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DIHYDROLIPOIC ACID—A UNIVERSAL ANTIOXIDANT BOTH IN THE MEMBRANE AND IN THE AQUEOUS PHASE

REDUCTION OF PEROXYL, ASCORBYL AND CHROMANOXYL RADICALS

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Abstract—Thioctic (lipoic) acid is used as a therapeutic agent in a variety of diseases in which enhanced free radical peroxidation of membrane phospholipids has been shown to be a characteristic feature. It was suggested that the antioxidant properties of thioctic acid and its reduced form, dihydrolipoic acid, are at least in part responsible for the therapeutic potential. The reported results on the antioxidant efficiency of thioctic and dihydrolipoic acids obtained in oxidation models with complex multicomponent initiation systems are controversial. In the present work we used relatively simple oxidation systems to study the antioxidant effects of dihydrolipoic and thioctic acids based on their interactions with: (1) peroxyl radicals which are essential for the initiation of lipid peroxidation, (2) chromanoxyl radicals of vitamin E, and (3) ascorbyl radicals of vitamin C, the two major lipid- and water-soluble antioxidants, respectively. We demonstrated that: (1) dihydrolipoic acid (but not thioctic acid) was an efficient direct scavenger of peroxyl radicals generated in the aqueous phase by the water-soluble azoinitiator 2,2′-azobis(2-amidinopropane)-dihydrochloride, and in liposomes or in microsomal membranes by the lipid-soluble azoinitiator 2,2′-azobis(2,4-dimethylvaleronitrile); (2) both dihydrolipoic acid and thioctic acid did not interact directly with chromanoxyl radicals of vitamin E (or its synthetic homologues) generated in liposomes or in the membranes by three different ways: UV-irradiation, peroxyl radicals of 2,2′-azobis(2,4-dimethylvaleronitrile), or peroxyl radicals of linolenic acid formed by the lipoxygenase-catalyzed oxidation; and (3) dihydrolipoic acid (but not thioctic acid) reduced ascorbyl radicals (and dehydroascorbate) generated in the course of ascorbate oxidation by chromanoxyl radicals. This interaction resulted in ascorbate-mediated dihydrolipoic acid-dependent reduction of the vitamin E chromanoxyl radicals, i.e. vitamin E recycling. We conclude that dihydrolipoic acid may act as a strong direct chain-breaking antioxidant and may enhance the antioxidant potency of other antioxidants (ascorbate and vitamin E) in both the aqueous and the hydrophobic membraneous phases.

Lipid peroxidation is an important component of oxidative damage [1, 2]. Oxidative modification of phospholipid polyunsaturated fatty acid residues has been linked with altered membrane structure, barrier function and catalytic activity [3]. Thiols are thought to play a pivotal role in protecting cells against lipid peroxidation. McCay *et al.* [4] demonstrated that lipid peroxidation in rat liver microsomes is inhibited by a GSH-dependent enzymic liver cytosolic factor not identical to glutathione peroxidase. These observations were confirmed by data from different laboratories which reported the presence in liver cytosol of a heat-labile, GSH-dependent factor containing sulfhydryl groups necessary for its activity [5–10]. Later experiments showed that GSH protection of rat liver microsomes is independent of the selenium status of the rats but is dependent upon their vitamin E status [6, 11, 12]. It was suggested that the GSH-dependent enzymic factor prevents irreversible oxidation of vitamin E (tocopherol) by regenerating it from its phenoxyl (tocopheroxyl) radical [5–14]. Ascorbate was shown to enhance the GSH-dependent protection of liver microsomes isolated from vitamin E-supplemented rats [14]. Based on these findings a concept of "free radical reductase" was put forward to emphasize the existence of a specific thiol-dependent enzymic mechanism for vitamin E free radical reduction in membranes [4–18].

Dihydrolipoic acid is a potent sulfhydryl reductant: redox potential of the dihydrolipoic acid/thioctic (lipoic) acid couple is −0.32 V (compared to −0.24 V for the GSH/GSSG couple) [19, 20]. It was demonstrated recently that dihydrolipoic acid shows vitamin E- and GSH-related antioxidant activity [21, 22]. Thioctic acid is presently used as a therapeutic agent in a variety of diseases, including liver and neurological disorders [23–26]. Patients diagnosed with liver cirrhosis, diabetis mellitus, atherosclerosis and polyneuritis have been found to contain a reduced level of endogenous thioctic acid [25–27]. Since enhanced free radical peroxidation of membrane phospholipids has been shown to be a characteristic feature of these pathologies, studies

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of the molecular mechanism(s) of antioxidant effects of dihydrolipoic acid/thioctic acid have been carried out recently in various systems [21, 22, 28–31]. However, the results of these experiments are controversial.

Bast and Haenen [21] and Pruijn *et al.* [28] reported that neither dihydrolipoic acid nor thioctic acid itself displayed protection against microsomal lipid peroxidation induced by Fe^{2+} + ascorbate. Dihydrolipoic acid even has a pro-oxidant activity presumably due to reduction of Fe(III). In contrast to that, Muller [25] and Muller and Menzel [29] showed that both thioctic acid and dihydrolipoic acid are able to inhibit Cd(II)-stimulated lipid peroxidation and protect isolated hepatocytes against acute Cd(II) toxicity. Bonomi *et al.* [32, 33] found that dihydrolipoic acid and dihydrolipoamide are potent iron chelators. However, the efficiency of dihydrolipoic acid– and dihydrolipoamide–iron complexes in the promotion/inhibition of lipid peroxidation is unknown. Thus, it is not surprising that both stimulatory and inhibitory effects of dihydrolipoic acid have been found in systems with transition metal-induced lipid peroxidation [30, 31]. Suzuki *et al.* [34] demonstrated that dihydrolipoic acid (but not thioctic acid) is efficient in quenching superoxide radicals. However, both dihydrolipoic acid and thioctic acid are almost equally efficient in their interaction with hydroxyl radicals.

Studies from several laboratories demonstrated that dihydrolipoic acid exerts an antioxidant activity, but not as a direct radical scavenger. This antioxidant activity has been observed only in the presence of other compounds which act as antioxidant mediators. Bast and Haenen [21] and Pruijn *et al.* [28] reported that the protective antioxidant effect is detected only when the combination of dihydrolipoic acid and oxidized glutathione (GSSG) is present. This protective effect of dihydrolipoic acid is due to its ability to reduce GSSG to GSH. Similarly, Scholich *et al.* [22] found that dihydrolipoic acid by itself is not efficient as an inhibitor or NADPH/iron/ADP-induced lipid peroxidation (in microsomes from vitamin E-deficient animals) but demonstrates high antioxidant activity in combination with vitamin E (in microsomes from vitamin E-sufficient animals). Lipoate does not inhibit microsomal lipid peroxidation.

Thus, all possible effects of dihydrolipoic/thioctic acid have been observed under conditions of oxidative stress: from a strong antioxidant protection to an enhancement of oxidative damage. This is probably due to a multiplicity of pathways by which dihydrolipoic/thioctic acid can affect peroxidation during its induction and propagation in complex biological systems: transition metal reduction and/or chelation, interaction with other thiols, antioxidants and their radicals, direct interaction with initiating oxygen and lipid radicals. The variety of possible interactions of dihydrolipoic/thioctic acid with different components of the lipid peroxidation induction system(s) hinders the deciphering of the mechanism(s) of its action.

Hence, the goal of this work was to study the antioxidant effects of dihydrolipoic and thioctic acids in relatively simple oxidation systems in which the initiation of oxidation was not dependent on the presence of transition metals. In particular, our experiments were aimed at studying the interactions of dihydrolipoic acid and thioctic acid with three kinds of radicals:

(i) peroxyl radicals generated in the aqueous phase, in liposomes or in membranes by the water-soluble azoinitiator 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH)* or the lipid-soluble azoinitiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN);

(ii) chromanoxyl radicals of vitamin E (α-tocopherol, or its synthetic homologues) generated in liposomes or in the membranes by three different ways: UV-irradiation, peroxyl radicals of AMVN, or peroxyl radicals of linolenic acid formed by the lipoxygenase-catalyzed oxidation; and

(iii) ascorbyl radicals generated in the course of ascorbate oxidation by chromanoxyl radicals.

MATERIALS AND METHODS

Preparation of liposomes

Dioleoylphosphatidylcholine (DOPC) or dilinoleoylphosphatidylcholine (DLPC) liposomes with incorporated α-tocopherol or its synthetic homologue with a shorter side-chain, chromanol-α-C6, were obtained by dissolving the phospholipid and the antioxidant in chloroform, evaporating under a stream of nitrogen to dryness, and subsequent sonicating lipid dispersion in the phosphate buffer (0.1 M, pH 7.4, at 37°) under nitrogen at 4°. Dihydrolipoic acid or thioctic acid was added in ethanolic solutions.

Preparation of microsomal membranes

Microsomes were prepared by perfusing the livers of Sprague–Dawley female rats (120–150 g) with ice-cold 1.15% KCl. The liver was removed and then homogenized, followed by a 10 min 10,000 *g* centrifugation. The supernatant from this fraction was centrifuged at 105,000 *g* for 60 min.

Phycoerythrin fluorescence-based assay of radical scavenging activity

For this assay we used the continuous generation of peroxyl radicals by AAPH, according to the procedure of DeLange and Glazer [35]. AAPH-induced peroxyl radicals oxidatively modified B-phycoerythrin. This was monitored by the loss of its characteristic fluorescence (λ_{ex} = 540 and λ_{em} = 575). The reaction mixture (2 mL) consisted of 50 nM phycoerythrin, 9.4 mM AAPH (added in methanolic solution) in 0.1 M sodium phosphate buffer, pH 7.4. Antioxidants were dissolved in absolute methanol and added to the reaction mixture after the initiation of peroxidation.

Lipid peroxidation determination

The accumulation of lipid peroxidation products

* Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); DPLC, dilinoleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; MDA, malonyldialdehyde; and TBARS, thiobarbituric acid reactive substances.

(diene conjugates) formed during the oxidation of DPLC liposomes was measured spectrophotometrically by following the absorbance of the sample at 233 nm. The incubation medium (3 mL) contained 0.2 mg/mL DLPC, 1.0 mM AMVN in 50 mM sodium-phosphate buffer, pH 7.4, at 40°. Thioctic acid, dihydrolipoic acid or α-tocopherol was added to the incubation medium in ethanolic solutions.

Accumulation of secondary lipid peroxidation products reacting with 2-thiobarbituric acid (TBARS) was measured spectrophotometrically by the absorbance at 535 nm, as described by Buege and Aust [36]. Lipid peroxidation was induced in rat tissue homogenates or in liver microsomes by AMVN at 40°. The incubation medium contained: 5 mM AMVN, 2.0 mg/mL protein in sodium-phosphate buffer, pH 7.4, at 40°. Thioctic acid or dihydrolipoic acid was added to the incubation medium in ethanolic solutions.

HPLC measurements of chromanol content

Chromanol-α-C6 was assayed by reverse phase HPLC using a C-18 column (Waters, Inc.) with an in-line electrochemical detector. The eluent was methanol:ethanol (1:9, v/v, 20 mM lithium perchlorate. Chromanol-α-C6 was extracted into hexane from sodium dodecyl sulfate-treated samples as described earlier [37].

Generation of chromanoxyl radicals

Chromanoxyl radicals from α-tocopherol and its shorter hydrocarbon chain homologue were generated using: (1) UV-irradiation, (2) an enzymic oxidation system (soybean 15-lipoxygenase + linolenic acid), and (3) AMVN. When the enzymic oxidation system was used, the incubation medium (100 μL) contained microsomal (20 mg/mL protein) or liposomal (10 mg/mL DOPC) suspension in 0.1 M phosphate buffer, pH 7.4, at 37°. Linolenic acid (1.4 mM) and lipoxygenase (3 U/μL) were added subsequently to the microsomal/liposomal suspension. With the azoinitiator the incubation medium was essentially the same but AMVN (5.0 mM) was added instead of (lipoxygenase + linolenic acid) and the reaction was carried out at 40°.

ESR spectroscopy

ESR measurements were made on a Varian E-109E spectrometer at room temperature, in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan, NJ, U.S.A.). The permeable tube (approximately 8 cm in length) was filled with 60 μL of the mixed sample, folded into quarters and placed in an open 3.0 mm internal diameter EPR quartz tube such that all of the sample was within the effective microwave irradiation area. The sample was flushed with oxygen. Spectra were recorded at 100 mW power and 2.5 G modulation at 100 kHz and a time constant of 0.064 sec. Spectra were recorded at room temperature (at 40° with AMVN), under aerobic conditions by flowing oxygen through the ESR cavity.

Irradiation

Irradiation was by a solar stimulator (Solar Light Co., model 14S), whose output closely matches the solar spectrum in the wavelengths 290–400 nm. The samples were illuminated directly in the ESR resonator cavity; the distance between the light source and the sample was 30 cm. The intensity of the light at the sample surface in the spectral region 310–400 nm was 1.5 mW/cm^2 and was 10% of this value at 290 nm.

Reagents used

Linolenic acid, soybean 15-lipoxygenase (101,000 U/mg protein), DOPC, DLPC, dehydroascorbate, ascorbate, dihydrolipoic acid, and 2-thiobarbituric acid were from the Sigma Chemical Co., St. Louis, MO; potassium phosphate dibasic and phosphate monobasic from Mallinckrodt, Inc., Paris, KY; AMVN and AAPH from PolySciences, Inc., Warrington, PA; and HPLC grade ethanol and methanol from Fischer Scientific, Fair Lawn, NJ. 2*R*,4*R*′,8*R*-α-Tocopherol was a gift from the Henkel Co., and thioctic acid was a gift from Asta Pharma. α-Tocopherol homologue with a 6-carbon side chain (chromanol-α-C6) and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) were gifts from Prof. R. P. Evstigneeva (Institute for Fine Chemical Technology, Moscow, USSR).

RESULTS

Interaction of dihydrolipoic acid and thioctic acid with peroxyl radicals

Efficiency of peroxyl radical scavenging by thioctic and dihydrolipoic acids in the aqueous phase. To evaluate the peroxyl radical scavenging efficiency of thioctic and dihydrolipoic acids in the aqueous phase, the decay of the phycoerythrin fluorescence was followed, using a continuous generation of peroxyl radicals by the water-soluble azoinitiator, AAPH. Exposure of the phycoerythrin to AAPH-derived peroxyl radicals induced an immediate and linear decrease in its fluorescence intensity (Fig. 1). Thioctic acid had no effect on the phycoerythrin fluorescence decay, i.e. failed to show any activity to quench peroxyl radicals in the aqueous phase. In contrast to that, when dihydrolipoic acid was added to the system, a complete protection of phycoerythrin against oxidative modification was achieved. The phycoerythrin fluorescence intensity remained constant until the antioxidant added has been consumed, after which the fluorescence decay had the same slope as in the absence of dihydrolipoic acid. Dihydrolipoic acid produced a concentration-dependent quenching of AAPH radicals (Fig. 1). Based on the constant rate of AAPH radical generation (1.6×10^{-6} [AAPH] sec^{-1} at 40° [38]) and the duration of the lag-period during which dihydrolipoic acid protected phycoerythrin against oxidative damage, the stoichiometry of peroxyl radical quenching by dihydrolipoic acid can be evaluated. The estimated stoichiometry of peroxyl radical quenching by dihydrolipoic acid was 1.5 (mol of peroxyl radicals per mol of dihydrolipoic acid) (Table 1).

Efficiency of peroxyl radical scavenging by thioctic and dihydrolipoic acids in the membraneous phase. To estimate the efficiency of peroxyl radical

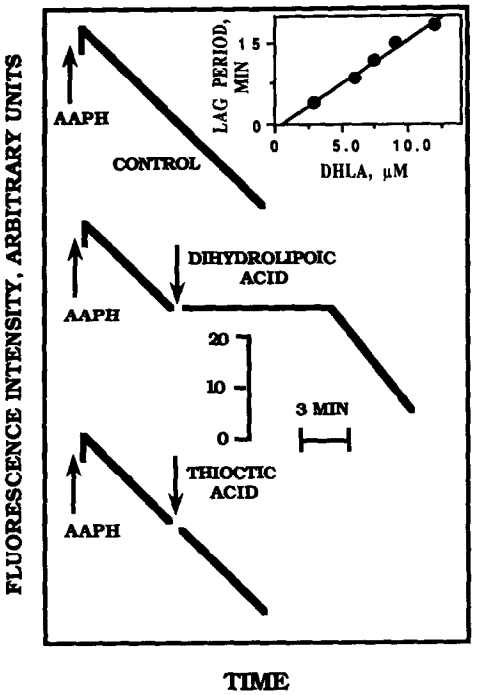

Fig. 1. Effect of dihydrolipoic acid and thioctic acid on the B-phycoerythrin fluorescence decay induced by AAPH. Incubation conditions: B-phycoerythrin (50 nM), AAPH (9.4 mM) in 0.1 M sodium-phosphate buffer (pH 7.4 at 40°). The fluorescence intensity was measured with 540 nm excitation and 565 nm emission (5 nm slits). The concentrations of dihydrolipoic acid and thioctic acid were 9.0 and 30 μM, respectively. Inset: The dependence of the lag period in AAPH-induced fluorescence decay of B-phycoerythrin on the concentration of dihydrolipoic acid (DHLA).

scavenging by thioctic and dihydrolipoic acids in the hydrophobic membraneous phase, their effects on lipid peroxidation induced by the lipid-soluble azoinitiator of peroxyl radicals, AMVN, were studied. The time-course of lipid hydroperoxides with diene conjugation in DPLC liposomes during AMVN-initiated oxidation was followed (Fig. 2). AMVN induced linear accumulation of conjugated dienes over 50 min of incubation. Dihydrolipoic acid produced inhibition of DLPC peroxidation (Fig. 2, Table 2). The inhibitory effect was concentration dependent, and at concentrations higher than 50 μM accumulation of conjugated dienes in DLPC liposomes was not observed over a 50-min incubation with AMVN. In contrast, AMVN-induced peroxidation in DLPC liposomes was insensitive to thioctic acid.

Similarly, dihydrolipoic acid inhibited AMVN-induced lipid peroxidation in rat tissue homogenates (brain, liver, heart, skin) (Fig. 3) and rat liver microsomes as assayed by the accumulation of TBARS (Fig. 4). This inhibition was concentration dependent and was not tissue specific. Thioctic acid was inefficient as the inhibitor of AMVN-induced lipid peroxidation in rat tissue homogenates and liver microsomes.

Interaction of dihydrolipoic and thioctic acids with chromanoxyl radicals

Interaction of dihydrolipoic and thioctic acids with 2,2,5,7,8-pentamethyl-6-hydroxychromane in quenching peroxyl radicals in the aqueous phase. The vitamin E homologue devoid of the hydrophobic hydrocarbon side chain, PMC, showed interaction with AAPH-derived peroxyl radicals in the aqueous phase with a stoichiometry of 0.3 (of peroxyl radicals per mol of PMC). A combination of dihydrolipoic acid and PMC did not give any synergistic effect (Table 1). In the presence of both dihydrolipoic acid and PMC, the quenching of radicals seemed to be less than additive. In the presence of thioctic acid and PMC, the inhibitory effect was solely due to the latter.

Interaction of dihydrolipoic and thioctic acids with α-tocopherol in quenching peroxyl radicals in the membraneous phase. Vitamin E (α-tocopherol) suppressed AMVN-induced accumulation of hydroperoxides with conjugated dienes in DLPC liposomes (Fig. 2). Under conditions used, AMVN-induced oxidation of DLPC liposomes was inhibited completely by α-tocopherol concentrations higher than 15 μM. To examine whether a synergistic antioxidant interaction of α-tocopherol and di-

Table 1. Reaction stoichiometry of dihydrolipoic acid, thioctic acid and 2,2,5,7,8-pentamethyl-6-hydroxychromane with peroxyl radicals generated by 2,2′-azobis-(2-amidinopropane)-dihydrochloride as detected by the phycoerythrin fluorescence decay assay

Substance	Reaction stoichiometry (mol peroxyl radicals quenched/mol antioxidant)
Dihydrolipoic acid	1.5 ± 0.2
Thioctic acid	0
2,2,5,7,8-Pentamethyl-6-hydroxychromane	0.3 ± 0.1
Dihydrolipoic acid + 2,2,5,7,8-pentamethyl-6-hydroxychromane	1.3 ± 0.4
Thioctic acid + 2,2,5,7,8-pentamethyl-6-hydroxychromane	0.3 ± 0.1

Data are means ± SD, N = 4.

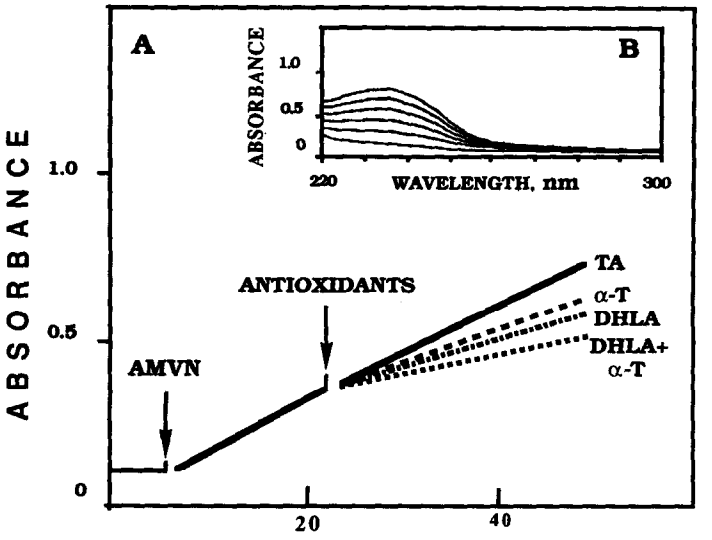

Fig. 2. Effects of dihydrolipoic acid, thioctic acid and α-tocopherol on AMVN-induced oxidation of DLPC liposomes. Incubation conditions: DLPC (0.2 mg/mL), AMVN (1.0 mM) in 50 mM sodium-phosphate buffer (pH 7.4 at 40°). α-Tocopherol (α-T) (3.3 μM), dihydrolipoic acid (DHLA) (16.7 μM) or thioctic acid (TA) (16.7 μM) was co-sonicated with DLPC in the buffer (5 min at 4°). Inset: Serial UV-spectra of DLPC liposomes incubated in the presence of AMVN. Spectra were recorded in 10-min intervals. Other conditions as above.

Table 2. Inhibition of AMVN-induced DLPC liposome oxidation by dihydrolipoic acid, thioctic acid or α-tocopherol

Substance	% inhibition
Dihydrolipoic acid (16.7 μM)	40 ± 3
Thioctic acid (16.7 μM)	0
α-Tocopherol (3.3 μM)	29 ± 3
Dihydrolipoic acid + α-tocopherol	67 ± 6
Thioctic acid + α-tocopherol	30 ± 4

Incubation conditions: 0.2 mg/mL dilinoleoylphosphatidylcholine, 1.0 mM 2,2'-azobis(2,4-dimethylvaleronitrile), 50 mM sodium-phosphate buffer, (pH 7.4, at 40°). Data are means ± SD, N = 5. In the control, the rate of AMVN-induced DLPC peroxidation was 6.2 ± 0.7 nmol hydroperoxides/mg DLPC/min.

hydrolipoic acid occurs in liposomes, concentrations of these compounds were chosen which produced only 30–40% inhibition of AMVN-induced DLPC oxidation. A combination of α-tocopherol and dihydrolipoic acid gave additive but not synergistic inhibitory effects (Fig. 2, Table 2). When both thioctic acid and α-tocopherol were present, the inhibition of AMVN-induced lipid peroxidation in DLPC liposomes was only produced by α-tocopherol.

Direct effects of thioctic and dihydrolipoic acids on chromanoxyl radicals in liposomes. Direct ESR detection of chromanoxyl radicals of vitamin E (α-tocopherol) and of its homologues with a shorter hydrocarbon side-chain was employed [39]. We used two different approaches to generate chromanoxyl radicals from vitamin E and its homologue—(i) oxidation by peroxyl radicals [40]:

$$\text{ChR-OH} + \text{LOO}^{\cdot} \rightarrow \text{ChR-O}^{\cdot} + \text{L-OO-H} \quad (1)$$

or (ii) exposure to irradiation by ultraviolet light absorbed by vitamin E:

$$\text{ChR-OH} + h\nu \rightarrow \text{ChR-O}^{\cdot} + \text{e}^{-} + \text{H}^{+}. \quad (2)$$

To produce the peroxyl radicals necessary in reaction (1), two different sources were chosen: (i) the hydrophobic azoinitiator, AMVN, or (ii) soybean 15-lipoxygenase, which produces peroxyl radicals of polyunsaturated fatty acids (linolenic acid in our experiments) [41]. Incubation of either of these peroxyl radical generators with DOPC liposomes containing either α-tocopherol or its homologue with a 6-carbon side-chain (chromanol-α-C6) resulted in the appearance of a characteristic chromanoxyl radical ESR signal with g values for the five resolved components of 2.0122, 2.0092, 2.0061, 2.0028 and 1.993 (Fig. 5). This signal was not observed in the absence of the oxidizing systems. Chromanol-α-C6 which is more uniformly distributed in the lipid bilayer gave the same but a better resolved ESR signal of its chromanoxyl radical.

Constant UV-irradiation of liposomal suspensions containing chromanol-α-C6 or α-tocopherol resulted in the appearance of a characteristic chromanoxyl radical ESR signal which was not detected in the dark.

In DOPC liposomes loaded with chromanol-α-C6 in which chromanoxyl radicals were generated by

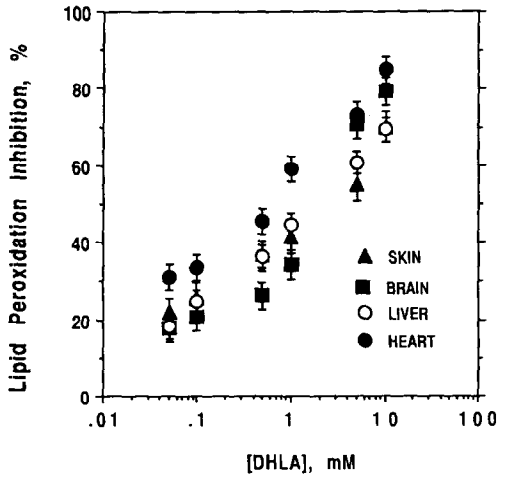

Fig. 3. Inhibitory effect of dihydrolipoic acid on AMVN-induced lipid peroxidation (TBARS formation) in rat tissue homogenates. Incubation conditions: AMVN (5.0 mM), homogenate (2.0 mg protein/mL), in 0.1 M sodium-phosphate buffer (pH 7.4 at 40°). Dihydrolipoic acid (DHLA) in ethanolic solution was added to the homogenates. Homogenates were incubated in the presence of AMVN for 40 min. In controls, AMVN-induced peroxidation (40 min) resulted in the following accumulation of malonyldialdehyde (MDA): skin 3.5 ± 0.3 nmol MDA/mg protein; brain 4.8 ± 0.3 nmol MDA/mg protein; liver 6.8 nmol MDA/mg protein; and heart 7.5 ± 0.6 nmol MDA/mg protein. Data are means ± SD, N = 5.

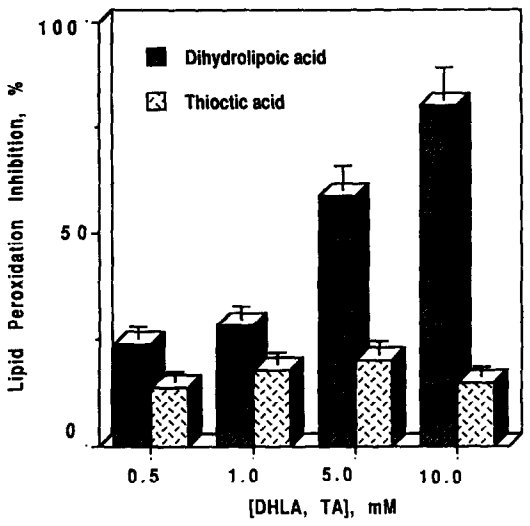

Fig. 4. Inhibitory effects of dihydrolipoic acid and thioctic acid on AMVN-induced lipid peroxidation (TBARS formation) in rat liver microsomes. Incubation conditions; AMVN (5.0 mM), microsomes (0.3 mg protein/mL) in 0.1 M sodium-phosphate buffer (pH 7.4 at 40°). Dihydrolipoic acid and thioctic acid in ethanolic solutions were added to the microsomal suspension. Microsomes were incubated in the presence of AMVN for 40 min. In the control, AMVN-induced peroxidation (40 min) resulted in the accumulation of 9.4 ± 0.7 nmol MDA/mg protein. Data are means ± SD, N = 5.

AMVN or by lipoxygenase + linolenic acid, i.e. by the reaction with peroxyl radicals (reaction 1), dihydrolipoic acid markedly suppressed the ESR signal (Fig. 6). In contrast to that, UV-induced ESR signal of the chromanoxyl radical was not changed by the addition of dihydrolipoic acid. Thioctic acid did not affect the ESR signal of chromanoxyl radical generated from α-tocopherol or chromanol-α-C6 by either the peroxyl radicals or by UV-irradiation.

Interaction of dihydrolipoic and thioctic acids with ascorbyl radicals

Ascorbate-mediated effects of thioctic and dihydrolipoic acids on chromanoxyl radicals in liposomes. When ascorbate was added to the DOPC liposome suspension, the chromanoxyl radical ESR signal generated from α-tocopherol or chromanol-α-C6 by either peroxyl radicals or by UV-irradiation could not be observed. Instead, the characteristic ESR signal of ascorbyl radical was detected (Fig. 7). This signal decreased in time and was substituted by the progressive appearance of the chromanoxyl radical ESR signal (Figs. 7 and 8).

Dihydrolipoic acid added to the chromanol-α-C6-loaded liposomes in combination with ascorbate synergistically enhanced the effect of ascorbate: substantially increased the delay in the reappearance of the chromanoxyl radical ESR signal. During this period of time, only the ascorbyl radical ESR signal could be found in the ESR spectra. This effect of dihydrolipoic acid was concentration dependent (Fig. 8). Thioctic acid did not affect chromanol-α-C6 radical ESR signal in combination with ascorbate.

Ascorbate-mediated effects of thioctic and dihydrolipoic acids on chromanoxyl radicals in rat liver microsomes. Ascorbate was able to reduce chromanoxyl radicals in microsomes with incorporated α-tocopherol or chromanol-α-C6 and to cause a transient disappearance of the chromanoxyl radical ESR signal at the expense of ascorbate oxidation as was reported earlier [13, 16, 42]. This was accompanied by a transient appearance of a characteristic ascorbyl radical ESR signal which decreased in time and was substituted by the reappearing chromanoxyl radical signal of α-tocopherol (Fig. 9).

Dihydrolipoic acid added to the rat liver microsomal suspension alone decreased the chromanoxyl radical ESR signal generated from α-tocopherol by either lipoxygenase + linolenic acid or by AMVN but did not cause a transient disappearance of the chromanoxyl radical ESR signal. In some microsomal preparations in which the addition of AMVN or lipoxygenase + linolenic acid produced both a faint chromanoxyl radical signal from α-tocopherol and a scanty ascorbyl radical ESR signal from endogenous ascorbate entrapped by microsomal vesicles, dihydrolipoic acid was able to markedly increase the ascorbyl radical signal and increase its life-time. However, when dihydrolipoic acid was added to the microsomal suspension in combination with exogenous ascorbate, only ascorbyl radical signals could be found in the

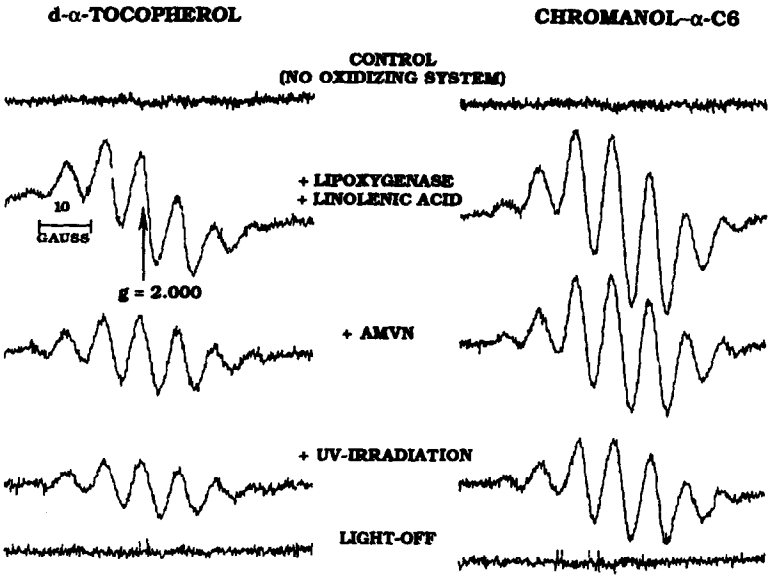

Fig. 5. ESR spectra of chromanoxyl radicals generated in dioleoylphosphatidylcholine liposomes from *d*-α-tocopherol or chromanol-α-C6. Chromanoxyl radicals were generated using an enzymic oxidation system (lipoxygenase + linolenic acid), an azoinitiator of peroxyl radicals (AMVN), or UV-irradiation. Incubation conditions: DOPC liposomes (10 mg/mL) in 0.1 M sodium-phosphate buffer (pH 7.4 at 25°). Chromanols (2.8 mM) were co-sonicated with DOPC during liposome preparation. Linolenic acid (1.4 mM) + lipoxygenase (3 U/μL)-induced reaction was carried out at 25°. AMVN (5 mM)-induced reaction was carried out at 40°. Linoleic acid and lipoxygenase or AMVN were added subsequently to the liposomal suspension. UV-irradiation was performed at 25°.

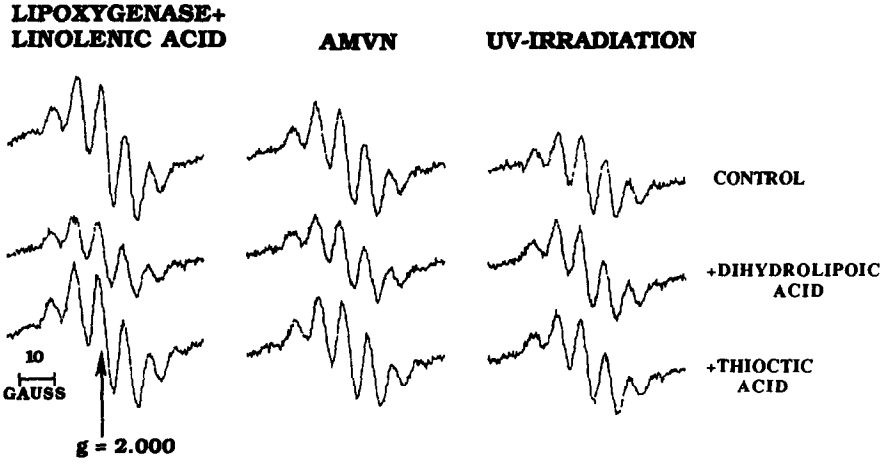

Fig. 6. Effects of dihydrolipoic acid and thioctic acid on ESR spectra of chromanoxyl radicals generated in dioleoylphosphatidylcholine liposomes from the vitamin E homologue, chromanol-α-C6. Chromanoxyl radicals were generated using an enzymic oxidation system (lipoxygenase + linolenic acid), an azoinitiator of peroxyl radicals (AMVN) or UV-irradiation. Dihydrolipoic acid (7.5 mM) and thioctic acid (7.5 mM) in ethanolic solution were added to DOPC liposomal suspension. Other conditions were as given in the legend of Fig. 5.

ESR spectra. The delay in the reappearance of the chromanoxyl radical ESR signal was dependent on the concentration of dihydrolipoic acid (Fig. 9). Thioctic acid did not affect the chromanoxyl radical ESR signal of α-tocopherol either alone or in combination with ascorbate. Similar results were obtained with chromanol-α-C6 (data not shown).

NADPH- and NADH-mediated effects of thioctic and dihydrolipoic acids on chromanoxyl radicals in rat liver microsomes. Earlier we reported that rat

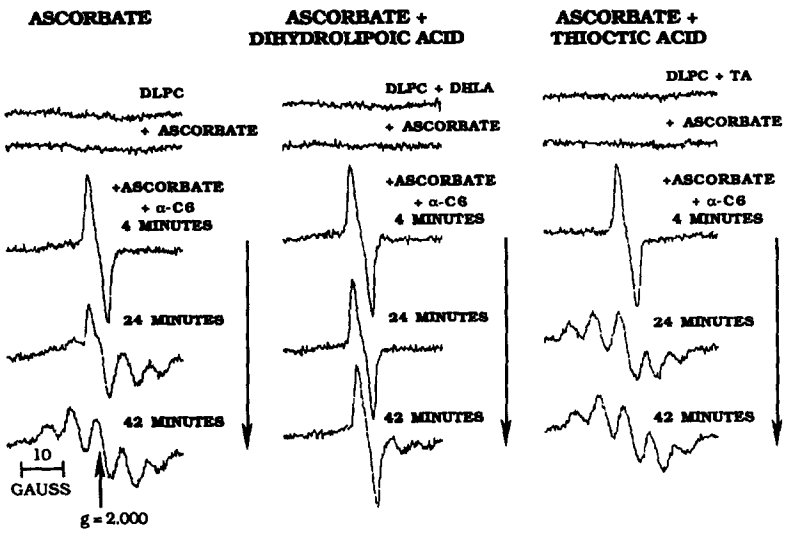

Fig. 7. Effects of ascorbate, dihydrolipoic acid, and thioctic acid on UV-induced ESR signals of chromanoxyl radicals generated in dioleoylphosphatidylcholine liposomes from the vitamin E homologue, chromanol-α-C6. All ESR spectra were recorded under continuous UV-irradiation of the samples in the ESR cavity. Dihydrolipoic acid (6.0 mM) and thioctic acid (6.0 mM) in ethanolic solution and ascorbate (0.4 mM) in sodium-phosphate buffer were added to DOPC liposomal suspension. Other conditions were as given in the legend of Fig. 5.

liver microsomes can reduce chromanoxyl radicals of vitamin E and its homologues by both NADPH- and NADPH-dependent electron transport [16, 39]. NADPH and NADH produced a transient decrease (but not complete disappearance) of the chromanoxyl radical ESR signal (Fig. 10). We tested whether dihydrolipoic acid and thioctic acid can synergistically enhance the reduction of chromanoxyl radicals generated from chromanol-α-C6 by lipoxygenase + linolenic acid by the microsomal electron transport.

The effects of dihydrolipoic acid on chromanoxyl radical reduction in rat liver microsomes were similar to those in liposomes. Partial quenching of the ESR chromanoxyl radical signal was observed in the presence of dihydrolipoic acid only (Fig. 10). However, when both NADPH or NADH and dihydrolipoic acid were present, a complete transient quenching of the chromanoxyl radical ESR signal was observed. Thioctic acid was unable to enhance NADH- or NADPH-dependent reduction of chromanoxyl radicals in microsomes.

HPLC determinations of the content of chromanol-α-C6 indicated that dihydrolipoate in combination with NADPH produced a strong sparing effect on the loss of chromanol-α-C6 caused by lipoxygenase + linolenic acid oxidation. In contrast, the sparing of chromanol-α-C6 in the presence of NADPH and thioctic acid was only due to the effect of NADPH (Table 3).

DISCUSSION

Action of dihydrolipoic acid/thioctic acid as an antioxidant couple

Thioctic (lipoic) acid is an essential cofactor (as lipoamide) in the oxidative decarboxylation of α-keto acids, e.g. pyruvate and α-keto-glutarate. Recently, it was suggested that in addition to this well recognized catalytic function, the thioctic/dihydrolipoic acid couple is involved in the thiol-dependent protection against oxidative damage interaction with other intracellular antioxidants [21, 22, 28, 29]. Using simple oxidation systems, in this work we studied the interaction of dihydrolipoic/thioctic acid with two antioxidants, vitamin E and vitamin C, and their radicals formed in the course of peroxidation.

The results show that thioctic acid is not effective in scavenging peroxyl radicals, inhibiting lipid peroxidation, or in recycling the chromanoxyl radicals. This is in agreement with earlier reported results [21, 22, 28]. However, the reduced form of thioctic acid, dihydrolipoic acid, exerted direct radical scavenging activity interacting with both water-soluble and lipid-soluble peroxyl radicals in the aqueous phase or in hydrophobic domains of the lipid bilayer of liposomes or membrane fractions, respectively. Since transition metals were not used for peroxyl radical generation, these effects were not due to the chelation of transition metals by dihydrolipoic acid.

There is no requirement for either vitamin E or glutathione to display this direct antiradical activity of dihydrolipoic acid as was suggested earlier [21, 22, 28]. In contrast to many other antioxidants, dihydrolipoic acid may function as a universal free radical quencher which can scavenge peroxyl radicals both in the cytosol and in the hydrophobic membrane domains. Thus we can suggest that mitochondrial reduction of thioctic acid to dihydrolipoic acid by keto-acid dehydrogenases may constitute the source of physiologically important direct chain breaking

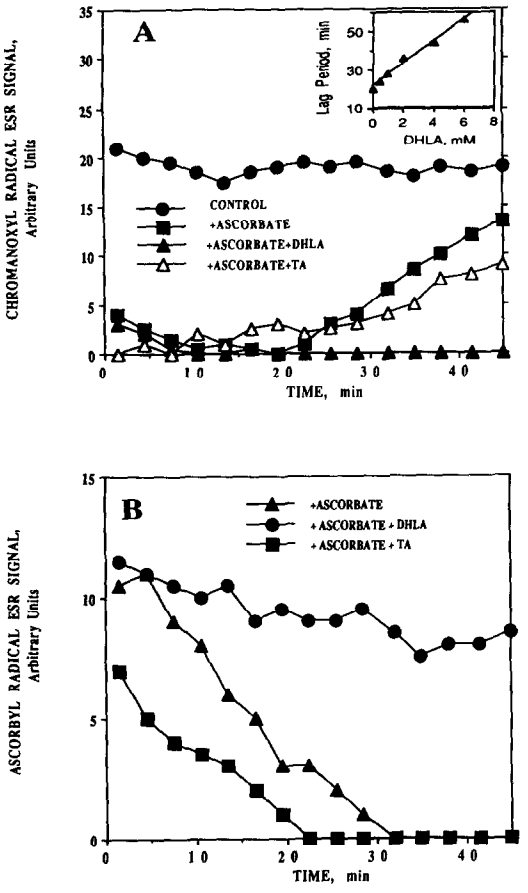

Fig. 8. Effects of ascorbate, dihydrolipoic acid (DHLA) and thioctic acid (TA) on the time-course of UV-induced chromanoxyl and ascorbyl radical ESR signals in chromanol-α-C6-containing DOPC liposomes. Chromanol-α-C6 (2.8 mM) was co-sonicated with DOPC during liposomal preparation. DHLA (6.0 mM) and TA (6.0 mM) in ethanolic solution and ascorbate (0.4 mM) in sodium-phosphate buffer were added to the DOPC liposomal suspension. Other conditions were as given in the legend of Fig. 5. (A) Chromanoxyl radical ESR signal. Inset: The dependence of the lag period for the reappearance of the chromanoxyl radical on the concentration of DHLA (ascorbate concentration was 0.4 mM). (B) Ascorbyl radical ESR signal.

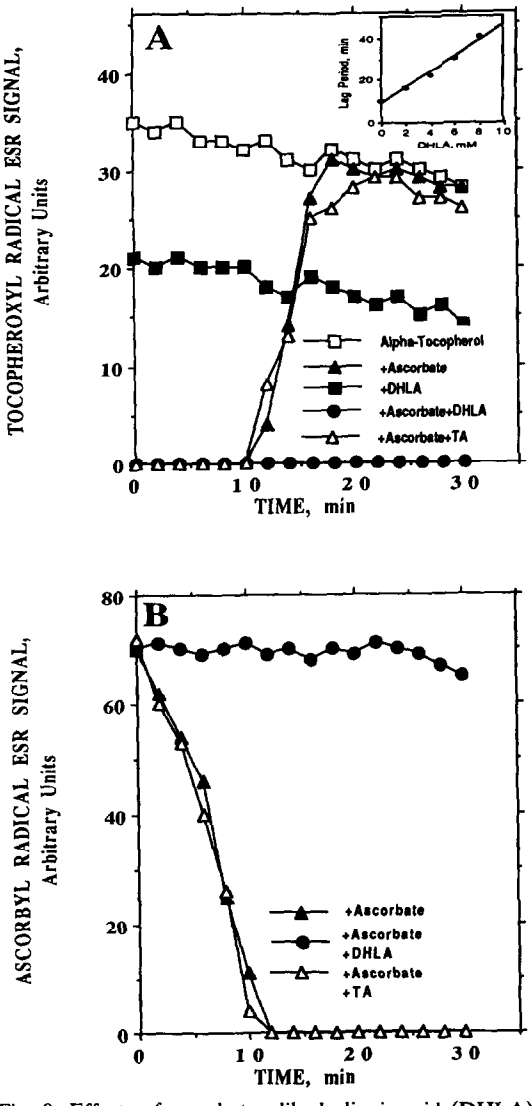

Fig. 9. Effects of ascorbate, dihydrolipoic acid (DHLA) and thioctic acid (TA) on the time-course of α-tocopheroxyl and ascorbyl radical ESR signals in rat liver microsomes in the presence of lipoxygenase + linolenic acid. α-Tocopherol (3.6 mM) in ethanolic solution was added to the microsomal suspension (20 mg protein/mL). The concentration of endogenous α-tocopherol in microsomes was 0.54 nmol/mg protein. The linolenic acid (1.4 mM) + lipoxygenase (3 U/μL)-induced reaction was carried out at 25°. Linolenic acid and lipoxygenase were added subsequently to the microsomal suspension. DHLA (6.0 mM) and TA (6.0 mM) in ethanolic solution and ascorbate (0.8 mM) in sodium-phosphate buffer were added to the microsomal suspension. (A) Tocopheroxyl radical ESR signal. Inset: The dependence of the lag period for the reappearance of the tocopheroxyl radical on the concentration of DHLA (ascorbate concentration was 0.8 mM). (B) Ascorbyl radical ESR signal.

antioxidants. It is known that intracellular concentrations of thioctic acid are within the range of 5–30 nmol/g [43]. Normal human urine contains 200 nmol/mL lipoic acid in a conjugate form [43]. In addition, the thioctic/dihydrolipoic acid antioxidant couple is presently used for treatment in a variety of diseases, including liver and neurological disorders [23–25]. In this case, elevated intracellular levels of dihydrolipoic acid may occur which may be sufficient for a direct antioxidant effect.

Significance of direct interaction of dihydrolipoic acid with vitamin E radical

The direct free radical scavenging effects of dihydrolipoic acid is not the only mode of its antioxidant action. It was suggested that dihydrolipoic acid can synergistically enhance antioxidant activity of membranes by reducing glutathione, GSSG, and/

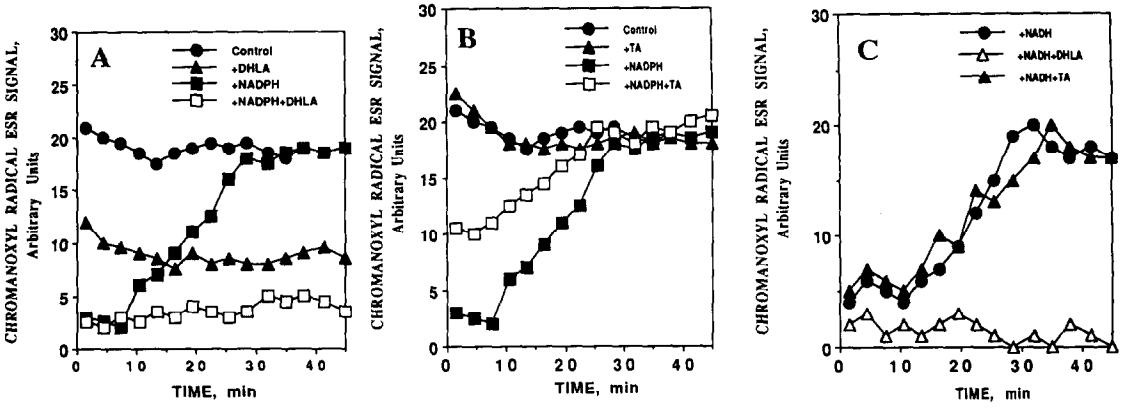

Fig. 10. Effects of NADPH, NADH, and dihydrolipoic acid (DHLA) and thioctic acid (TA) on the time-course of chromanoxyl radical ESR signals in rat liver microsomes in the presence of lipoxygenase + linolenic acid. Chromanol-α-C6 (1.8 mM) in ethanolic solution was added to the microsomal suspension. The concentration of endogenous α-tocopherol in microsomes was 0.54 nmol/mg protein. The linolenic acid (1.4 mM) + lipoxygenase (3 U/μL)-induced reaction was carried out at 25°. Linolenic acid and lipoxygenase were added subsequently to the microsomal suspension. DHLA (6.0 mM) and TA (6.0 mM) in ethanolic solution and NADPH (1.1 mM) or NADH (1.1 mM) in sodium-phosphate buffer were added to the microsomal suspension. NADPH + DHLA; (B) NADPH + TA; and (C) NADH + DHLA and NADH + TA.

Table 3. Sparing effects of dihydrolipoic acid, thioctic acid and NADPH on chromanol-α-C6 oxidation by lipoxygenase + linoleic acid in rat liver microsomes

Substance	Chromanol α-C6* (mM)	% Sparing
Control 1 (no lipoxygenase)	1.8	
Control 2 (+ lipoxygenase and linolenic acid)	0.4 ± 0.1	0
Thioctic acid (6.0 mM)	0.5 ± 0.2	7.1
Dihydrolipoic acid (6.0 mM)	0.8 ± 0.2	28.6
NADPH (1.1 mM)	1.2 ± 0.3	57.2
Thioctic acid + NADPH	1.1 ± 0.4	50.0
Didhydrolipoic acid + NADPH	1.7 ± 0.3	92.9

Incubation conditions: chromanol-α-C6 (1.8 mM) in ethanolic solution was added to the microsomal suspension (20 mg protein/mL). The concentration of endogenous α-tocopherol in microsomes was 0.54 nmol/mg protein. The linolenic acid (1.4 mM) + lipoxygenase (3 U/μL)-induced reaction was carried out at 25°. Linolenic acid and lipoxygenase were added subsequently to the microsomal suspension. Dihydrolipoic acid (6.0 mM) and thioctic acid (6.0 mM) in ethanolic solution and NADPH (1.1 mM) in sodium-phosphate buffer were added to the microsomal suspension.

* Values are means ± SD, N = 5.

or vitamin E radical possibly via a "free radical reductase" mechanism [21, 22, 28]. However, no direct ESR data on the interaction of dihydrolipoic acid with tocopheroxyl radical were reported.

Our ESR measurements showed that neither dihydrolipoic acid nor thioctic acid was efficient in direct reduction of UV-induced chromanoxyl radicals in liposomes. The rate constant for the reaction of hydrogen abstraction from the fatty acid methylene group by chromanoxyl radicals has been shown to be three orders of magnitude lower for monounsaturated oleic acid than for polyunsaturated fatty acids (linoleic, linolenic, arachidonic) [44]. Thus, we employed monounsaturated DOPC liposomes in these experiments to avoid any possible interference with the chromanoxyl radical prooxidant effect—the interaction of UV-induced chromanoxyl radicals with liposomal lipids.

However, dihydrolipoic acid (but not thioctic acid) substantially decreased the magnitude of the ESR

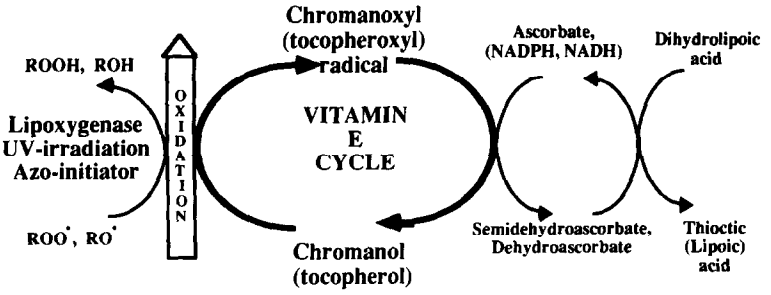

Fig. 11. Scheme explaining the interactions of DHLA with ascorbate, NADPH and NADH in reducing chromanoxyl radicals.

signals from radicals generated by reaction with peroxyl radicals (AMVN or lipoxygenase + linolenic acid). This quenching effect of dihydrolipoic acid is probably caused by its ability to reduce peroxyl radicals:

$$2RSH + 2LOO^{\cdot} \rightarrow R\text{-}SS\text{-}R + 2LOOH. \quad (3)$$

Thus, the steady-state concentration of the peroxyl radicals necessary for the generation of chromanoxyl radicals by reaction mechanism (1) will be reduced. As a result of this interaction, the magnitude of the observed chromanoxyl radical ESR signal in liposomes or in microsomes should be decreased. These data suggest that dihydrolipoic acid is not efficient in direct reduction (recycling) of vitamin E (chromanol-α-C6) chromanoxyl radicals generated in liposomes or membranes. This result corresponds to the earlier data of Rao *et al.* [45] who reported the low efficiency of the reaction between acetoaminophen phenoxyl radicals and reduced thiols.

We could not find any synergistic interaction of dihydrolipoic acid with vitamin E or with its more hydrophilic homologue, PMC, in the scavenging peroxyl radicals in either the aqueous or the hydrophobic phase. Clearly, the oxidized form, thioctic acid, was not able to reduce chromanoxyl radicals of vitamin E or its homologue.

Thus, we conclude that the thioctic/dihydrolipoic acid couple does not recycle vitamin E by the mechanism of direct reduction of the tocopheroxyl radical.

Ascorbate-, NADPH- or NADH-mediated interaction of dihydrolipoic acid with vitamin E radical

Dihydrolipoic acid however, can bolster the antioxidant systems by interacting with other redox couples (e.g. ascorbate/dehydroascorbate, components of NADPH- or NADH-dependent electron transporting chains) to synergistically enhance their potency in the vitamin E recycling.

In both liposomes and microsomes, dihydrolipoic acid (but not thioctic acid) was able to synergistically enhance the ascorbate-driven reduction of chromanoxyl radicals and recycling of vitamin E (chromanol-α-C6). This probably is due to the ability of dihydrolipoic acid to maintain a high steady-state concentration of ascorbate by reducing it from semidehydroascorbate and dehydroascorbate [17, 46]. This suggestion is supported by our ESR data showing that in the presence of dihydrolipoic acid the magnitude of the ascorbyl radical ESR signal did not change significantly over time, i.e. the steady-state concentration of the ascorbate oxidation intermediate, ascorbyl radical, was maintained by its continuous recycling by dihydrolipoic acid. Rao *et al.* [45] have shown that ascorbate is much more reactive with phenoxyl radicals than reduced thiols. There is no requirement in glutathione for the efficient dihydrolipoic acid-dependent vitamin E recycling in the presence of ascorbate. Thus, the reducing cascade with dihydrolipoic acid as the source of reducing equivalents may operate to recycle vitamin E, as shown in Fig. 11.

Similarly, dihydrolipoic acid (but not thioctic acid) synergistically interacted with NADPH- and NADH-driven electron transport in microsomes in enzymic reduction of chromanoxyl radicals. However, the mediator(s) of this synergistic interaction is still to be elucidated. One possible candidate is ubiquinone which is an intrinsic component of microsomal electron transporting chains and was shown recently to participate in vitamin E recycling in mitochondria [47]. However, we could not reduce ubiquinone Q10 by dihydrolipoic acid in ethanolic solution although it could be readily reduced by sodium borohydride. The other possibility is that residual ascorbate (dehydroascorbate) trapped inside microsomal vesicles may act as the mediator of dihydrolipoic acid-dependent chromanoxyl radical reduction. However, even in the absence of an ascorbyl radical ESR signal (which indicates that no endogenous ascorbate was present in the microsomal suspension), dihydrolipoic acid synergistically enhanced both NADPH- and NADH-dependent reduction of vitamin E radicals in liver microsomes.

Our findings appear to leave little doubt about the antioxidant effectiveness of dihydrolipoic acid to directly scavenge peroxyl radicals and/or enhance other water- or lipid-soluble antioxidants (ascorbate, vitamin E) by regenerating them via the reduction of their radicals. However, only the reduced but not the oxidized form of thioctic acid exerts these outstanding antioxidant properties. Information is still lacking on the mechanisms of reduction of exogenously introduced thioctic acid to form dihydrolipoic acid *in vivo*.

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REFERENCES

1. Halliwell B and Gutteridge JMC, *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford, 1989.
2. Sies H (Ed.), *Oxidative Stress: Oxidants and Antioxidants*. Academic Press, London, 1991.
3. Kagan VE, *Lipid Peroxidation in Biomembranes*. CRC Press, Boca Raton, FL, 1988.
4. McCay PB, Gibson DD, Fong K-L and Hornbrook KR, Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. *Biochim Biophys Acta* **431**: 459–468, 1976.
5. Burk RF, Trumble MJ and Lawrence RA, Rat hepatic cytosolic glutathione-dependent enzyme protection against lipid peroxidation in the NADPH-microsomal lipid peroxidation system. *Biochim Biophys Acta* **618**: 35–41, 1980.
6. Reddy CC, Scholz RW, Thomas CE and Massaro EJ, Vitamin E dependent reduced glutathione inhibition of rat liver microsomal lipid peroxidation. *Life Sci* **31**: 571–576, 1982.
7. Haenen GRMM and Bast A, Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. *FEBS Lett* **159**: 24–28, 1983.
8. Burk RF, glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. *Biochim Biophys Acta* **757**: 21–28, 1983.
9. Bast A and Haenen GRMM, Cytrochrome P-450 and glutathione. What is the significance of their interrelationship in lipid peroxidation? *Trends Biochem Sci* **9**: 510–513, 1984.
10. McCay PB, Brueggemann G, Lai EK and Powell SR, Evidence that alpha-tocopherol functions cyclically to quench free radicals in hepatic microsomes. Requirement for glutathione and a heat-labile factor. *Ann NY Acad Sci* **570**: 32–45, 1989.
11. Hill KE and Burk RF, Influence of vitamin E and selenium on glutathione-dependent protection against microsomal lipid peroxidation. *Biochem Pharmacol* **33**: 1065–1068, 1984.
12. Scholz RW, Graham KS, Gumpricht E and Reddy CC, Mechanism of interaction of vitamin E and glutathione in the protection against membrane lipid peroxidation. *Ann NY Acad Sci* **570**: 514–517, 1989.
13. Niki E, Tsuchiya J, Tanimura R and Kamiya Y, Regeneration of vitamin E from α-chromanoxyl radical by glutathione and vitamin C. *Chem Lett* 789–792, 1982.
14. Wefers H and Sies H, The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur J Biochem* **174**: 353–357, 1988.
15. Bando M and Obazawa H, Ascorbate free radical reductase and ascorbate redox cycle in the human lens. *Jpn J Ophthalmol* **32**: 176–186, 1988.
16. Packer L, Maguire JJ, Melhorn RJ, Serbinova EA and Kagan VE, Mitochondria and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical accumulation. *Biochem Biophys Res Commun* **159**: 229–235, 1989.
17. Rose RC, Ascorbic acid metabolism in protection against free radicals: A radiation model. *Biochem Biophys Res Commun* **169**: 420–436, 1990.
18. Melhorn R, Ascorbate- and dehydroascorbic acid-mediated reduction of free radicals in the human erythrocytes. *J Biol Chem* **266**: 2724–2731, 1991.
19. Searls RL and Sanadi DR, α-Ketoglutaric dehydrogenase. 8. Isolation and some properties of a flavoprotein component. *J Biol Chem* **235**: 2485–2491, 1960.
20. Scott I, Duncan W and Ekstrand V, Purification and properties of glutathione reductase of human erythrocytes. *J Biol Chem* **238**: 3928–3939, 1963.
21. Bast A and Haenen GRMM, Interplay between lipoic acid and glutathione in the protection against microsomal lipid peroxidation. *Biochim Biophys Acta* **963**: 558–561, 1988.
22. Scholich H, Murphy ME and Sies H, Antioxidant activity of dihydrolipoate against microsomal lipid peroxidation and its dependence on α-tocopherol. *Biochim Biophys Acta* **1001**: 256–261, 1989.
23. Altenkirch H, Stoltenburg-Didinger H, Wagner M, Herrmann J and Walter G, Effects of lipoic acid in hexacarbon-induced neuropathy. *Neurotoxicol Teratol* **12**: 619–622, 1990.
24. Piering WF and Bratanow N, Role of the clinical laboratory in guiding treatment of *Amanita virosa* mushroom poisoning: Report of two cases. *Clin Chem* **36**: 571–574, 1990.
25. Müller L, Protective effects of DL-α-lipoic acid on cadmium-induced deterioration of rat hepatocytes. *Toxicology* **58**: 175–185, 1989.
26. Prehn JHM, Karkoutly C, Nuglisch J, Peruche B and Krieglstein J, Dihydrolipoate reduces neuronal injury after cerebral ischemia. *J Cereb Blood Flow Metab* **12**: 78–87, 1992.
27. Jorg J, Metz F and Scharafinski H, Drug treatment of diabetic polyneuropathy with alpha-lipoic acid or vitamin B preparations. A clinical and neurophysiologic study. *Nervenarzt* **59**: 36–44, 1988.
28. Pruijn FB, Haenen GRMM and Bast A, Interplay between vitamin E, glutathione and dihydrolipoic acid in protection against lipid peroxidation. *Fat Sci Technol* **93**: 216–221, 1991.
29. Müller L and Menzel H, Studies on the efficacy of lipoate and dihydrolipoate in the alteration of cadmium^{2+} toxicity in isolated hepatocytes. *Biochim Biophys Acta* **1052**: 386–391, 1990.
30. Jonas SK, Riley PA and Willson RL, Hydrogen peroxide cytotoxicity. Low-temperature enhancement by ascorbate or reduced lipoate. *Biochem J* **264**: 651–655, 1989.
31. von Zgliniciki T, Wiswedel I, Trumper L and Augustin W, Morphological changes of isolated rat liver mitochondria during Fe^{2+}/ascorbate-induced peroxidation and the effect of thioctic acid. *Mech Ageing Dev* **57**: 233–246, 1991.
32. Bonomi F, Cerioli A and Pagani S, Molecular aspects of the removal of ferritin-bound iron by DL-dihydrolipoate. *Biochim Biophys Acta* **994**: 180–186, 1989.
33. Bonomi F and Pagani S, Uptake of iron by aproferritin from a ferric dihydrolipoate complex. *Eur J Biochem* **199**: 181–186, 1991.
34. Suzuki YJ, Tsuchiya M and Packer L, Thioctic acid and dihydrolipoic acid are novel antioxidants which interact with reactive oxygen species. *Free Rad Res Commun* **15**: 255–264, 1992.
35. DeLange RJ and Glazer AN, Phycoerythrin fluorescence-based assay for peroxy radicals: A screen for biologically relevant protective agents. *Anal Biochem* **177**: 300–306, 1989.
36. Buege JA and Aust SD, Microsomal lipid peroxidation. In: *Methods in Enzymology* (Ed. L. Packer), Vol. 52: pp. 302–310. Academic Press, San Diego, 1978.
37. Lang J, Gohil K and Packer L, Simultaneous determination of tocopherols, ubiquinols and ubiquinones in blood, plasma, tissue homogenates and subcellular fractions. *Anal Biochem* **157**: 106–116, 1986.
38. Terao K and Niki D, Damage to biological tissues induced by radical initiator 2,2′-azobis(2-amidinopropane)dihydrochloride and its inhibition by chain-

breaking antioxidants. *Free Rad Biol Med* **2**: 193–201, 1986.
39. Kagan VE, Serbinova EA and Packer L, Recycling and antioxidant activity of tocopherol homologs of differing hydrocarbon chain lengths in liver microsomes. *Arch Biochem Biophys* **282**: 221–225, 1990.
40. Kagan VE, Witt E, Goldman R, Scita G and Packer L, Ultraviolet light-induced generation of vitamin E radicals and their recycling. A possible photosensitizing effect of vitamin E in skin. *Free Rad Res Commun* **16**: 51–64, 1992.
41. Chamulitrat W and Mason R, Lipid peroxyl radical intermediates in the peroxidation of polyunsaturated fatty acids by lipoxygenase. *J Biol Chem* **264**: 20968–20973, 1989.
42. Packer JE, Slater TF and Wilson RL, Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature* **278**: 737–738, 1979.
43. Bonomi F and Pagani S, Removal of ferritin-bound iron by DL-dihydrolipoate and DL-dihydrolipoamide. *Eur J Biochem* **155**: 295–300, 1986.
44. Nagaoka S-I, Okauchi Y, Urano S, Nagashima U and Mukai K, Kinetic and *ab initio* study of the prooxidant effect of vitamin E. Hydrogen abstraction from fatty acid esters and egg yolk lecithin. *J Am Chem Soc* **112**: 8921–8924, 1990.
45. Rao RDN, Fischer V and Mason RP, Glutathione and ascorbate reduction of the acetaminophen radical formed by peroxidase. *J Biol Chem* **265**: 844–847, 1990.
46. Winkler BS, *In vitro* oxidation of ascorbic acid and its prevention by GSH. *Biochim Biophys Acta* **925**: 258–264, 1987.
47. Kagan VE, Serbinova EA and Packer L, Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. *Biochem Biophys Res Commun* **169**: 851–857, 1990.