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The extraordinary antioxidant activity of vitamin E phosphate

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Abstract

The antioxidant activities of RRR-vitamin E (VE), all-*rac*-vitamin E (all-*rac*-VE), trolox, RRR-vitamin E acetate (VEA), all-*rac*-vitamin E phosphate (VEP) and RRR-vitamin E succinate (VES) were compared. In this study, the rank order in the inhibition of lipid peroxidation (LPO) of VE and its derivatives was trolox>VE ≈ all-*rac*-VE>VEA>VES. VE and trolox inhibited LPO in non-heated and heated rat liver microsomes. It has generally been accepted that this is due to scavenging of free radicals by these antioxidants, and during this protection the antioxidants are oxidized. VEA and VES have to be converted into VE by esterases to obtain antioxidant activity against LPO. VEP, however, had a potent antioxidant effect of its own without conversion to VE. In contrast to VE, VEP is not consumed during this protection. Of the compounds tested, VEP is the most potent in induction of hemolysis of erythrocytes. EPR experiments using the spin label 16-doxylstearic acid showed that VEP reduces membrane fluidity, in contrast to VE. This indicates that VEP acts as a detergent and forms a barrier that might inhibit the transfer of radicals from one polyunsaturated fatty acid to another. This new mechanism may form the basis for a new class of antioxidants.
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Keywords: Vitamin E; Trolox; Vitamin E acetate; Vitamin E phosphate; Vitamin E succinate; Antioxidant activity; Microsome

1. Introduction

Free radicals are continuously produced within living cells as a result of metabolic reactions. In addition, numerous exogenous sources, including xenobiotics and radiation, can induce free radicals. Although cells have an elaborate defense system that provides protection against these free radicals, the development of cancer, cardiovascular and neurological diseases, and other oxidative stress-mediated dysfunctions occur [1–3].

Vitamin E (VE) is one of the most important lipid-soluble primary defense antioxidants. Vitamin E is a generic term used for several naturally occurring tocopherols and tocotrienols. The VE molecule can be divided into two parts, a hydroxyl-bearing aromatic system (one phenolic and one heterocyclic ring, called the chroman head) that is responsible for its antioxidant properties, and either a saturated (tocopherols) or polyunsaturated (tocotrienols) hydrocarbon tail for the orientation of VE in the lipid membrane [4].

In its function as a chain-breaking antioxidant, VE rapidly transfers its phenolic H-atom to a lipid peroxyl radical, converting it into a lipid hydroperoxide and a VE radical [5,6]. The VE radical can be reduced to VE by vitamin C or reduced glutathione or alternatively it is further oxidized to VE quinone [6]. VE is often supplemented as a chemically stable ester derivative such as RRR-vitamin E acetate (VEA), all-*rac*-vitamin E phosphate (VEP) or RRR-vitamin E succinate (VES). These esters are expected to possess no antioxidant effect due to the shielding of the phenolic active antioxidant group by acetate, phosphate or succinate, respectively (Fig. 1).

The aim of this study is to compare the intrinsic antioxidant activity of VE and its derivatives, trolox, VEA, VEP and VES, in non-heated and heated microsomes.

2. Materials and methods

2.1. Chemicals

The tested compounds, RRR-α-tocopherol (VE), all-*rac*-α-tocopherol (all-*rac*-VE), RRR-α-tocopherol succinate (VES), RRR-α-tocopherol acetate (VEA), all-*rac*-α-tocopherol phosphate (VEP) and trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), in addition to alkaline

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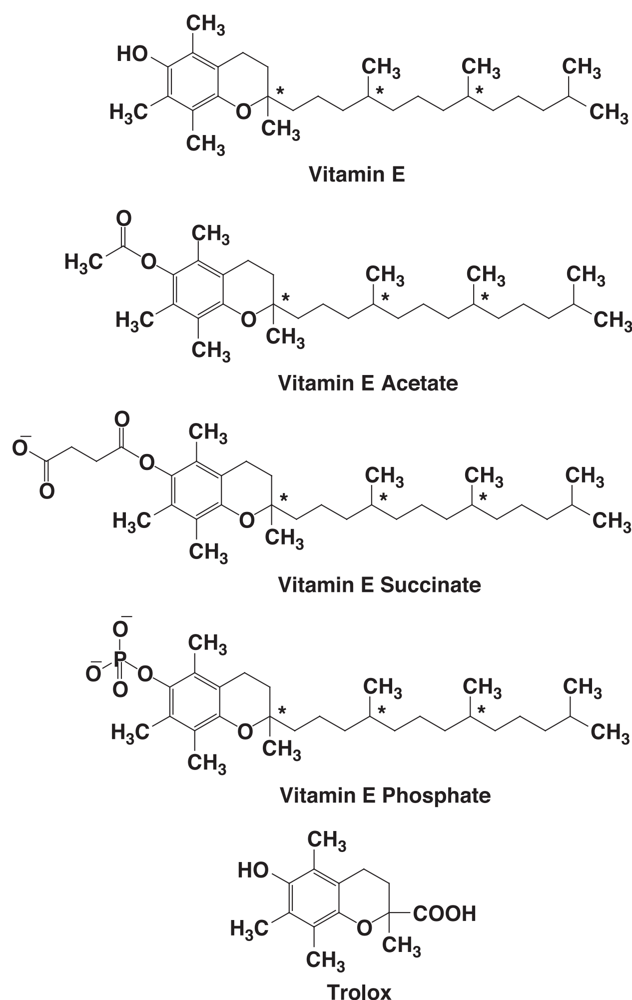

Fig. 1. Chemical structure of VE and its derivatives VEA, VES, VEP and Trolox. The asymmetrical carbon atoms are indicated by an asterisk.

phosphatase (orthophosphoric-monoester phosphohydrolase[alkaline optimum]: EC 3.1.3.1), 16-doxylstearic acid and ABTS (2,2′ -azinobis(3-ethylbenzthiazoline-6-sulfonic acid), were obtained from Sigma (St. Louis, MO, USA). All other used chemicals were of analytical grade purity.

2.2. Isolation of rat liver microsomes

Male Lewis rats, 200–250 g, were killed by decapitation. Microsomes were prepared according to Haenen and Bast [7] with a slight modification. Livers were removed and homogenized (1:2 w/v) in ice-cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA. The homogenate was centrifuged at 10,000×*g* for 30 min at 4 °C. Subsequently, the supernatant was centrifuged at 65,000×*g* for 60 min at 4 °C. The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml), corresponding with 1–1.3 mg protein/ml, and stored at −80 °C. Before use, the microsomes were thawed and diluted fivefold with ice-cold Tris–HCl buffer (50 mM, pH 7.4) containing 150 mM KCl and washed twice followed by centrifugation at 65,000×*g* for 40 min at 4 °C. Finally, the pellet was resuspended in 4-ml Tris–HCl buffer and then used. The protein concentration was measured according to Smith et al. [8].

2.3. Lipid peroxidation (LPO) assay

2.3.1. Incubation conditions

At 37 °C, 2150 μl of Tris–HCl/KCl buffer was added to 1-ml non-heated microsomes or heated microsomes (3 min at 90 °C); the final concentration was 0.5 g liver tissue/ml. Twenty-five microliters of a stock solution of a test compound was added, giving a final concentration of 5, 20, or 40 μM (VE, trolox, VEA, and VES were dissolved in absolute ethanol, and VEP was dissolved in deionized water). Control experiments showed that at the used concentration, the solvent has no effect on the LPO. Five hundred microliters of an ascorbate solution was added, giving a final concentration of 0.2 mM. The reaction started by adding 300 μl of a freshly prepared ferrous sulfate solution giving a final concentration of 10 μM. The reaction was stopped after 20 min of incubation as described in Section 2.3.2. The concentration that reduces LPO by 50% (IC_{50}) was calculated using the lowest concentration tested giving a protection more than 50% and the concentration tested that was just below that concentration. A linear relationship between the concentration of the antioxidant and the inhibition of LPO was used in the calculation.

2.3.2. Spectral measurements

LPO was assayed by measuring thiobarbituric acid (TBA) reactive material as described previously [7]. An aliquot of the incubation mixture (0.3 ml) was stopped by adding 2 ml of an ice-cold TBA–trichloroacetic acid–HCl–butylhydroxytoluene (BHT) solution. The mixture was heated for 15 min at 90 °C and then centrifuged for 5 min. The absorbance was determined at 535 vs. 600 nm. The TBA–trichloroacetic acid–HCl–BHT solution was prepared by dissolving 41.6 mg TBA/10 ml trichloroacetic acid (16.8 w/v in 0.125 N HCl). To 10-ml TBA–trichloroacetic acid–HCl, 1-ml BHT (1.5 mg/ml ethanol) was added. The added chemicals did not interfere with the assay in the concentrations used.

2.4. The trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay described by Re et al. [9] was used with minor modifications. The ABTS stock solution was prepared by dissolving 30 mg of ABTS in 7.8 ml of 2.46 mM potassium peroxodisulfate. The ABTS stock solution was diluted with 100 mM phosphate buffer (pH 7.4). The absorbance of this radical solution at 734 nm had to be between 0.68 and 0.73 at 37 °C. For measuring the antioxidant capacity, 50 μl of the test compounds was mixed with 950 μl of the ABTS solution. Absorbance was monitored at 734 nm for 6 min. The antioxidant capacity was calculated according to van den Berg et al. [10].

2.5. VE determination

2.5.1. Conversion of VEP into VE

VEP (500 μg/ml) was incubated with alkaline phosphatase (3 U/ml) at 37 °C. The reaction was carried out in a solution, containing 8.3 mM sodium carbonate, 5.5 mM glucose, 1.2 mM magnesium chloride, 0.5 mM potassium chloride and 0.2 mM zinc sulfate (pH 7.4). To stop the reaction, 250 μl of the reaction mixture was added to a cold solution of absolute ethanol (2 ml) with 750 μl of deionized water on ice. VE was extracted and determined as described in Section 2.5.3.

2.5.2. VE content of microsomes

One milliliter or non-heated and heated microsomes (1–1.3-mg protein) was incubated with 100 μM of the VE derivatives for 20 min at 37 °C. The reaction was stopped by addition of 100 μl of 2.5% SDS, and 2 ml ethanol containing 1 mg BHT to prevent further oxidation. VE was extracted and determined as described in Section 2.5.3. Vitamin E nicotinate (200 μg/ml) was used as an internal standard.

2.5.3. VE extraction and HPLC determination

The mixture (from Sections 2.5.1, 2.5.2 or 2.6) was shaken for 5 min, then 3 ml of hexane was added. Again the mixture was shaken for 15 min. After centrifugation, the upper hexane layer was aspirated in a conical glass tube and evaporated under nitrogen. The residue was dissolved in 200 μl isopropyl alcohol for HPLC analysis according to Burton et al. [11] using a Nucleosil C-18 column and absolute methanol as a mobile phase**,** with a flow rate of 2 ml/min. VE was detected at 295 nm.

2.6. VEP content of microsomes

One hundred micromolar of VEP was added to microsomes as described in Section 2.3.1. After 1 h incubation, the samples were centrifuged at 65,000×*g* for 40 min. The pellet was resuspended in 4 ml Tris–HCl and 250 μl of 4 M KOH and then samples were heated at 90 °C for 3 min for complete rapture of microsomal membrane. After cooling (room temperature), the samples were neutralized (pH 7.4). At 37 °C, 10 U/ml alkaline phosphatase was added and the samples were incubated for 30 min to convert VEP into VE. Extraction and determination of VE was carried out by the procedure described above in Section 2.5.3. Experiments without the addition of alkaline phosphatase were performed as blank.

2.7. Membrane fluidity of human erythrocytes

The membrane fluidity of erythrocytes from healthy donors was measured by means of electron paramagnetic resonance (EPR) spectroscopy. Blood samples were obtained by venipuncture. Heparin was used as anti-coagulant. The plasma and buffy coat were carefully removed after centrifugation at 2000×*g* for 10 min at 4 °C. Erythrocytes were washed three times with phosphate-buffered saline (PBS) and finally resuspended in PBS at a haematocrit of 45%. Half milliliter of PBS with the test compound (final concentration; 50 μM) was added to 0.5 ml of the erythrocyte containing PBS. After 90 min incubation at 37 °C, spin labeled 16-doxylstearic acid was added and the samples were incubated for 90 min at 37 °C. Finally, EPR measurements were performed using spectrometer (Bruker EMX, GmbH, Freiburg, Germany) under the following conditions: microwave power, 2 mW; modulation amplitude, 1 G; scan width, 50 G; modulation frequency, 100 kHz; and temperature, 30 °C. Membrane fluidity was calculated from the peak height ratio (h_0/h_{-1}) as described previously [12].

2.8. Erythrocyte hemolysis

Erythrocyte hemolysis was assessed as described by Neuzil et al. [13] with minor modifications. Erythrocytes were prepared from fresh heparin-treated human blood. One milliliter blood was diluted 80 times with saline, followed by centrifugation at 2000×*g* for 10 min at 4 °C. The pellet was resuspended with 45 ml saline. One milliliter of the erythrocyte suspension was incubated with different concentrations of the test compounds at 37 °C. After 2 h incubation, the samples were centrifuged for 10 min at 2000×*g*. Supernatant was diluted two times with saline, and the absorbance at 546 nm was determined.

2.9. Statistics analysis

All results are expressed as mean±S.D. Differences were tested using Student *t*-test with a *P* value of 0.05.

3. Results

The TEAC of trolox is by definition 1. The TEAC of VE and all-*rac*-VE were 0.97±0.05 and 0.96±0.03, respective-

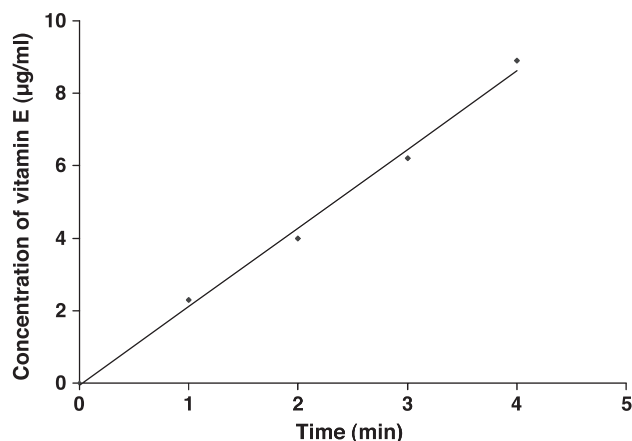

Fig. 2. Hydrolysis of VEP by alkaline phosphatase. Experimental conditions are given in Section 2.5.1.

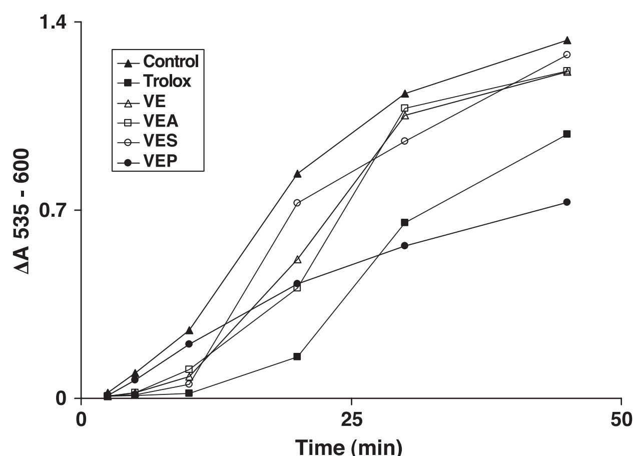

Fig. 3. Time course of the effect of 20 μM of VE, Trolox, VEA, VES, and VEP on LPO in non-heated microsomes. Experimental conditions are given in Section 2.3. Values are presented as mean of at least three separate experiments.

ly. The TEAC of VEA, VES, and VEP were 0.01±0.02, 0.01±0.01 and 0.01±0.01, respectively.

Alkaline phosphatase (3 U/ml) can hydrolyze VEP (500 μg/ml) into VE (Fig. 2) and thus unshield the OH moiety. The rate of VE formation was 0.718±0.048 μg min^{-1} U^{-1}.

VE, trolox, VEA, and VES induced a lag time in the time course of LPO in non-heated microsomes (Fig. 3). The antioxidant activities of VE, VEA and VES were approximately the same. The antioxidant activity of trolox was superior to that of VE, VEA or VES. VEP was also a potent antioxidant but showed another profile, i.e. it reduced the extent of LPO without inducing a lag time (Fig. 3).

The protection provided by the test compounds in non-heated microsomes against LPO was dose-dependent. The IC_{50} values of VEP, trolox, VE, VEA, and VES were 25±2, 20±1, 30±2, 35±2, and >40 μM, respectively.

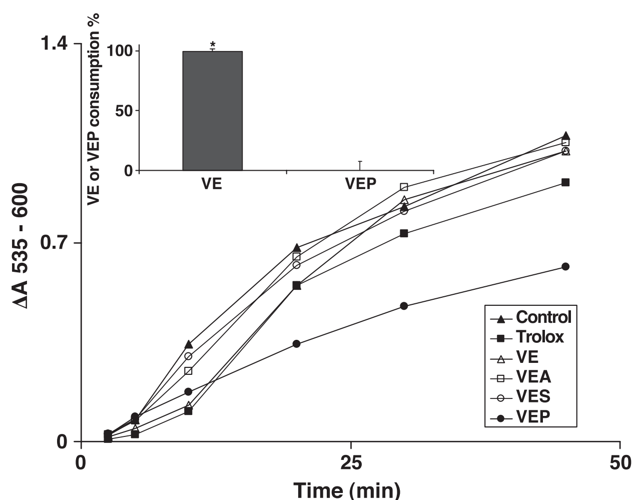

Fig. 4. Time course of the effect of 20 μM of VE, Trolox, VEA, VES, and VEP on LPO in heated microsomes. Experimental conditions are given in Section 2.3. The insert shows the percentage of VE and VEP consumption. Values are presented as mean of at least three separate experiments.

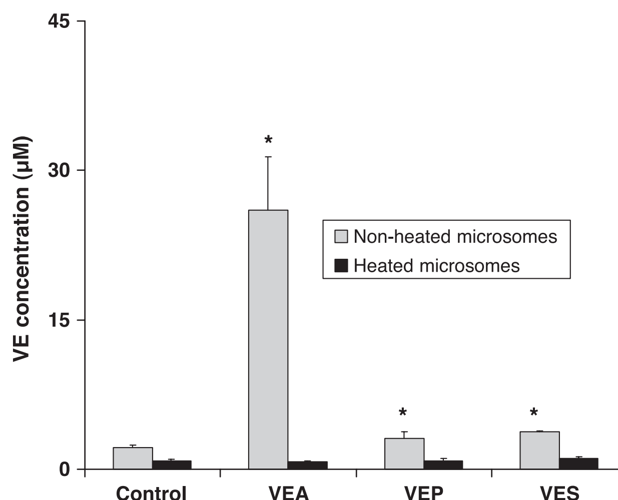

Fig. 5. Formation of VE from VEA, VES and VEP in non-heated and heated microsomes after 20-min incubation. Experimental conditions are given in Section 2.5.2. Values are presented as mean±S.D. of at least three separate experiments. (*) indicates a significant change vs. control ($P<0.05$).

Heating of the microsomes did not greatly affect the antioxidant activity of VE and trolox (Fig. 4), whereas the antioxidant activity of VEA and VES almost completely disappeared. The potent antioxidant effect of VEP in heated microsomes is comparable to that in non-heated microsomes. In heated microsomes, the IC_{50} values of VEP, trolox, VE, VEA, and VES were 25±1, 23±1, >40, ≫40 and ≫40 μM, respectively.

Formation of VE from VEA, VEP and VES in non-heated and heated microsomes was also measured. VEA showed a larger conversion into VE in non-heated microsomes compared to VES and VEP. No conversion of the esters into VE in heated microsomes could be observed (Fig. 5).

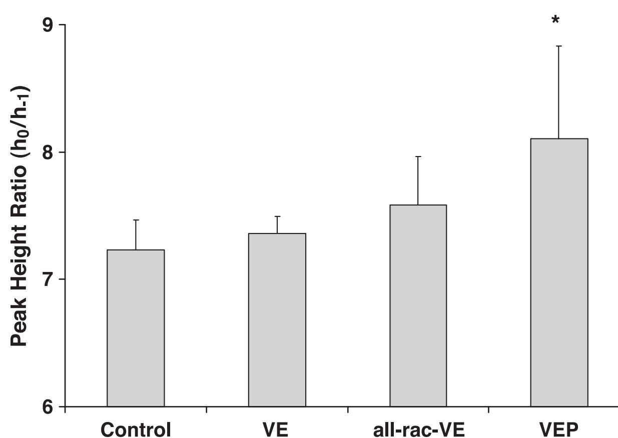

Fig. 6. Effect of VE, all-*rac*-VE and VEP on membrane fluidity of erythrocytes. Membrane fluidity is expressed as the ratio of the peak height (h_0/h_{-1}) [12]. The higher the peak height ratio, the lesser the freedom of motion of the spin label in biomembrane bilayer, indicating a lower membrane fluidity [12]. Experimental conditions are given in Section 2.7. The data are presented as mean±S.D. of at least three separate experiments. (*) indicates a significant change of VEP vs. control ($P<0.05$).

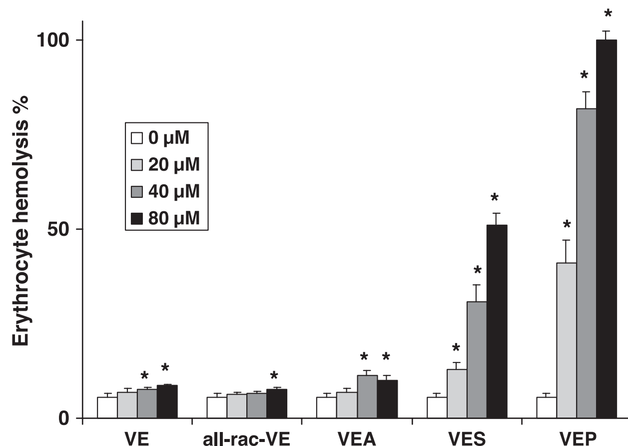

Fig. 7. Induction of hemolysis by VE and VE esters. Isolated erythrocytes were incubated for 2 h with different concentrations of compounds at 37 °C. The data are presented as mean±S.D. of at least three separate experiments. (*) indicates a significant change vs. control ($P<0.05$).

To further investigate the mechanisms of protection, the consumption of VE and VEP during the protection against LPO was assessed. It was found that addition of 100 μM VE to the microsomes was fully consumed after 1 h incubation. In contrast, 100 μM VEP was not consumed during the 1 h incubation with heated microsomes (Fig. 4, insert).

To determine the effect of VEP on biomembranes, membrane fluidity of erythrocytes was monitored. Membrane fluidity is determined using EPR by calculating the ratio of h_0/h_{-1} [12]. At a concentration of 50 μM, VEP induced a significant decrease in membrane fluidity. The peak height ratio with VEP is 8.1 ± 0.7 compared to that of control, h_0/h_{-1} is 7.2 ± 0.2. Both VE and all-*rac*-VE at concentration 50 μM have no significant effect on the erythrocyte membrane fluidity (Fig. 6).

Hemolyses of erythrocytes by VE, all-*rac*-VE, VEA, VES and VEP were also determined (Fig. 7). At a concentration of 80 μM, VEP induced 100% erythrocyte hemolysis, whereas the same concentration of VES induced only 50% erythrocyte hemolysis. VE, all-*rac*-VE and VEA were not effective in inducing erythrocyte hemolysis.

4. Discussion

In the present study, the antioxidant action of trolox, VE and several VE esters was compared. First, the ability of these VE-derivatives to scavenge a stable ABTS radical was measured. The obtained data were normalized to trolox, which has a TEAC of 1. The TEAC value of VE was also approximately 1 for either VE or all-*rac*-VE. This was expected since VE and all-*rac*-VE contain the same antioxidant moiety as trolox. In contrast, the TEAC of VEA, VES, and VEP was almost 0. This could be anticipated because the antioxidant moiety in these compounds is shielded by acetate, succinate and phosphate, respectively. The small antioxidant capacity of the esters is possibly due to a small fraction (<1%) of free VE.

Our results showed that the phosphate group of VEP can be removed by alkaline phosphatase, which results in the formation of VE (Fig. 2) as has previously been demonstrated by Nakayama et al. [14]. Esterases can hydrolyze VEA and VES into VE [15,16].

As expected, VE and trolox decreased LPO induced by the iron/ascorbate system, both in non-heated and heated rat liver microsomes (Figs. 3 and 4). This is due to the free radical scavenging property of the free hydroxyl group. Once VE and trolox are consumed, LPO starts. During the antioxidant activity, the chroman head is finally converted into a quinone [6]. The higher efficacy of trolox may be due to the absence of the hydrocarbon tail, which makes it less lipophilic in comparison with VE. Similarly, Kagan et al. [17] showed that decreasing the length of the phytyl chain of α-tocopherol from 16 carbon atoms to 11, 6 or 1 decreased the concentration needed to inhibit LPO in vitro. No difference in antioxidant effect was found between VE and all-*rac*-VE, indicating that the stereochemistry of the hydrocarbon tail has no major influence. This is of importance since VEP is only available as all-*rac*-VEP and the stereochemistry of its tail is probably of minor importance in the in vitro experiments.

The inhibition of LPO by VEA and VES in non-heated microsomes (Fig. 3) occurs as a secondary event following the enzymatic conversion of VEA and VES into VE (Fig. 5) [15,16]. In non-heated microsomes, the higher antioxidant activity of VEA in comparison to VES is probably due to a more extensive conversion of VEA into VE compared to VES. The lack of inhibition of LPO by VEA and VES in heated microsomes (Fig. 4) corroborates this explanation because denaturation of the enzymes in the microsomes by heating prevented hydrolysis of VEA and VES into VE.

The rate of conversion of VEP into VE was less than that of VEA in non-heated microsomes (Fig. 5). VEP had no direct radical scavenging activity as demonstrated in the TEAC assay. Nevertheless, VEP was a more efficient antioxidant than the other esters and VE, not only in non-heated microsomes but also in heated microsomes. Also, the profile of the time course of inhibition of LPO points to a different mechanism of inhibition. VE, trolox, VEA, and VES induced a lag time in the onset of LPO. VEP induced no lag time, it reduced the extent of LPO. Additionally, in heated microsomes, VEP is not consumed during the LPO (Fig. 4, insert). Apparently, the antioxidant effect of VEP is not due to scavenging of free radicals but rather to another mechanism. A clue for this mechanism may be found in the chemical structure of VEP. VEP has a hydrophilic polar phosphate group attached to the chroman head and the hydrophobic hydrocarbon tail (Fig. 1). This might enable VEP to be incorporated into biomembranes in such a way that its polar lipophobic phosphate group is

in the water–membrane interface, and that the lipophilic hydrocarbon chain tail is embedded inside the lipid membrane. In this way, VEP could act as a barrier for the transfer of radicals from one polyunsaturated fatty acid to another one. This may shorten the propagation phase of LPO, resulting in less peroxidation. This is in line with the finding that VEP reduces membrane fluidity (Fig. 6). Results from this study also revealed that VEP induces hemolysis in erythrocytes much more efficiently than the other compounds (Fig. 7).

Mabile et al. [15] reported that trolox exhibited a more potent cytoprotective effect than VE against oxidized LDL-induced endothelial cell toxicity, whereas VEA and VES were almost completely ineffective. In contrast, Carini et al. [16] emphasized that VES exerted a more effective antioxidant protection than an equivalent amount of VE against iron/ascorbate-mediated peroxidation in microsomes. In the present study, the rank order of the antioxidant activity of VE and its derivatives in microsomal LPO is trolox>VE≈all-*rac*-VE>VEA>VES. Recently, Nakayama et al. [14] reported that pretreatment of cultured mouse skin with VEP provided significant protection greater than VEA against UV-B-induced skin damage characterized by reduced LPO, sunburn cell formation, and DNA degradation. Nakayama et al. [14] hypothesized that the potent protective effect of VEP might be due to the high conversion of VEP into VE in cultured mouse skin compared to that of VEA. Our study showed that in lipid membranes, VEP has a potent antioxidant effect of its own which is not due to the conversion into VE. The differences in antioxidant activity of the compounds and different mechanisms in the reported studies are probably due to differences in the experimental conditions.

With respect to the antioxidant effect of VES, it should be noted that in control microsomes, VES and VEP yielded the same amount of VE. VES did induce a small lag time in the time course of LPO. VEP did not induce a lag time. This indicates that the antioxidant effect of VES cannot be explained only by its conversion to VE [15,16]. Possibly, a moderate detergent-like effect of VES, as observed in the hemolysis of erythrocytes, is involved. An antioxidant effect of VES was, however, not found in heated microsomes. The reduction of membrane fluidity by VEP can be explained both by the protection against LPO and induction of erythrocyte hemolysis. In various pathological conditions, LPO plays a major role in the etiology. It is speculated that the extraordinary antioxidant effect of VEP against LPO might be of importance in the treatment of these pathologies. Data on bioavailability and metabolism are needed to support this possible use.

In conclusion, VEP has a potent antioxidant effect of its own in contrast to VEA and VES. The antioxidant mechanism differs from that of VE. The mechanism used by VEP may lead to the development of a new class of antioxidants that might strengthen our therapeutic arsenal against free radical-mediated disorders.

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