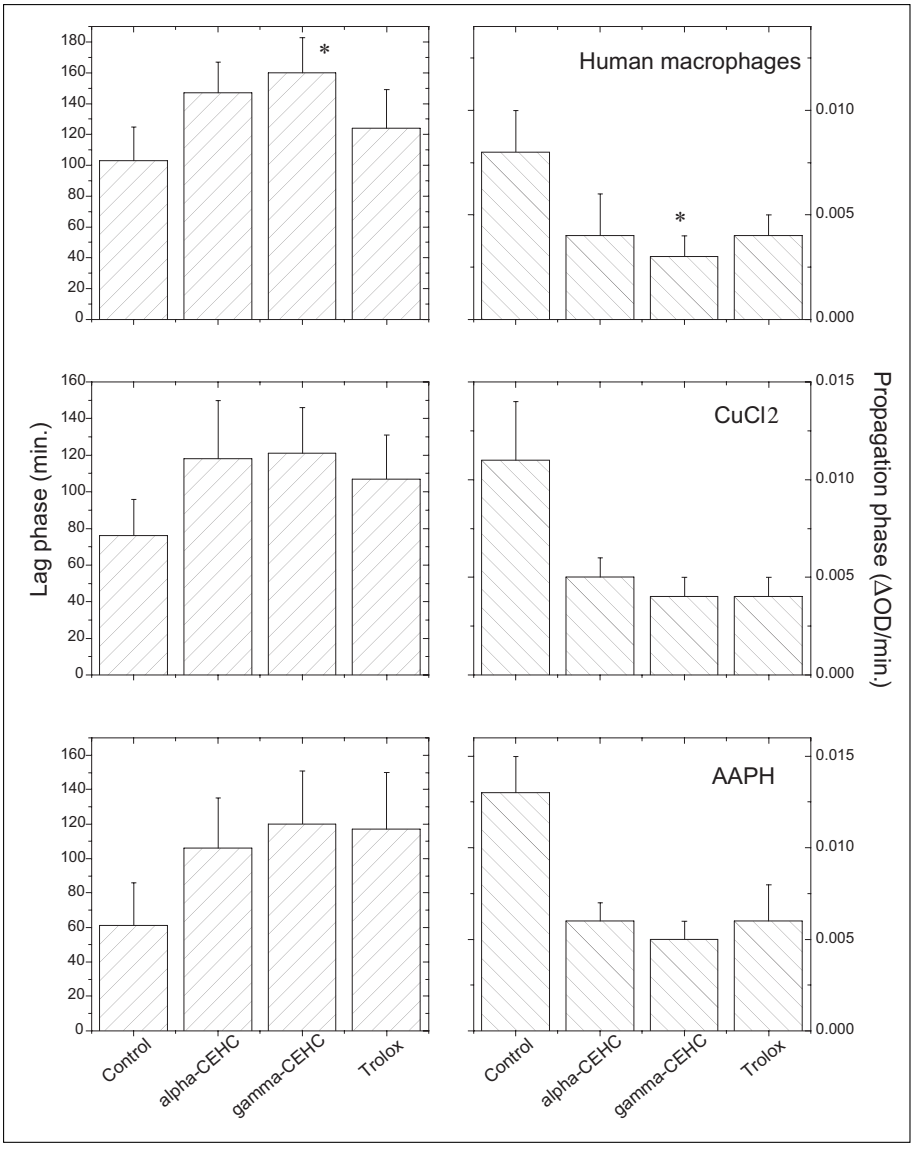

Figure 3: CEHC-related inhibition of LDL oxidation elicited by human macrophages, Cu^{2+}, and AAPH. A suspension of LDL (100 μg protein/mL) in the presence or absence (control) of CEHC or Trolox (final concentration = 1 μM) was incubated at 37°C in the presence of 105 MØ, 2.5 μM $CuCl_2$, 0.5 mM AAPH, or PBS (blank). Lipid oxidation was followed by recording the absorbance at 245 nm and the values of lag phase (left panels) and propagation rate (or ΔOD/minute, right panels) of the reaction were calculated [26]. Values are means ± SD of 3 different experiments carried out in duplicate. All the data when compared to control experiments showed p values < 0.05 or greater. * $p < 0.05$ vs. Trolox or α-CEHC.

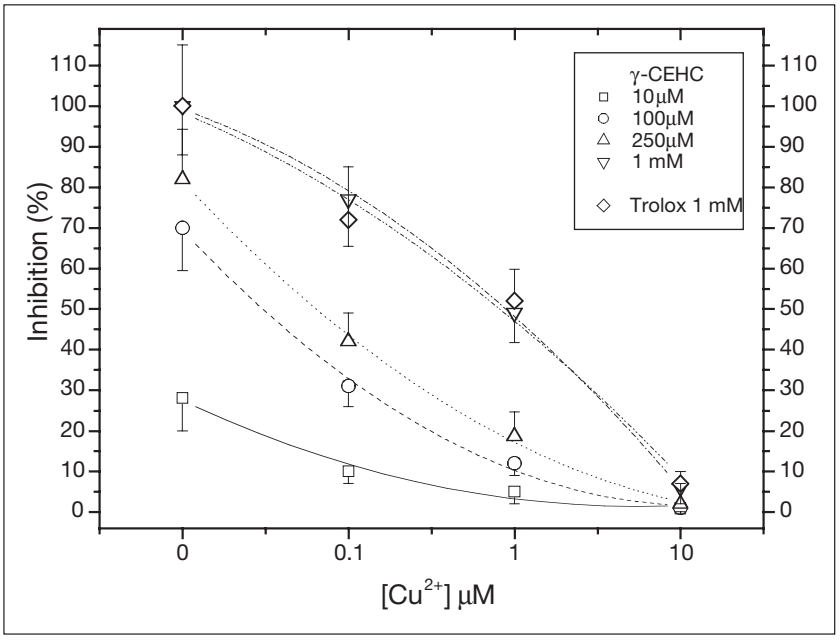

Figure 4: Inhibition of Cu^{2+}-induced LDL oxidation by γ-CEHC. A suspension of LDL (100 µg of proteins/mL) was incubated in duplicate with the concentrations of $CuCl_2$ shown in figure, in the presence or absence (control) of γ-CEHC (concentration range 0.01–1 µM), or 1 µM Trolox. The oxidation reaction was left to proceed for 180 minutes and the absorbance at 245 nm was recorded at the beginning and at the end of the reaction (time 0 and 180 minutes). Data were means ± SD of three different assays performed in duplicate and were expressed as % of inhibition of the oxidation reaction measured as increase of OD_{245nm} at 180 minutes in the control test (without antioxidants) plotted against the concentrations of copper ions and antioxidant compounds. In the presence of Cu^{2+} < 10 µM, values of $p < 0.05$ or greater vs. the control experiment were observed for all the concentrations of γ-CEHC.

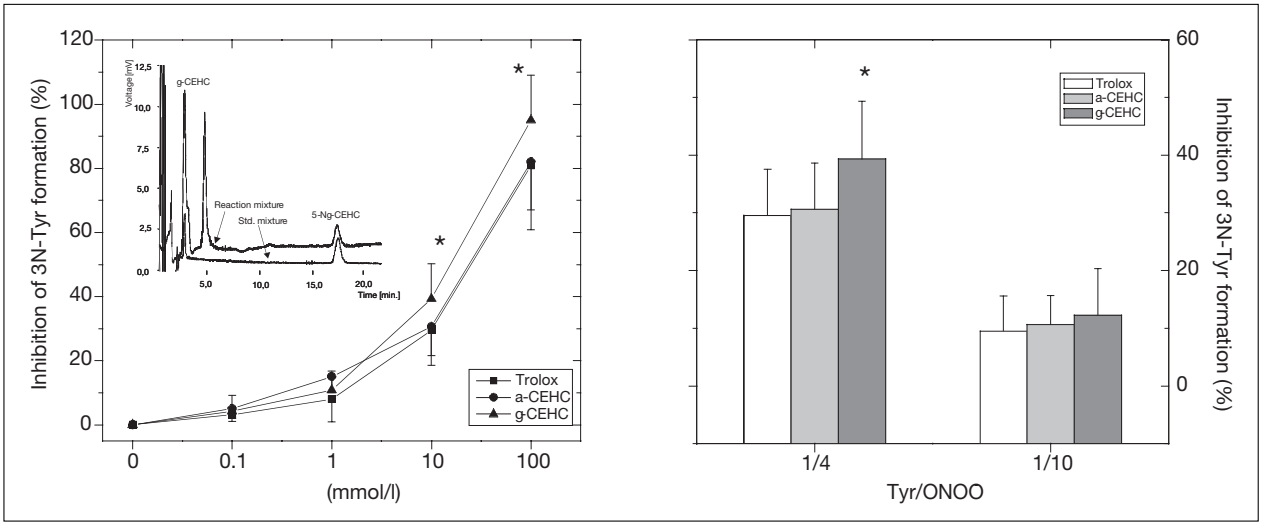

Figure 5: Inhibition by CEHC of the peroxynitrite-mediated nitration of Tyr. A 100 µM solution of Tyr in 100 mM PBS (pH 7.4) was exposed to 400 µM $ONOO^-$ as described in the text (left panel) in the presence or absence of α-CEHC, γ-CEHC, or Trolox (concentration range 0.1–100 µM). In the right panel the inhibitory activity of these antioxidants at the concentration 10 µM was measured in the presence of a Tyr/$ONOO^-$ ratio of ¼ (the same as in the left panel) or 1/10 (1 mM $ONOO^-$). The formation of 3N-Tyr was followed measuring the absorbance of the solution at 430 nm and the specificity of the test was confirmed by HPLC analysis. The results were expressed as % of inhibition in the 3N-Tyr formation vs. the control (sample without antioxidant compounds). Data were means ± SD of 4 different experiments run in duplicate. In the experiment carried out with Tyr/$ONOO^-$ molar ratio of ¼ and 100 µM γ-CEHC the formation of 5N-γ-CEHC was assessed by HPLC analysis (insert in the left panel) using the method described in the text. A volume of 100 µL of the reaction mixture was injected for the analysis and compared with the chromatographic profile of a same volume of a 1.5 µM standard solution of 5N-γ-CEHC (bottom trace). * $p < 0.05$ vs. α-CEHC.

panel). Under these experimental conditions, γ-CEHC showed a significantly ($p < 0.05$) higher inhibitory effect than γ-CEHC at either the concentration of 10 or 100 µM (on average +9 and +14%, respectively), and in the reaction mixture there was formation of 5N-γ-CEHC (insert in the left panel of Fig. 5). As measured in four separate experiments, the concentration of 5N-γ-CEHC formed in the presence of 100 µM γ-CEHC, was between 0.8 and

1.3 μM (median value = 1.2 μM), i.e., 12% of the γ-CEHC added.

The antioxidant power of these two compounds was influenced by the Tyr/$ONOO^-$ molar ratio (Fig. 5, right panel). In the presence of a Tyr/$ONOO^-$ molar ratio of ¼, CEHC, or Trolox at the final concentration of 10 μM showed an average inhibition of 3N-Tyr formation between 30 and 35% while in the presence of 1/10 ratio, the same concentration of these antioxidants showed an average inhibition of approximately 10%.

ESR analysis of the radical formed by the CEHC and tocopherols

In this study we also investigated by ESR analysis the mechanism of antioxidant action of CEHC. Using a chemical system in which peroxyl radicals were generated by ferrous sulfate-induced breakdown of TBH in the presence of α-T or α-CEHC, the production of corresponding radical species was observed (i.e., chromanoxyl and tocopheroxyl radicals, respectively). These radicals when measured and in the case of the alpha homologues at g = 2.0046 gave identical spectra (Fig. 6), with hyperfine splitting constants: a_H ($5CH_3$) = 5.70 G, a_H ($7CH_3$) = 4.48 G, a_H ($4CH_3$) = 1.5 G, a_H ($8CH_3$) = 0.9 G. This confirms the expected result that the antioxidant mechanism of CEHC metabolites is equivalent to that of parent tocopherols.

Discussion

Early *in vitro* and *in vivo* experiments provided evidence supporting the role of CEHC metabolites as antioxidants. In fact, γ-CEHC was observed to protect against metal-induced kidney damage in the rat [14]. These authors suggested that this effect could be due to the antioxidant activity of this metabolite since it was observed to inhibit *in vitro* reactions of lipid oxidation catalyzed by either peroxyl radicals or iron with a higher potency than α-T. Moreover, γ-CEHC could play a specific role as NOx scavenger by the formation of the nitration derivative 5N-γ-CEHC that is homologous to the nitration product formed during the reaction of its precursor γ-T with reactive nitrogen species such as $ONOO^-$ or its byproducts [7, 8, 21, 23]. Again, functional homology between γ-CEHC and its parent vitamer could explain the same inhibitory effect of these compounds on the activity of the pro-oxidant enzyme COX-2 in macrophages and epithelial cells, while the α-T was a less effective inhibitor [20].

Recently Betancor-Fernandez *et al* [15], found that α-CEHC, similarly to the structurally related compound Trolox, can act as hydrosoluble scavenger of peroxyl radicals and inhibitor of the $ONOO^-$-induced nitration of Tyr. Although the chemistry of this reaction was not investigated, it could be easily supposed that α-CEHC might inhibit the nitration of low molecular weight phenols

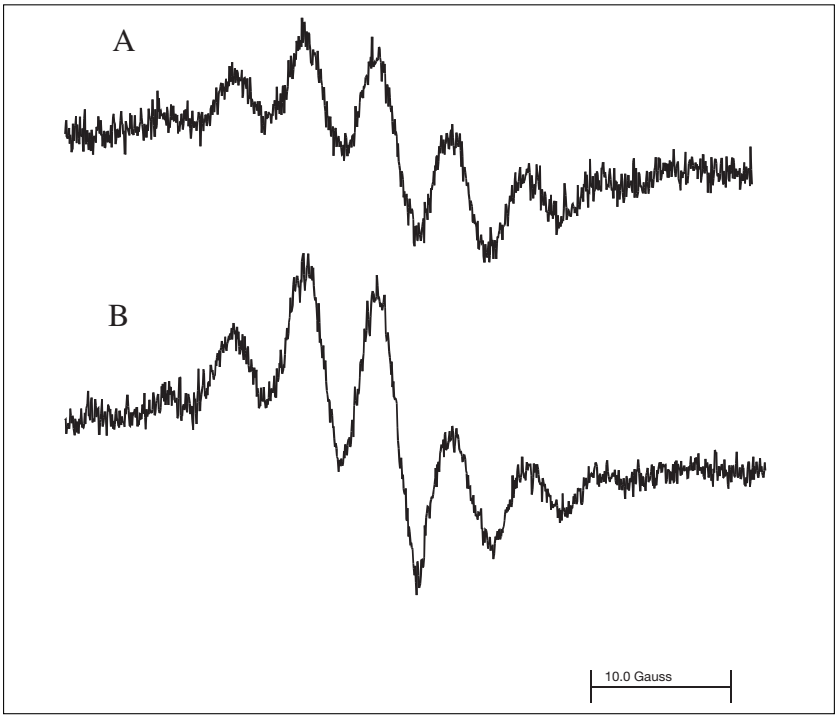

Figure 6: ESR analysis of chromanoxyl radicals of α-tocopherol (signal A) and α-CEHC (signal B) incubated in the presence of TBH/Fe^{2+} as a generator of peroxyl radicals. ESR spectra of the chromanoxyl radicals formed were determined as described in detail in the text and [27].

through the classical electron-transferring mechanism already reported for tocopherols and Trolox [28].

Now, these pieces of evidence highlight the importance to make a direct comparison between the antioxidant and NOx scavenging functions of the two main metabolic products of tocopherols and tocotrienols found in biological fluids [11, 17, 18].

Results in this study confirmed that micromolar concentrations of both α-CEHC and γ-CEHC, as well the hydrosoluble analogue Trolox, could provide a substantial antioxidant effect in terms of inhibition of the Cu^{2+}-induced lipid oxidation in whole plasma.

Conversely, in this test CEHC showed a higher potency than parent tocopherols, of which they maintain the same chroman moiety and reaction chemistry when exposed to peroxyl radicals in an aqueous milieu. This suggests that CEHC metabolites act essentially as hydrophilic chain breakers through the formation of the corresponding chromanoxyl radicals, herein demonstrated by ESR analysis. As already investigated for the α-T [1, 2, 27, 29], this reaction chemistry is based on the electron transferring to quench free radicals that, for instance, may form during the transition metal-catalyzed activation of the molecular oxygen or in the oxidation of polyunsaturated lipids.

Accordingly, with the finding of a higher inhibition of plasma lipid oxidation by CEHC as compared with their tocopherol precursors, previous studies showed that "short-tailed" tocopherol homologues behave as amphiphilic compounds that could be more effective chain-breakers than "long-tailed" homologues when added as a bulk to LDL suspensions [29]. In this model, the shortening of the pythyl chain (that is known to have little if any effect on the reactivity of the chromanoxyl group [30]) was proposed to favor the inter-particle "shuttling" of radical species formed into the LDL particles, the vitamin E-derived radicals included. Then, a rapid rate of inter-particle diffusion and escape of radicals is supposed to hasten their termination. CEHC could fit with this model as they may interface with the hydrophobic components of LDL and the hydrophilic milieu, thus providing an efficient system for shuttling peroxyl radicals outside the LDL particle. Thanks to its amphiphilic character, CEHC might also contribute to transfer reducing equivalents from hydrosoluble co-antioxidants (such as vitamin C) to intra-particle liposoluble antioxidants as vitamin E.

Since this mechanistic view of the antioxidant function of CEHC, sorted out from experiments on total plasma, could be biased by the interference of several factors such as the presence of other hydrosoluble antioxidants (vitamin C and uric acid), high concentrations of copper required to induce lipid oxidation, and other factors [24], further experiments were performed using as an oxidation substrate purified LDL. These were exposed to different oxidation systems such as low-micromolar concentrations of Cu^{2+}, the peroxyl radical generator AAPH, and MØ. Low micromolar (Fig. 3) and submicromolar concentrations of CEHC (Fig. 4) showed a protective effect on the oxidative challenge that all these oxidation systems may cause to LDL. The two CEHC metabolites and Trolox showed similar inhibitory effects on oxidation experiments carried out with copper and AAPH, but in the MØ-induced LDL oxidation, γ-CEHC was a more effective inhibitor than α-CEHC and Trolox. This difference could be the consequence of the production by MØ of more reactive species than could be selectively scavenged only by the γ-CEHC, such as NO-derived species and particularly $ONOO^-$ [31]. In fact, similarly to other phenolic compounds such as the amino acid Tyr, the gamma configuration of the chroman moiety can undergo nucleophilic attack by a NO_2 group in the position 5'. This reaction has been demonstrated to occur in the case of γ-T both *in vitro* in insulinoma cells [7] and *in vivo* in patients suffering from atherosclerotic cardiovascular disease [8]. As expected, this reaction is possible *in vitro* also in the case of γ-CEHC when exposed to $NaNO_2$ or $ONOO^-$ with the formation of the homologous 5N-γ-CEHC.

We also investigated whether this reaction could provide a reasonable mechanism for a specific inhibition by γ-CEHC of the $ONOO^-$-mediated nitration of Tyr. Previously, it was shown that α-CEHC, similarly to Trolox but with a lower potency than (–)-epicatechin, inhibits this reaction in a concentration-dependent way (range tested 10–80 μM) [15]. Our results are in agreement with these findings, but we showed that γ-CEHC exerts a higher inhibitory effect than α-CEHC when low Tyr/$ONOO^-$ ratios are used. During the reaction there was the formation of 5N-γ-CEHC in a proportion sufficient to explain the different inhibitory effect observed between the two homologues.

However, under the experimental conditions of this study, γ-CEHC nitration was clearly less relevant to the scavenging of $ONOO^-$-derived reactive species [22] than the classical electron-transfer mechanism. The results demonstrate also that the scavenging of $ONOO^-$ by CEHC, either occurring through the formation of 5N-γ-CEHC or electron transfer, was a function of the quantitative relationship between CEHC, other nitration substrates (such as the same Tyr), and the nitrating species. Obviously, the possible physiological roles of γ-CEHC nitration could be sensibly constrained by the presence in the reaction milieu of effective electron donors and radical species as factors known to strongly affect the chemistry of $ONOO^-$ [32].

Importantly, when the *in vitro* concentrations of CEHC decreased to levels << 1 μM (i.e., those found in the blood

of non-supplemented individuals [11, 18]), the inhibitory effect observed either on plasma or LDL oxidation experiments and in the $ONOO^-$-mediated formation of 3N-Tyr was rather feeble. This makes difficult to suppose that CEHC could play such an antioxidant role in the blood, particularly in the presence of a sustained pro-oxidant challenge. However, previously some of us and others [11, 18] showed that even a moderate increase of the intake of less retained vitamin E homologues, such as γ-T and γ-tocotrienol, may bring the corresponding CEHC metabolites to reach low μM concentrations in plasma, i.e., the levels at which they were observed to exert appreciable in vitro antioxidant function.

In conclusion, this study shows that CEHCs behave as hydrophilic antioxidants through the same chemistry of their lipophilic precursors (e.g., they are able to form chromanoxyl radicals). In the low μM range, α-CEHC and γ-CEHC are effective inhibitors of the oxidation of plasma and LDL lipids induced by transition metals, hydrosoluble peroxyl radicals, and MØ. Moreover, both the CEHC homologues inhibit the $ONOO^-$-induced formation of 3N-Tyr. α-CEHC was observed to exert a moderately higher inhibition of the MØ-induced LDL oxidation and $ONOO^-$ scavenging than α-CEHC. This latter effect was most likely due to the sequestration of NO_2 through the formation of the nitration product 5N-γ-CEHC.

However, at the concentrations found in the blood of subjects with normal dietary intake of vitamin E (low nanomolar range) CEHC show feeble *in vitro* antioxidant potency. This suggests a minor role of CEHC within the antioxidant network of plasma, also considering the possible interference by factors such as conjugation with glucuronidides and sulfates that might further limit the *in vivo* antioxidant role of these metabolites. Further studies are required to ascertain this aspect and to assess whether increasing CEHC concentrations by a high vitamin E intake could contribute to reinforcing the antioxidant defense of biological fluids and tissues.

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