Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 19 (2008) 320–327
Journal of Nutritional Biochemistry

Effects of tocopherols and 2,2′-carboxyethyl hydroxychromans on phorbol-ester-stimulated neutrophils

Zsuzsa Varga[a,*], Eszter Kosaras[a], Edina Komodi[b], Monika Katko[a], Istvan Karpati[a], Jozsef Balla[a], Gyorgy Paragh[a], Maria C. Aisa[b], Francesco Galli[b]

[a]First Department of Medicine, Health and Medical Science Centre, University of Debrecen, P.O. Box 19, H-4012 Debrecen, Hungary
[b]Department of Internal Medicine, Section of Applied Biochemistry and Nutritional Sciences, University of Perugia, Perugia, Italy

Received 15 November 2006; received in revised form 22 March 2007; accepted 10 May 2007

Abstract

Tocopherol vitamers [e.g., alpha-, gamma- and delta-tocopherol (α-TOC, γ-TOC and δ-TOC, respectively)] and their water-soluble 2,2′-carboxyethyl hydroxychroman metabolites (e.g., α-, γ- and δ-CEHC) all possess antioxidant properties. As a consequence, and similarly to other natural antioxidants, vitamin E compounds may be useful in preventing inflammatory and oxidative-stress-mediated diseases. In this study, we investigated the concentration-dependent effect of tocopherols and water-soluble metabolites on a key event in oxidative stress, for example, the oxidative burst in neutrophils. It was found that not only α-TOC but also γ-TOC and δ-TOC as well as α-, γ- and δ-CEHC at physiological concentrations inhibit superoxide anion ($O_2^{\bullet-}$) production in phorbol-ester-stimulated neutrophils. This effect was mediated by the inhibition of the translocation and activation of protein kinase C (PKC) enzyme, which is the key event in the phorbol-ester signaling. Importantly, CEHCs were stronger inhibitors of PKC as compared with the vitamer precursors, and the gamma forms of both tocopherol and CEHC showed the highest inhibitory activities. Tocopherols, but not CEHCs, directly inhibit the fully activated nicotine–adenine–dinucleotide phosphate (NADPH) oxidase. However, none of the test compounds was able to directly scavenge $O_2^{\bullet-}$ when tested in a cell-free system. In conclusion, vitamin E compounds can control the neutrophil oxidative burst through the negative modulation of PKC-related signaling and NADPH oxidase activity. As an original finding, we observed that CEHC metabolites might contribute to regulate PKC activity in these cells. These results may have important implications in the anti-inflammatory and antioxidant role of vitamin E compounds.

Keywords: Vitamin E; Tocopherols; CEHC; Neutrophils; Protein kinase C; NADPH oxidase; Xanthine oxidase

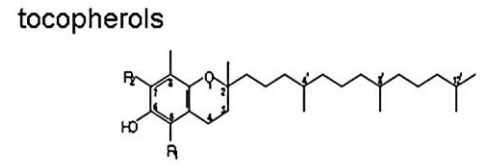
1. Introduction

Vitamin E, since its discovery as one of the most important naturally occurring lipophilic antioxidants, was recognized to possess a number of biological functions that may justify its use in prevention and therapy of chronic-degenerative diseases associated with inflammation and aging, such as atherosclerosis, cardiovascular diseases or immune dysfunction [1]. This background has been supported by several observational and epidemiological studies, which almost exclusively investigated the alpha homologue of tocopherols (α-TOC) [1,2]. It is a chain-breaking antioxidant commonly reported to physiologically protect polyunsaturated lipids in cell membranes and lipoproteins from injury induced by reactive oxygen species (ROS).

However, vitamin E can also exert an important antioxidant role through the control of inflammatory cell activation such as neutrophils and monocytes, which are important sources of ROS such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide [3,4]. In these processes, the antioxidant activity of α-TOC is mainly due to the inhibition of protein kinase C (PKC) enzyme activity, which controls, among them, thyrosine phosphorylation-dependent signaling [5] and $O_2^{\bullet-}$ production [6] in inflammatory cells. However, during the past few years, increasing evidence has suggested that not only α-TOC but also gamma-tocopherol (γ-TOC) can exert significant antioxidant activity and other intriguing biological functions, which have increased the general interest of the scientific community on this hypomethylated form [7–9]. Thus, the common and long-lasting belief that it

* Corresponding author. Fax: +36 52 414 951.
E-mail address: vargazs@internal.med.unideb.hu (Z. Varga).

0955-2863/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2007.05.002



R1	R2	homologue
CH3	CH3	α
CH3	H	β
H	CH3	γ
H	H	δ

Fig. 1. Structure of tocopherols and 2,2′-carboxyethyl hydroxychromans.

could be of minor importance as compared with the alpha form because of its being less effective in some bioactivity tests and less abundant in some tissues has been overcome, and now, several lines of evidence suggest that it may play a role in preventing some oxidative-stress-mediated conditions [10–12]. γ-TOC was found to be a more potent anti-inflammatory agent as compared with α-TOC [11,12], and under some experimental circumstances, not only γ-TOC but also delta-tocopherol (δ-TOC) proved to be superior to the well-characterized α-TOC as a lipid antioxidant [13].

Recently, it was demonstrated that the water-soluble metabolites 2,2′-carboxyethyl hydroxychromans (α-CEHC, γ-CEHC and δ-CEHC), which are formed during the hepatic catabolism of tocopherols and tocotrienols, retain the scavenging function of peroxyl radicals and the inhibitory effect against peroxynitrite-mediated tyrosine nitration [14,15]. Moreover, CEHCs can inhibit not only the proliferation of some human cancer cell lines via down-regulation of cyclin-related signaling [16,17] but also inflammatory pathways [18].

However, the effect of γ-TOC and δ-TOC as well as their water-soluble CEHC metabolites on PKC activity and $O_2^{\bullet-}$ formation in neutrophils remains unexplored, leading to the hypothesis that other vitamin E compounds might contribute to the overall antioxidant-dependent protective role of α-TOC. Previously, we observed that γ-TOC can inhibit total PKC activity more effectively than α-TOC in C6 murine glioma cells [17]. To explain how just a small structural difference could produce such biological difference, recent studies carried out by our group on other antioxidants have confirmed that even a small change in the molecular structure of the test compounds can markedly affect their in vitro antioxidant properties, and the magnitude of this structure–activity relationship strongly depended on which kind of oxidative stress route or type of initiator is examined [15,19,20].

In this study, we investigated the effect of tocopherol vitamers (α-TOC, γ-TOC and δ-TOC) and their water-soluble metabolites (α-CEHC, γ-CEHC and δ-CEHC) on the oxidative burst response of phorbol–myristate–acetate (PMA)-stimulated human neutrophils. This was assessed by measuring $O_2^{\bullet-}$ formation, nicotine–adenine–dinucleotide phosphate (NADPH) oxidase activity and PKC activity and translocation. The antioxidant potency of the test compounds was also compared by assessing their ability to inhibit xanthine oxidase activity and to scavenge $O_2^{\bullet-}$ radicals.

2. Materials and methods

2.1. Chemicals

Cytochrome *c* (Type IV); xanthine; xanthine oxidase; Histopaque 1077; Hanks' Balanced Salt Solution (HBSS); α-, γ- and δ-tocopherol; dimethyl sulfoxide (DMSO); reduced NADPH; and PMA were from Sigma (St. Louis, MO, USA). CEHCs were a kind gift to Dr. Galli by Eisai Co., Japan. Stock and working solutions of tocopherols and their water-soluble metabolites were prepared in DMSO to achieve appropriate final concentrations in the reaction mixtures. All controls and test samples contained the same volume of DMSO (≤0.1%). The structure of tocopherols and water-soluble metabolites is presented on Fig. 1.

2.2. Neutrophil separation

Neutrophils were separated from fresh heparinized blood of 15 healthy volunteers after an overnight fasting according to the method of Boyum [21] using Histopaque 1077 gradient density centrifugation. None of the healthy volunteers was a smoker or was supplemented by vitamin E, but they had to have a sufficient dietary intake of vitamin E as assessed by a 72-h recall of diet (avarage α-TOC content in diet was 6–12 mg/day). Neutrophil purity over 95% and viability greater than 95% were ascertained by microscopic examination using Giemsa staining and trypan blue exclusion test, respectively. All volunteers gave their informed consent to participate in the study, which followed the guidelines of the Ethical Committee of the University of Debrecen.

2.3. Determination of tocopherol incorporation rate in neutrophils

Three milliliters of 1×10^6 cells/ml neutrophils was resuspended in HBSS (Sigma) containing 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$ and 10 mM glucose. Cell suspension was incubated with individual tocopherols at 37°C in a humidified CO_2 incubator. Final compound concentrations in the cell media were set in order to simulate physiological condition in

human plasma (α-TOC: 25 μmol/L, γ-TOC: 4 μmol/L, δ-TOC: 1 μmol/L) [16]. After 20, 40 and 60 min of incubation, 1 ml of cell suspension was removed and centrifuged (5 min, 500×*g*, 4°C). The cell pellet was washed once with ice-cold phosphate buffer (pH 7.4). Thus, tocopherols were extracted by the addition of 1 vol of ethanol/methanol (1:1 v/v) and 10 vol of *n*-hexane. The organic layer was collected and evaporated under a stream of N_2. The dried samples were resuspended in methanol. Samples were injected into an HPLC apparatus (Hitachi-Merck) coupled to a fluorescence detector (F1050) set at 292 nm for excitation and 325 nm for emission [22]. Compounds were identified by spiking the samples with known amounts of individual pure tocopherols, which were used also to calibrate the assay. Three independent experiments were performed in duplicate for each test compound.

2.4. Assay of superoxide generation

The production of $O_2^{\bullet-}$ was assessed spectrophotometrically by measuring the reduction of cytochrome *c* with the methods of Babior et al. [23], as published in detail previously [24]. Briefly, the cells, resuspended in HBSS, were preincubated with various concentrations of test molecules (α-TOC: 10–50 μmol/L, γ-TOC: 0.1–4 μmol/L, δ-TOC: 0.1–4 μmol/L, α-CEHC: 0.05–5 μmol/L, γ-CEHC: 0.05–5 μmol/L, δ-TOC: 0.05–5 μmol/L) for 30 min at 37°C and then were stimulated with PMA at the final concentration of 10^{-7} M. Cytochrome *c* reduction was monitored at 550 nm with baseline correction at 492 nm. Each experiment was run in triplicate. The results were expressed as % inhibition in $O_2^{\bullet-}$ production by controls using the following equation: % inhibition=$(A_s/A_c-A_s)\times100$, where A_c is the absorbance in controls and A_s is the absorbance in samples. Results are expressed as mean±S.D. of five independent experiments. All experiments were run in triplicate.

2.5. PKC activity in the cytosol

This activity was determined in the cytosol of PMA-activated neutrophils as previously published [25] using an ELISA kit (Calbiochem-Novabiochem Co., San Diego, CA, USA). Cell-free cytosol samples were prepared from a suspension of 5×10^6 cells/ml after 30 min preincubation with test compounds at 37°C in HBSS. Afterward, neutrophils were stimulated with PMA at 10^{-7} M for 3 min at 37°C. Until this point, test compounds were present in the incubation media. The reaction was stopped by the addition of 10 vol of ice-cold phosphate buffer. After centrifugation, cells were resuspended in sample preparation buffer (50 mM Tris–HCl, 50 mM β-mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM PMSF, 10 mM benzamidine, pH 7.5) and disrupted by three cycles of freezing in liquid nitrogen (1 min) and thawing at room temperature. The cell lysate was centrifuged (13,000×*g* for 1 h at 4°C), and the supernatant was used immediately to determine PKC activity. The assay was performed in the reaction mixture containing 25 mM Tris–HCl (pH 7.0), 0.3 mM $MgCl_2$, 0.1 mM ATP, 2 mM $CaCl_2$, 50 μg/ml phosphatidylserine, 0.5 mM EDTA, 1 mM EGTA and 5 mM β-mercaptoethanol in final concentrations. Protein content in the cytosol was determined using the method of Lowry et al. [26]. Results were expressed as ratio to control, for example, optical density per milligram of protein in samples compared to optical density per milligram of protein in PMA control. All experiments were performed in duplicate using neutrophils from three different donors.

2.6. NADPH oxidase activity

NADPH oxidase activity was determined in the cell-free system, for example, in PMA-stimulated neutrophil lysates [25,27]. Fully activated NADPH oxidase was prepared in intact cell suspensions in HBSS (1×10^7 cells/ml) after stimulation with PMA (10^{-7} M) for 3 min at 37°C. Thus, the cells were disrupted and kept on ice until use. Tocopherols and their water-soluble metabolites were added to the cell lysate at the final concentrations indicated and incubated for 10 min at room temperature. The reaction was started by the addition of NADPH. Enzymatic activity was determined by measuring cytochrome *c* reduction rate at 550 nm for 5 min with correction of baseline absorbance of 492 nm. NADPH oxidase activity was calculated as the difference of optical density per minute, and final data were expressed as % inhibition using the following equation: % inhibition=$(\delta V_s/\delta V_c-\delta V_s)\times100$, where δV_c is the reduction rate of cytochrome *c* in control and δV_s is the reduction rate of cytochrome *c* in samples. All experiments were performed in triplicate using neutrophils of five different subjects.

2.7. Xanthine oxidase inhibition and superoxide anion scavenging

Xanthine oxidase activity was determined by monitoring uric acid formation at 295 nm for 3 min at room temperature [28]. The reaction mixture contained xanthine as substrate (at a final concentration of 60 μM) and xanthine oxidase (0.044 U/ml) in the presence and the absence of test molecules at the concentrations shown. The reaction was started by the addition of xanthine oxidase, and uric acid formation was followed for 3 min. Inhibition of xanthine oxidase activity was calculated, for example, as the percentage of total uric acid formed per minute in test samples with respect to control samples. In another series of experiments, cytochrome *c* (100 μmol/L) was also added to the reaction mixture, and its reduction rate was followed. Results were compared to inhibition of uric acid formation. Three independent experiments were performed in triplicate for each test compound.

2.8. Statistical analysis

Data were expressed as means±S.D. Differences between the results obtained in test and control samples were assessed using the paired Student's *t* test or the ANOVA test for nonparametric data. $P<.05$ was considered significantly different.

3. Results

An aspect that is yet to be characterized, the uptake rate of tocopherols in neutrophils was determined in a preliminary experiment (Fig. 2). Results showed that a plateau in the concentration of the incorporated tocopherols was achieved between 20 and 40 min of incubation. During this time, neutrophils incorporated individual tocopherols with different efficacies. In the case of α-TOC, 25% of the vitamer added to the culture media was incorporated into the cells, while this value increased to 50% and 100% in the case of γ-TOC and δ-TOC, respectively. At steady state, supplemented cells did not show any significant decrease in viability. Based on this, 30 min of preincubation was considered to be sufficient to achieve maximal cell concentrations of tocopherols, and it was used in all experiments with intact neutrophils.

The concentration-dependent effect of tocopherols and their water-soluble CEHC metabolites on PMA-induced $O_2^{\bullet-}$ production is shown in Panels A and B of Fig. 3, respectively. All tocopherols and CEHC metabolites inhibited $O_2^{\bullet-}$ production in PMA-stimulated human neutrophils, although to different extents. Importantly, all compounds produced maximal in vitro inhibitory effect at concentrations close to physiological ones in human serum. However, we also observed that cells isolated from the blood of one subject who had elevated plasma levels of α-TOC (49.1 μmol/L) responded up to threefold higher superoxide anion production than cells of subjects with normal plasma α-TOC levels (~30 μmol/L), when they were exposed with 40 and 50 μmol/L extracellular α-TOC (110% vs. 43±4.6% and 130% vs. 40±5.8%, respectively), suggesting that at given circumstances, α-TOC might behave as a pro-oxidant, at least in vitro.

Since phorbol ester develops its effect through direct activation of PKC [29–31], we assessed the changes in the activity of this enzyme in neutrophils preincubated with vitamin E compounds. All vitamin E compounds inhibited PKC activity, and this inhibition reached a peak near the physiological concentrations of tocopherols and CEHC metabolites (Fig. 3C and D, respectively). In detail, maximal inhibitory effects were observed for α-TOC near 25 μmol/L, for γ-TOC between 2 and 3 μmol/L and for δ-TOC below 1 μmol/L (Fig. 3C). Between tocopherol homologues, α-TOC was found to be the less potent inhibitor of PMA-stimulated PKC activation. For all CEHCs, higher inhibitory activity was observed at concentrations between 0.1 and 0.3 μmol/L, although δ-CEHC showed weaker inhibitory activity as compared with the other CEHC homologues, particularly γ-CEHC (Fig. 2D). Importantly, among the entire groups of test compounds, gamma homologues showed the highest inhibitory activity on PKC translocation.

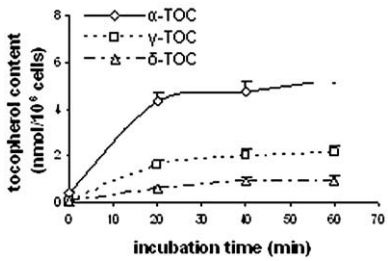


Fig. 2. Time course of in vitro uptake of tocopherols by neutrophils supplemented with their physiological concentrations. Cell samples (1×10^6 cells/ml) were incubated with tocopherols (α-TOC: 25 μmol/L, γ-TOC: 4 μmol/L, δ-TOC: 1 μmol/L). After the time indicated, one portion of the cells was removed and tocopherol content was determined as described in Section 2. Data were mean±S.D. of three independent experiments.

Given that $O_2^{\bullet-}$ production in stimulated neutrophils is due to NADPH oxidase activity, the direct effect of tocopherols and CEHC metabolites on the activity of this enzyme was also examined. All tocopherols inhibited NADPH oxidase activity in the lysate of PMA-stimulated neutrophils, with IC_{50} values of 24.5 μmol/L for α-TOC, 8.9 μmol/L for γ-TOC and 2.9 μmol/L for δ-TOC (Fig. 3E). On the contrary, CEHC metabolites did not inhibit NADPH oxidase activity; rather, they produced a slight pro-oxidant effect at the highest concentrations assessed (Fig. 3F).

To explore whether the direct $O_2^{\bullet-}$ scavenging properties of test compounds may influence their effect on NADPH oxidase activity, we investigated it using the xanthine/xanthine oxidase reaction system, which produces $O_2^{\bullet-}$ and uric acid in equimolar ratio. We found that tocopherols inhibited uric acid formation through the activity of xanthine oxidase with similar IC_{50} values (~28 μmol/L). In contrast, none of the CEHC metabolites produced a significant inhibitory effect. Furthermore, neither tocopherols (Fig. 4A) nor their water-soluble metabolites (Fig. 4B) showed $O_2^{\bullet-}$ scavenging properties when tested by measuring cytochrome *c* reduction rate in the xanthine/xanthine oxidase system. Accordingly with the effect observed on NADPH oxidase activity, in the case of CEHC metabolites, a slight increase in cytochrome *c* reduction was observed when they were used at concentrations higher than physiological ones (Fig. 4B).

Finally, the strong correlation between $O_2^{\bullet-}$ production and the remaining PKC activity in the cytosol, found in intact cells exposed to physiological concentrations of tocopherols and CEHC metabolites (Fig. 5), suggests that the different effects of tocopherols and CEHC metabolites on activated NADPH oxidase did not dominate in physiological circumstances, for example, in intact neutrophils. Thus, inhibition in $O_2^{\bullet-}$ production has to be considered essentially as a consequence of PKC inhibition.

4. Discussion

α-TOC is known to inhibit PKC activity in a wide variety of cells [32–34] including neutrophils [5,6,35]. In human neutrophils, the activation and translocation of PKC from its inactive form found in the cytosol to the cell membrane are key events in phorbol-ester-induced signaling and are required to induce $O_2^{\bullet-}$ production [36]. However,

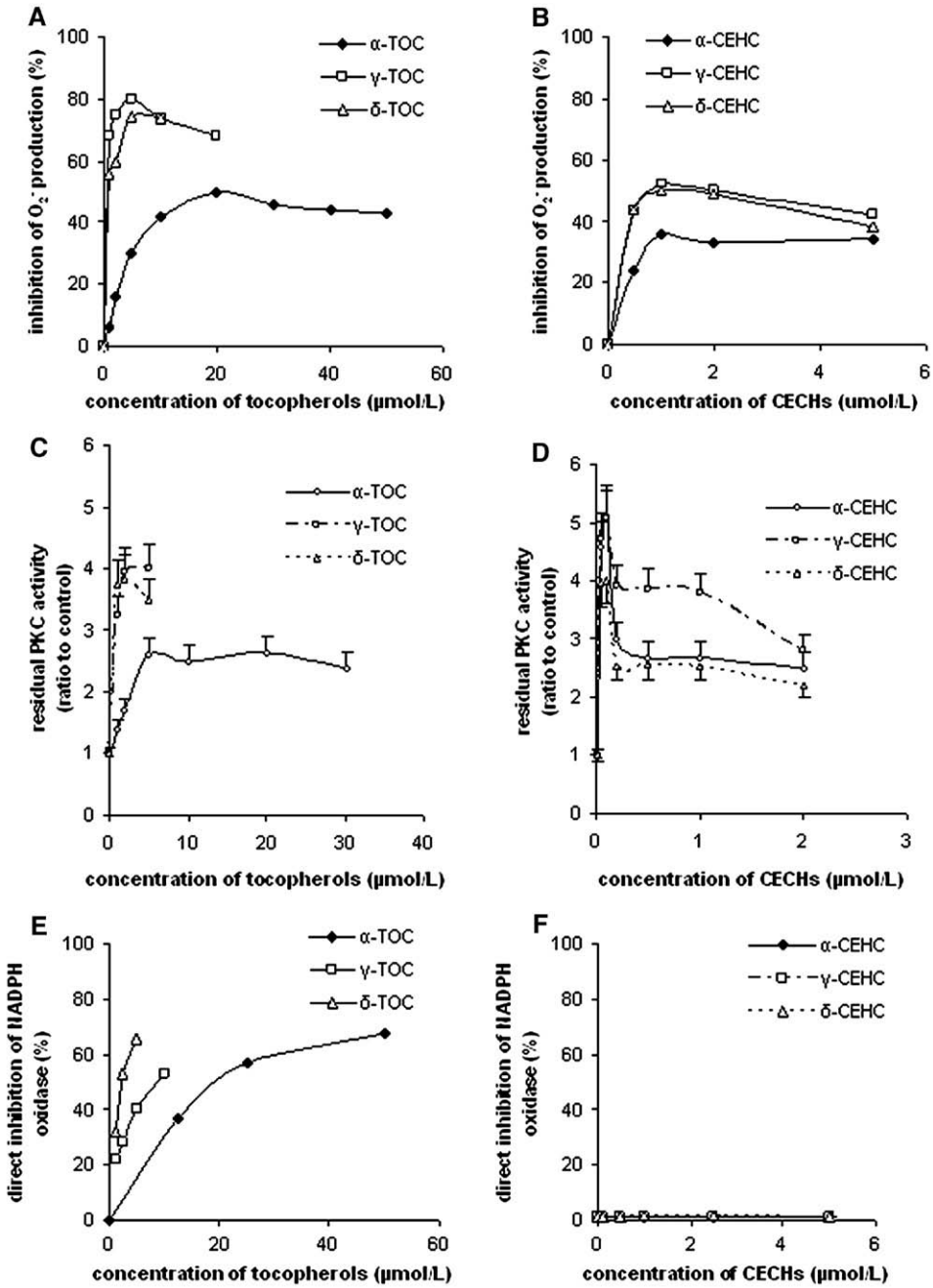


Fig. 3. Concentration-dependent effect of tocopherols (left panels) and CEHCs (right panels) on superoxide anion production (A and B), PKC activity in the cytosol measured as residual activity (C and D) and NADPH oxidase activity measured in cell lysate (E and F) in PMA-stimulated human neutrophils. Cells were treated as described in Section 2. Results were expressed as mean±S.D. of at least five independent experiments. All determination was performed in triplicate.

in this context, a comparative analysis considering the effect of hypomethylated tocopherols (namely, γ-TOC and δ-TOC) and water-soluble CEHC metabolites is missing, although it could be of relevance in understanding the overall anti-inflammatory and antioxidant properties of vitamin E compounds.

In this study, we showed that all tocopherols and CEHCs inhibited PMA-stimulated $O_2^{\bullet-}$ production via inhibition of PKC activity. Different homologues produced different degrees of inhibition, and importantly, physiological plasma concentrations of CEHC homologues (low micromolar range) produced a higher inhibition of PKC enzyme translocation and activity — and, as a consequence, of cell $O_2^{\bullet-}$ production — than physiological concentrations of the corresponding tocopherol precursors (micromolar range). These findings seem to go in the same direction of previous studies showing that even if supplemented in the extracellular milieu, CEHCs can interact with cell signaling

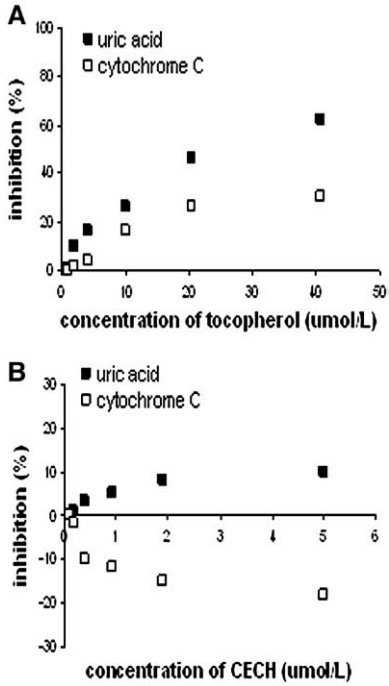


Fig. 4. Concentration-dependent effect of tocopherols (A) and CEHCs (B) on xanthine oxidase activity. Xanthine oxidase activity was determined using xanthine as substrate in the presence of the given concentration of tocopherols and their metabolites. Uric acid formation was followed at 295 nm for 3 min at room temperature (filled squares). Cytochrome *c* reduction showing superoxide anion formation was followed at 550 nm using 492 nm for baseline correction (open squares). Results are expressed as mean±S.D. of three independent experiments.

pathways, ultimately influencing key metabolic processes such as cell cycle, differentiation, inflammation and oxygen metabolism [14–18,37].

Another key finding of this study was the observation of the superiority of gamma forms (i.e., γ-TOC and γ-CEHC) as inhibitors of neutrophil PKC activation in comparison with their alpha counterparts. An aspect that may explain this result is the different uptake of individual forms and their possible interaction with cell membrane components, which can affect PKC activation. In agreement with previous findings obtained in other types of cells [15,38–40], neutrophils incorporated all tocopherols, but γ-TOC and δ-TOC are taken up to a greater extent (50% and 100% of the respective vitamer added to the extracellular milieu) than α-TOC (25%), and intracellular concentrations of all tocopherols in supplemented cells were between 1 and 4 μmol/L. Moreover, the fact that the methylation pattern of gamma conformation may directly or indirectly interact with PKC cannot be ruled out. This mechanism was proposed in the case of the inhibition of COX-2 activity in inflammatory cells and in the control of PPARγ of colon cancer cells by tocopherols (reviewed in Refs. [18,40]).

It is well known that NADPH oxidase plays a key role in the oxidative burst of stimulated neutrophils since it is the enzyme that catalyzes the one-electron reduction of O_2 to $O_2^{\bullet-}$. NADPH oxidase is dormant in resting cells because its components are divided between the cytosol and the membrane. Following neutrophil activation, cytosolic components (p40phox, p47phox and p67phox) translocate and join the membrane-bound part of the enzyme to form the fully functional form [41,42]. NADPH oxidases are the main source of $O_2^{\bullet-}$ production not only in circulation but also in the arterial wall. A correlation between enzyme activity and mRNA expression and between conventional risk factors and severity of atherosclerosis was observed [43–46]. At the same time, several natural occurring antioxidants, which include also α-TOC, exert a direct inhibitory effect on fully activated NADPH oxidase in cell lysates of PMA-stimulated neutrophils [19,20,25,47,48]. Accordingly, we also observed that all the tocopherols inhibit the activated NADPH oxidase in those conditions, and likely because of their effect on PKC, hypomethylated forms were more effective than α-TOC. Since NADPH oxidase was assessed in cell lysate after its activation by PMA in intact cells, it can be supposed that vitamin E compounds can counteract with the catalytic site and not prevent the assembly of enzyme subunits. Another possibility might be that vitamin E molecules inhibit or modify activation or binding of Rac GTPase, which is another factor essential for $O_2^{\bullet-}$ production by NADPH oxidase [49] together with the assembly of cytosolic phox proteins.

However, CEHCs did not inhibit cell lysate NADPH oxidase activity. Rather, they seem to activate the enzyme, suggesting that the presence of the phytyl chain on the chroman ring is essential for direct inhibition of NADPH oxidase enzyme activity. When the direct scavenging of $O_2^{\bullet-}$ by tocopherols and CEHCs was tested using the xanthine/xanthine oxidase system, it was found as expected [50] that none of the test molecules can be an effective $O_2^{\bullet-}$ scavenger. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid. During reoxidation of xanthine oxidase, molecular oxygen acts as an electron acceptor, thus leading to $O_2^{\bullet-}$ production [50]. As a consequence, this enzyme was reported to be involved in the pathogenesis of

Fig. 5. Relationship between superoxide anion production and the remaining PKC activity in phorbol-ester-stimulated neutrophils after preincubation with a physiologically relevant concentration of tocopherols and CEHC metabolites. Results are expressed as mean±S.D. of three independent experiments.

some oxidative-stress-mediated conditions, particularly in the endothelium after ischemia–reperfusion [51,52]. α-TOC [53] and many other natural compounds [19,20,25,28,54] are known to be in vitro inhibitors of xanthine oxidase enzyme, and this may have clinical relevance, especially with this enzyme activity being elevated in some conditions such as viral hepatitis infection [55] and acute myocardial infarction [56]. Accordingly, we showed that tocopherols (α-TOC, γ-TOC and δ-TOC) inhibit the activity of this enzyme (e.g., uric acid formation). However, and similar to the case of NADPH oxidase, the water-soluble metabolites did not affect the activity of this enzyme.

These data strongly suggest that a reduced $O_2^{\bullet-}$ production by tocopherols and CEHCs in intact neutrophils mostly occurs through the control of their metabolic activation via inhibition of the first step of PMA signaling, which is the activation of PKC, while direct inhibition of main ROS-generating enzymes such as NADPH oxidase and xanthine oxidase might play a secondary role.

Finally, the preliminary observation that neutrophils of a subject with high plasma concentration of α-TOC (near 50 μmol/L) responded by enhanced $O_2^{\bullet-}$ production when they were exposed with high levels of α-TOC could lead to the hypothesis that, at least, in vitro α-TOC might act as a pro-oxidant when its cellular concentrations surpass a certain threshold. This observation is in accordance with the findings of Kontush et al. [57], who also found that α-TOC behaves as a pro-oxidant in isolated LDL containing high α-TOC when oxidation was induced either with a low concentration of copper or with AAPH [57]. They hypothesized that it might be due to the prolonged residence of α-tocopheroxyl radical, which results in the pro-oxidant activity of α-TOC [57]. However, additional experiments are warranted to explain the pro-oxidant role of high α-TOC level in cells and biological fluids.

In conclusion, tocopherols and CEHC metabolites, when tested at extracellular concentrations resembling those found in human plasma, exerted a hypostimulatory effect on PMA-induced oxidative burst of human neutrophils. This effect correlated with the inhibition of PKC translocation and activity. The inhibitory potency of the different test compounds varied according to the degree of methylation of the chroman ring with the gamma forms as the most effective inhibitors. The fact that CEHC metabolites are as effective as, if not more potent than, their vitamer precursors suggests that the presence of the phytyl chain does not influence PKC-related signaling and metabolic activation of neutrophils. Individual properties of vitamin E compounds differ as concerns the direct activity inhibition of $O_2^{\bullet-}$ generating enzymes, NADPH oxidase and xanthine oxidase, tested under cell-free conditions. In this case, tocopherols but not CEHC metabolites were effective inhibitors. The control of PKC signaling, which is coupled with metabolic activation and $O_2^{\bullet-}$ production in neutrophils, may represent a key underlying mechanism for the antioxidant activity of vitamers and metabolites of the vitamin E family.

Acknowledgments

Research was sponsored by the Hungarian Scientific Research Found (Grant No. T 42550). We are indebted to Eisai Co. (Japan) for providing vitamin E metabolites.

References

[1] Packer L, Obermuller-Jevic UC. Vitamin E: an introduction. Parker L, Traber MG, Kraemer K, Frei B, editors.The antioxidant vitamins C and E. Champaign (Ill): USA AOCS Press; 2002. p. 133.
[2] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13:1145–55.
[3] Hiramatsu K, Arimori S. Increased superoxide production by mononuclear cells of patients with hypertriglyceridemia and diabetes. Diabetes 1988;37:832–7.
[4] Ludwig PW, Hunninghake DB, Hoidal JR. Increased leukocyte oxidative metabolism in hyperlipoproteinemia. Lancet 1982;2:348–52.
[5] Chan SS, Monteiro HP, Schindler F, Stern A, Junqueira VB. Alpha-tocopherol modulates tyrosine phosphorylation in human neutrophils by inhibition of protein kinase C activity and activation of tyrosine phosphatases. Free Radic Res 2001;35:843–56.
[6] Venugopal SK, Devaraj S, Yang T, Jialal I. Alpha-tocopherol decreases superoxide anion release in human monocytes under hyperglycemic conditions via inhibition of protein kinase C-alpha. Diabetes 2002;51:3049–54.
[7] Jiang Q, Christen S, Shigenaga MK, Ames BN. Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am J Clin Nutr 2001;74:714–22.
[8] Devaraj S, Traber MG. γ-Tocopherol. The new vitamin E? Am J Clin Nutr 2003;77:530–1.
[9] Wagner KH, Kamal-Eldin A, Elmadfa I. Gamma-tocopherol — an underestimated vitamin? Ann Nutr Metab 2004;48:169–88.
[10] Galli F, Lee R, Atkinson J, Floridi A, Kelly FJ. Gamma-tocopherol biokinetics and transformation in humans. Free Radic Res 2003;37:1225–33.
[11] Liu M, Wallmon A, Olsson-Mortlock C, Wallin R, Saldeen T. Mixed tocopherols inhibit platelet aggregation in humans: potential mechanism. Am J Clin Nutr 2003;77:700–6.
[12] Jiang Q, Elson-Schwab I, Courtemanche C, Ames BN. Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proc Natl Acad Sci U S A 2000;97:11494–9.
[13] Isnardy B, Wagner KH, Elmadfa I. Effects of alpha-, gamma-, and delta-tocopherols on the autoxidation of purified rapeseed oil triacylglycerols in a system containing low oxygen. J Agric Food Chem 2003;51:7775–80.
[14] Betancor-Fernandez A, Sies H, Stahl W, Polidori MC. In vitro antioxidant activity of 2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxychroman (alpha-CEHC), a vitamin E metabolite. Free Radic Res 2002;36:915–21.
[15] Galli F, Piroddi M, Jannone A, Pagliarani S, Tomasi A, Floridi A. A comparison between the antioxidant and peroxynitrite-scavenging functions of the vitamin E metabolites α- and γ-carboxyethyl-6-hydroxychromans. Int J Vitam Nutr Res 2004;74:362–73.
[16] Galli F, Stabile AM, Betti M, Conte C, Pistilli A, Rende M, et al. The effect of alpha- and gamma-tocopherol and their carboxyethyl hydroxychroman metabolites on prostate cancer cell proliferation. Arch Biochem Biophys 2004;423:97–102.
[17] Betti M, Minelli A, Canonico B, Castaldo P, Magi S, Aisa MC, et al. Antiproliferative effects of tocopherols (vitamin E) on murine glioma C6 cells: homologue-specific control of PKC/ERK and cyclin signaling. Free Radic Biol Med 2006;41:364–72.
[18] Hensley K, Benaksas EJ, Bolli R, Comp P, Grammas P, Hamdheydari L, et al. New perspectives on vitamin E: gamma-tocopherol and

carboxyelthylhydroxychroman metabolites in biology and medicine. Free Radic Biol Med 2004;36:1–15.
[19] Varga Z, Seres I, Nagy E, Ujhelyi L, Balla G, Balla J, et al. Structures prerequisite for antioxidant activity of silybin in different biochemical systems in vitro. Phytomedicine 2006;13:85–93.
[20] Varga Z, Nagy E, Katko M, Jeney V, Seres I, Paragh G, et al. Relationship of structure and antioxidant activity of synthetic silybin-derived molecules: identification of molecular structure responsible for antioxidant activity employing various models for inducing oxidative stress. In: Panglossi HV, editor. New developments in antioxidant research, chapter 6. Hauppauge, NY: Nova Science Publishers Inc; 2006. p. 113–51.
[21] Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968;21:77–89.
[22] Ruperez FJ, Barbas C, Castro M, Martiney S, Herrera E. Simplified method for vitamin E determination in rat adipose tissue and mammary glands by high-performance liquid chromatography. J Chromatogr 1998;823:483–7.
[23] Babior BM, Kipnes RS, Curnutte JJ. Biological defense mechanism. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 1970;52:791–4.
[24] Varga Z, Czompa A, Kakuk G, Antus S. Inhibition of superoxide anion release and hydrogen peroxide formation in PMNLs by flavonolignans. Phytother Res 2001;15:608–12.
[25] Varga Z, Czompa A, Újhelyi L, Kiss A, Balla J, Antus S. Effect of silybin on phorbol myristate actetate-induced protein kinase C translocation, NADPH oxidase activity, and apoptosis in human neutrophils. Phytomedicine 2004;11:206–12.
[26] Lowry OH, Rosenbrough NJ, Farr A, Randall RJ. Protein measurement with folin reagent. J Biol Chem 1951;125:265–73.
[27] Jones OT, Hancock JT. Assays of plasma membrane NADPH oxidase. In: Packer L, editor. Methods in enzymology, Vol. 233. Academic Press; 1994. p. 222–9.
[28] Sheu SY, Lai CH, Chiang HC. Inhibition of xanthine oxidase by purogallin and silymarin group. Anticancer Res 1998;18:263–7.
[29] Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 1982;257: 7847–51.
[30] Mosior M, Newton AC. Calcium-independent binding to interfacial phorbol esters causes protein kinase C to associate with membranes in the absence of acidic lipids. Biochemistry 1996;35:1612–23.
[31] Slater SJ, Ho C, Stubbs CD. The use of fluorescent phorbol esters in studies of protein kinase C–membrane interactions. Chem Phys Lipids 2002;116:75–91.
[32] Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, et al. Vitamin E mediates cell signaling and regulation of gene expression. Ann N Y Acad Sci 2004;1031:86–95.
[33] Ogru E, Libinaki R, Gianello R, West S, Munteanu A, Zingg JM, et al. Modulation of cell proliferation and gene expression by alpha-tocopheryl phosphates: relevance to atherosclerosis and inflammation. Ann N Y Acad Sci 2004;1031:405–11.
[34] Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, Gellert N, et al. Induction of cancer cell apoptosis by alpha-tocopheryl succinate: molecular pathways and structural requirements. FASEB J 2001;15: 403–15.
[35] Kanno T, Utsumi T, Takehara Y, Ide A, Akiyama J, Yoshioka T, et al. Inhibition of neutrophil-superoxide generation by alpha-tocopherol and coenzyme Q. Free Radic Res 1996;24:281–9.
[36] Nixon JB, McPhail LC. Protein kinase C (PKC) isoforms translocate to Triton-insoluble fraction in stimulated human neutrophils: correlation of conventional PKC with activation of NADPH oxidase. J Immunol 1999;163:4574–82.
[37] Aisa MC, Annetti C, Piroddi M, Galli F. Vitamin E and cell signaling. In: Preedy VR, Watson RR, editors. The encyclopedia of vitamin E. Oxfordshire (UK): CAB International; 2006. p. 343–58.
[38] Kaempf-Rotzoll DE, Traber MG, Arai H. Vitamin E and transfer proteins. Curr Opin Lipidol 2003;14:249–54.
[39] Tran K, Chan AC. Comparative uptake of alpha- and gamma-tocopherol by human endothelial cells. Lipids 1992;27:38–41.
[40] Campbell SE, Stone WL, Whaley SG, Qui M, Krishnan K. Gamma (γ) tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma (γ) expression in SW 480 human colon cancer cell lines. BMC Cancer 2003;3:25.
[41] Mariedonneau-Parini I, Tringale SM, Tauber AI. Identification of distinct activation pathways of the human neutrophil NADPH oxidase. J Immunol 1986;137:2925–9.
[42] Clark RA, Volpp BD, Leidal KG, Nauseef WM. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. J Clin Invest 1990;85:714–21.
[43] Dusi S, Della Bianca V, Grzeskowiak M, Rossi F. Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca(2+)-depleted human neutrophils. J Biochem 1993;290:173–8.
[44] Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res 2000;86:E85−E90.
[45] Sorescu D, Weiss D, Lassegue B, Clempus RE, Szocs K, Sorescu GP, et al. Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation 2002;105:1429–35.
[46] Guzik TJ, Sadowski J, Kapelak B, Jopek A, Rudzinski P, Pillai R, et al. Systemic regulation of vascular NAD(P)H oxidase activity and nox isoform expression in human arteries and veins. Arterioscler Thromb Vasc Biol 2004;24:1614–20.
[47] Cachia O, Benna JE, Pedruzzi E, Descomps B, Gougerot-Pocidalo MA, Leger CL. Alpha-tocopherol inhibits the respiratory burst in human monocytes. Attenuation of p47(phox) membrane translocation and phosphorylation. J Biol Chem 1998;273:32801–5.
[48] Chen X, Touyz RM, Park JB, Schiffrin EL. Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. Hypertension 2001;38:606–11.
[49] Bokoch GM, Zhao T. Regulation of the phagocyte NADPH oxidase by Rac GTPase. Antioxid Redox Signal 2006;8:1533–48.
[50] Halliwell B, Guttridge JM. Free radicals in biology and medicine. 3rd ed. New York: Oxford University Press; 1999. p. 13–4, 212–5, 617–783.
[51] McCord JM. Oxygen-derived radicals: a link between reperfusion injury and inflammation. Fed Proc 1987;46:2402–6.
[52] Kahl S, Elsasser TH. Endotoxin challenge increases xanthine oxidase activity in cattle: effect of growth hormone and vitamin E treatment. Domest Anim Endocrinol 2004;26:315–28.
[53] Irmak MK, Fadillioglu E, Sogut S, Erdogan H, Gulec M, Ozer M, et al. Effects of caffeic acid phenethyl ester and alpha-tocopherol on reperfusion injury in rat brain. Cell Biochem Funct 2003;21:283–9.
[54] Sanhueza J, Valdes J, Campos R, Garrido A, Valenzuela A. Changes in the xanthine dehydrogenase/xanthine oxidase ratio in the rat kidney subjected to ischemia–reperfusion stress: preventive effect of some flavonoids. Res Commun Chem Pathol Pharmacol 1992;78:211–8.
[55] Battelli MG, Musiani S, Valgimigli M, Gramantieri L, Tomassoni F, Bolondi L, et al. Serum xanthine oxidase in human liver disease. Am J Gastroenterol 2001;96:1194–9.
[56] Kojima S, Sakamoto T, Ishihara M, Kimura K, Miyazaki S, Yamagishi M, et al. Japanese Acute Coronary Syndrome Study (JACSS) Investigators. Prognostic usefulness of serum uric acid after acute myocardial infarction (the Japanese Acute Coronary Syndrome Study). Am J Cardiol 2005;96:489–95.
[57] Kontush A, Finckh B, Karten B, Kohlschutter A, Beisiegel U. Antioxidant and prooxidant activity of alpha-tocopherol in human plasma and low density lipoprotein. J Lipid Res 1996;37:1436–48.