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Coenzyme Q10, Vitamin E, and Dihydrothioctic Acid Cooperatively Prevent Diene Conjugation in Isolated Low-Density Lipoprotein

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ABSTRACT

Coenzyme Q (Q_{10}) and α-tocopherol cooperatively delay the onset of diene conjugation in isolated human low density lipoprotein if supplied in water-soluble preparations to blood serum. Both copper ions and morpholino sydnonimine (in the presence of glucose; SIN-1-glucose) -driven diene conjugation is measurable as soon as both reduced Q_{10} and tocopherol are oxidized, where tocopherol oxidation starts after 80–90% consumption of reduced Q_{10}. LDL-bound Q_{10} in turn can be rapidly reduced by dihydrolipoic acid (thioctic acid). This reaction is at least 10 times faster than reduction by ascorbic acid. Antiox. Redox Signal. 2, 327–333.

INTRODUCTION

OXIDIZED LOW-DENSITY LIPOPROTEIN (LDL) by macrophages is supposed to start atherosclerotic developments in the intima of blood vessels (Brown and Goldstein, 1979). Mechanisms protecting LDL from oxidation are thus of importance both for prophylaxis and therapy of atherosclerosis (Jessup *et al.*, 1990). It is well known from the literature (Thomas *et al.*, 1995, 1996) that coenzyme Q_{10} (Q_{10}) works cooperatively with vitamin E in this respect. This cooperation in the hydrophobic region of LDL, for example, is superimposed by interaction with vitamin C in the hydrophilic region, rendering all three antioxidants a powerful "team" subject to a certain "pecking order" (Buettner, 1993). Several different processes have been described as potentially responsible for LDL oxidation in the intima of blood vessels including: (1) different cell types such as monocytes, macrophages, endothelial cells, fibroblasts, and smooth muscle cells (Heinecke, 1997), (2) enzymes such as lipoxygenase, and finally (3) low-molecular-weight oxidants and reactive oxygen species (ROS) such as copper ions (Garner *et al.*, 1997) or peroxynitrite (Darley-Usmar *et al.*, 1992).

Recent reports support the concept that cellular redox activities after appropriate stimulation supply the reductants for the reduction of metal ions such as Cu^{2+} or Fe^{3+} (Garner and Jessup, 1996; Garner *et al.*, 1997) or ROS formation via thiols and superoxide (Heinecke *et al.*, 1993). Because such cell activations are characteristic for inflammatory events formation of nitric oxide (NO) is also indicated at such places (Mayer and Hemmens, 1997). In the presence of superoxide, NO reacts extremely fast under production of the strong oxidant, ONOOH

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TABLE 1. ANTIOXIDANT CONTENT OF LDL AFTER SERUM INCUBATION

	Content [Mol/Mol LDL]			
Antioxidants	*A*[a]	*B*[b]	*C*[c]	*D*[d]
α-Tocopherol	5.78 ± 0.1	5.64 ± 0.11	5.67 ± 0.09	**44.79 ± 1.21**
Ubiquinone-10	0.31 ± 0.02	**21.39 ± 0.87**	0.41 ± 0.05	0.21 ± 0.08
Ubiquinol-10	—	—	**5.10 ± 0.24**	—

[a]None.
[b]Ubiquinone-10, 1 m*M*.
[c]Ubiquinol-10, 1 m*M*.
[d]α-Tocopherol, 1 m*M*.

(Pryor and Squadrito, 1995; Hippeli *et al.*, 1997). Experimentally ONOOH is produced by morpholine sydnonimine (SIN-1), a compound that in aqueous solutions simultaneously releases NO as well as superoxide (Bohn and Schönafinger, 1989; Feelisch *et al.*, 1989).

It has been shown that peroxynitrite similar to Fenton systems is able to release ethene from α-keto-methylthiobutyric acid (KMB) (Pryor and Squadrito, 1995). In a similar way, KMB fragmentation is driven by SIN-1 or chemically synthesized ONOOH (Hippeli *et al.*, 1997).

In this study, we explored the influence of reduced coenzyme Q_{10} and α-tocopherol (αToc) on copper- and SIN-1-glucose-induced LDL-oxidation *in vitro*. In addition, we investigated on the reduction of LDL-bound ubiquinone by hydrophilic reductants such as ascorbic acid and dihydrolipoic acid.

MATERIAL AND METHODS

Materials

SIN-1 was given free of charge by Dr. R. Grewe (Fa. Hoechst AG, Frankfurt, Germany). All other chemicals were either purchased from SIGMA (Deisenhofen, Germany), Boehringer (Mannheim, Germany), or Merck (Darmstadt).

Methods

LDL-preparation: To accumulate lipid-soluble antioxidants (αToc, Q_{ox}, Q_{red}) in LDL, we in-

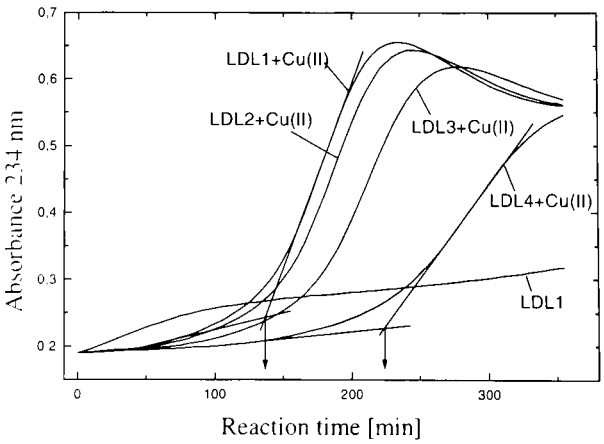

FIG. 1. Effects of ubiquinol-10 on copper-catalyzed LDL oxidation: 1.67 μ*M* Cu(II), 0.05 μ*M* LDL. LDL 1, 0 Mol Qred/Mol LDL, 5.78 Mol αToc/Mol LDL. LDL 2, 0.8 Mol Qred/Mol LDL, 5.15 Mol αToc/Mol LDL. LDL 3, 1.8 Mol Qred/Mol LDL, 5.32 Mol αToc/Mol LDL. LDL 4, 5.1 Mol Qred/Mol LDL, 5.53 Mol αToc/Mol LDL.

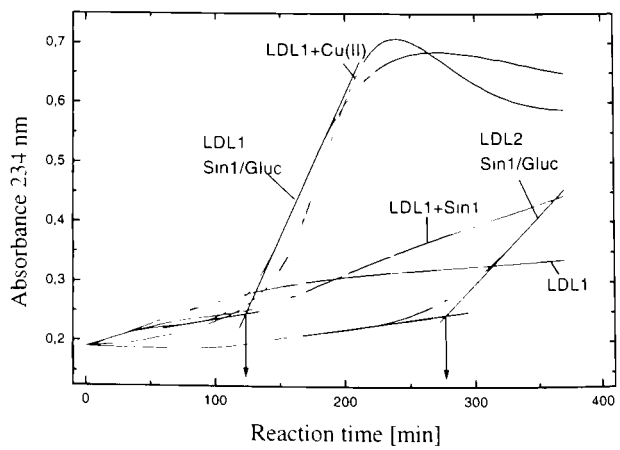

FIG. 2. Effects of ubiquinol-10 on SIN-1-induced and glucose-enhanced LDL oxidation: 10 μ*M* Sin-1, 20 m*M* glucose, 0.05 μ*M* LDL. LDL 1, 0 Mol Qred/Mol LDL, 5.78 Mol αToc/Mol LDL; LDL2, 5.1 Mol Qred/Mol LDL, 5.53 Mol αToc/Mol LDL.

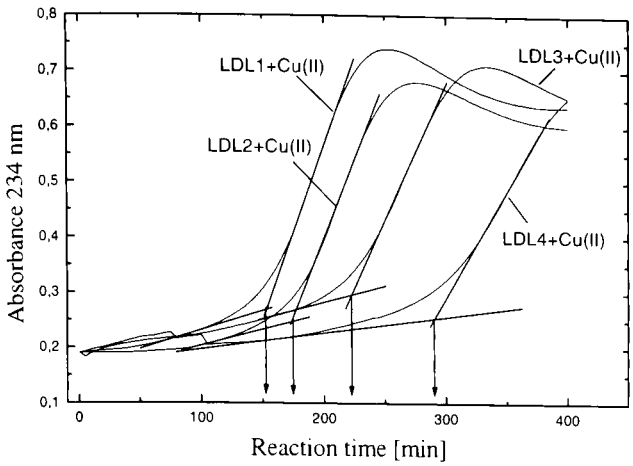

FIG. 3. Effects of ubiquinol-10 and α-tocopherol on copper-catalyzed LDL oxidation: 1.67 μM Cu(II), 0.05 mM LDL. LDL 1, 0.08 Mol Qred/Mol LDL, 3.60 Mol αToc/Mol LDL. LDL 2, 4.60 Mol Qred/Mol LDL, 2.98 Mol αToc/Mol LDL. LDL 3, 0.12 Mol Qred/Mol LDL, 9.90 Mol αToc/Mol LDL. LDL 4, 4.40 Mol Qred/Mol LDL, 9.71 Mol αToc/Mol LDL.

cubated human blood serum with a water-soluble preparation of antioxidants (International Patent Number PCT/EP9/06360) for 6 hr at 37°C. LDL was isolated from human blood serum (donor: healthy male, 58j., 88 kg) by ultracentrifugation as recently described (Kögl *et al.*, 1994; Schlüssel and Elstner, 1995). LDL was stored (4°C, in the dark) no longer than 2 weeks before use.

The αToc and ubiquinone/ubiquinole content of each LDL preparation was determined by organic extraction with *n*-hexane and high-performance liquid chromatography (HPLC) analysis (Nucleosil 300, methanol/isopropanol 92.5/7.5 vol/vol, UV detection at 280 nm). The αToc content was essentially the same for each LDL preparation (5.1 mol/mol LDL).

Oxidation of LDL: LDL oxidation was followed via diene conjugation (the Esterbauer method as modified by Schlüssel and Elstner, 1995). Formation of conjugated dienes was measured spectrophotometrically by monitoring the increase in absorbance at 234 nm (37°C) using 0.05 μM LDL and 1.67 μM Cu^{2+} ions or the indicated amounts of SIN-1 or ONOOH. Experiments with antioxidants—Q_{10}, αToc, dihydrolipoic acid (DHLA)—added to the isolated, not preincubated LDL just before starting the oxidative process are not shown due to lack of physiological relevance.

Reduction of ubiquinone: A total of 2 ml of a 10 m*M* Q_{ox} solution were treated for 1 hr at 37°C with 500 μl of sodium borohydrid solution (40 m*M* in aqueous solution) until the yellow color was completely lost. Reduction of oxidized Q_{10} in isolated LDL by either DHLA or ascorbic acid was measured after extraction of Q_{ox} and Q_{red} from LDL as outlined above.

Peroxynitrite and SIN-1: Peroxynitrite was either produced by aqueous solutions of SIN-1 (Feelisch *et al.*, 1989), or synthesized according to Beckmann *et al.* (1994) as follows: 0.7 *M* hydrogen peroxide solution in 5 ml of 0.6 *M* HCl was mixed with 5 ml of 0.6 *M* KNO_2 on ice for 1 sec. The reaction was quenched with 5 ml of ice-cold 1.2 *M* NaOH. This mixture was then frozen overnight (−20°C). The top layer was collected and stored as stock solution. The concentration was determined before each experiment by measuring the absorbance at 302 nm based on an absorption coefficient of E_{302} = 1670 $M^{-1}cm^{-1}$. A comparison of the oxidative capabilities measuring ethene release from ketomethylthiobutyrate (Hippeli *et al.*, 1997) indicated that SIN-1 on a "molar basis" had about double the oxidative power as compared to synthetic ONOOH, probably due to nitrate impurities in ONOOH also absorbing at 302 nm. Further experimental details are outlined in context with the individual tables and figures.

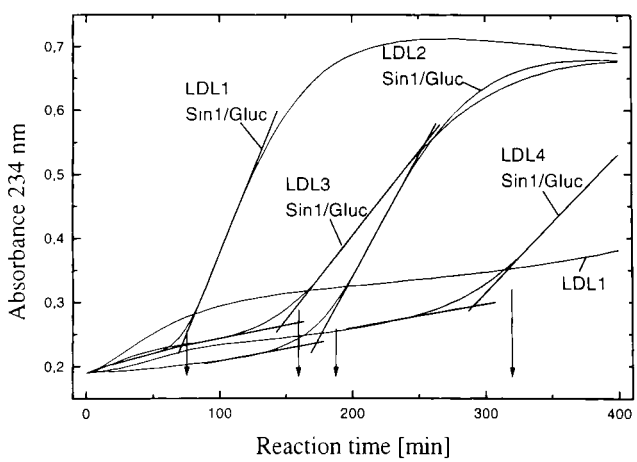

FIG. 4. Effects of ubiquinol-10 and α-tocopherol on SIN-1-induced and glucose-enhanced LDL oxidation: 10 μM SIN-1, 20 m*M* glucose, 0.05 -μM LDL. LDL 1, 0.08 Mol Qred/Mol LDL, 3.60 Mol aToc/Mol LDL. LDL 2, 4.60 Mol Qred/Mol LDL, 2.98 Mol aToc/Mol LDL. LDL 3, 0.12 Mol Qred/Mol LDL, 9.90 Mol aToc/Mol LDL. LDL 4, 4.40 Mol Qred/Mol LDL, 9.71 Mol aToc/Mol LDL.

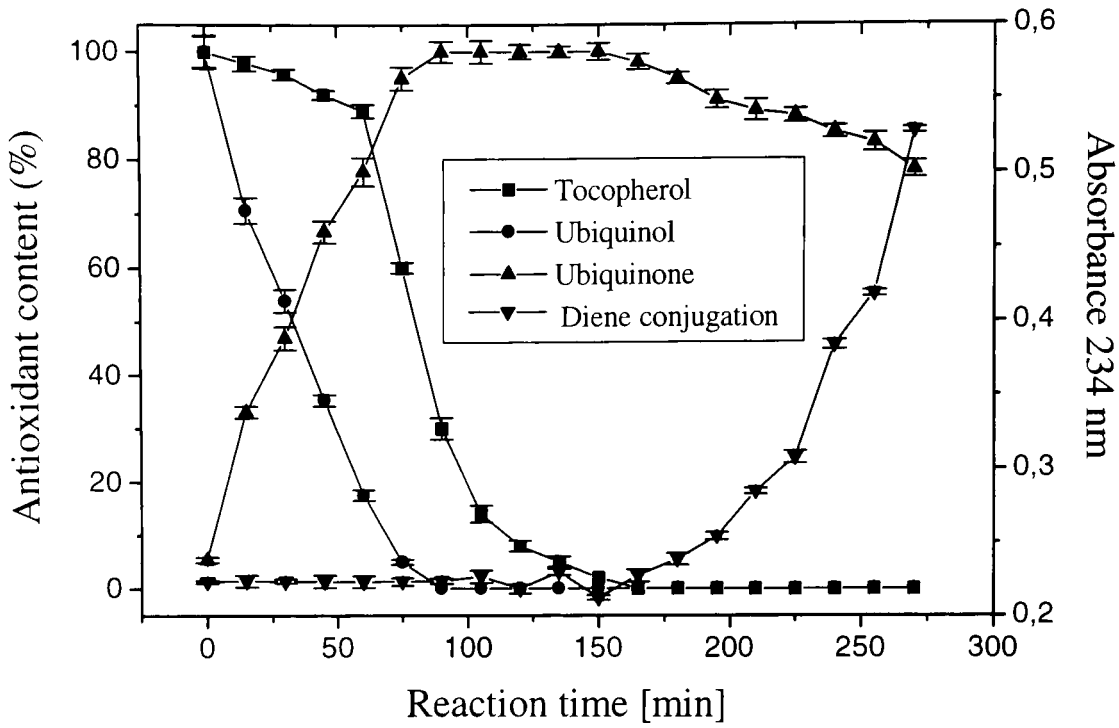

FIG. 5. Antioxidant content and diene conjugation during copper-induced LDL oxidation: Cu(II) 1 μM, LDL 1.6 μM. Antioxidant content at the beginning of the experiment: 5.1 Mol Qred/Mol LDL, 0.24 Mol Qox/Mol LDL, 5.53 Mol αToc/Mol LDL.

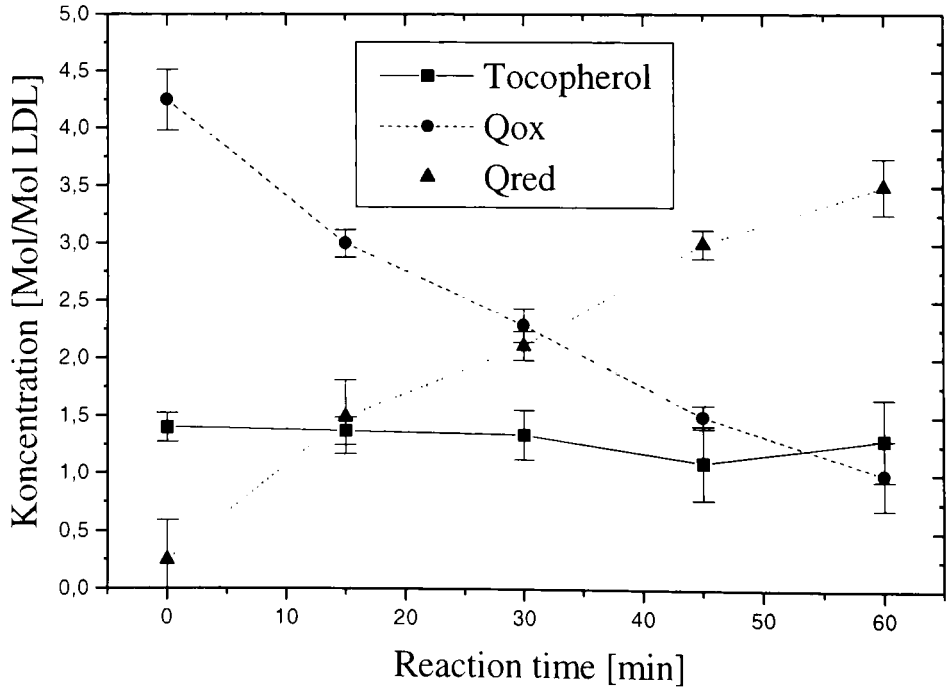

FIG. 6. Reduction of ubiquinone-10 bound to LDL by DHLA: 500 μM DHLA, 1.5 μM LDL (32,79 Mol Qox/Mol LDL, 3.43 Mol αToc/Mol LDL).

The presented results are means of three individual experiments undertaken on two different days ($n = 6$). Standard deviation are given as σ ($n - 1$). More experimental details are given in Koske (1998).

RESULTS

Coenzyme Q_{10} and vitamin E as water-soluble preparations accumulate in LDL after appropriate incubation with human blood serum (Table 1). Isolated LDL from these incubations contain different amounts of αToc and Q_{10}, thus exhibiting a prolonged lag phase of diene conjugation. The extension of the lag-phase corresponds directly with the internal Q_{10} content (Fig. 1). This also holds for the SIN-1/glucose (Schneider and Elstner, 1999) -initiated reaction (Fig. 2). Extension of the lag-phase of dienconjugation correlates directly with the concentrations of both αToc and Q_{red}, again observable in the copper system (Fig. 3) as well as in the SIN-1/glucose system (Fig. 4).

Rapid consumption of tocopherol bound to LDL is measured as soon as more than 90% of reduced Q_{10} has been converted into the oxidized form. Copper-catalyzed diene conjugation in LDL, in turn, is only measurable after disappearance of tocopherol (Fig. 5). This sequence is almost identical for LDL oxidation driven by either copper ions (Fig. 5) or by SIN-1, a compound that simultaneously releases both superoxide and nitrogen monoxide (data not shown).

Particle-bound oxidized Q_{10} can rapidly (complete reduction in ~1 hr) be re-reduced by DHLA (reduced thioctic acid) (Fig. 6) or in a slow process (represented by about 10% of the rate presented in Fig. 6) by ascorbic acid (Fig. 7). Lipoic acid has no effect on Q_{10} reduction and added NADH is also without effect, indicating that the LDL particle contains no NADH-Q_{10} reductase- (dehydrogenase-) activity (data not shown). Q_{10} reduction by DHLA in aqueous solution shows different kinetics as compared to particle-bound Q_{10} and proceeds only with one-half of this velocity (100% reduction in ~2 hr).

DISCUSSION

LDL-oxidation is thought to represent one critical step during initiation of atherosclerosis (Brown and Goldstein, 1979; Esterbauer *et al.*, 1988, 1990; Jürgens *et al.*, 1987; Steinbrecher *et al.*, 1989; Kuzuya *et al.*, 1991; Jessup *et al.*, 1990; Darley-Usmar *et al.*, 1992; Graham *et al.*, 1993; Hogg *et al.*, 1993; Moore *et al.*, 1995). As physiological oxidants, transition metal ions (see refs. in Hippeli and Elstner, 1999) and peroxynitrite have recently been discussed (Darley-Usmar *et al.*, 1992; Hogg *et al.*, 1993; Moore *et al.*, 1995). Reports on glucose-enhanced LDL oxidation by white blood cells (Rifici *et al.*, 1994; Kawamura *et al.*, 1994), referring to the diabetic situation, seem to include ROS such as superoxide.

The delay of the appearance of absorbance-increase at 234 nm in isolated LDL (diene con-

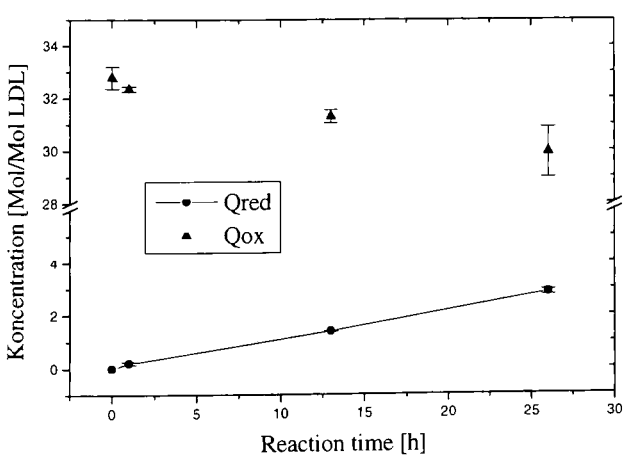

FIG. 7 Reduction of ubiquinone-10 bound to LDL by ascorbic acid: 4 m*M* ascorbic acid, 1.5 μM LDL (32,79 Mol Qox/Mol LDL, 3.43 Mol αToc/Mol LDL).

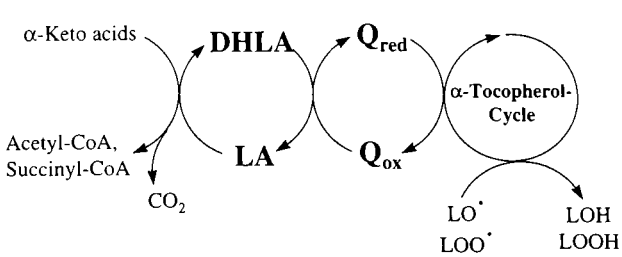

FIG. 8. Possible scheme of electron transport for the detoxification of fatty acid radicals ($LO^{\cdot}$, $LOO^{\cdot}$) at the expense of α-ketoacids. DHLA, Dihydrolipoic acid; LA, lipoic acid; Qred, Ubiquinol-10; Qox, Ubiquinone-10.

jugation) after oxidation by copper ions is a well-known method for determining the intrinsic antioxidative potential of LDL or the functions of external antioxidants. We used this method to study the protective effects of αToc and Q_{10} on LDL oxidation, driven by either copper ions or by SIN-1/glucose.

Different amounts of αToc and Q_{10} accumulate in LDL if blood serum is incubated with these antioxidant in aqueous solution (see Methods; Table 1). If we take preparations with different intrinsic antioxidant levels and compare the corresponding DC lag phases, we learn that best protection of LDL is achieved by both high αToc and Q_{10} content. This can be shown for both copper- and SIN-1/glucose-driven DC (Figs. 1–4). αToc and Q_{red} are consumed during diene conjugation (DC): while Q_{red} is oxidized into Q_{ox}, αToc decays into products that were not identified. This process occurs within a strict time scale: onset of DC occurs as soon as αToc is totally consumed, where disappearance of αToc is visible as soon as approximately 85% of total Q_{red} is converted into Q_{ox} (Fig. 5) indicating a clear pecking order of these antioxidants (Buettner, 1993). The question as to which component from the aqueous compartment reconstitutes Q_{red} from Qox was also addressed. We compared ascorbic acid with DHLA regarding to their potentials to reduce Q_{ox} in LDL and found that ascorbic acid reacts extremely slowly as compared to DHLA (Figs. 6 and 7).

This result is in agreement with results from Nohl's group (Kozlov *et al.*, 1999) reporting that DHLA reduces Q_{ox} by two-electron transfer, thus avoiding the potentially pro-oxidative semiquinone status and preventing biomembranes from peroxidation.

Our experiments, in agreement with other reports (Karlsson *et al.*, 1992; Alleva *et al.*, 1997), suggest cooperative LDL protection by intrinsic antioxidants, including thioctic acid and tocopherol where Q_{10} plays a pivotal role as mediator between aqueous- and lipid-phase redox events. The detoxification of fatty acids radicals by this electron transport may finally work at the expense of α-keto acids (Fig. 8).

ABBREVIATIONS

αToc, α-tocopherol; DC, dien conjugation; DHLA, dihydrolipoic acid; LA, lipoic acid; HPLC, high-performance liquid chromatography; KMB, α-keto-methylthiobutyric acid; LDL, low-density lipoprotein, ONOOH, peroxynitrite; NO, nitric oxide; Q_{10}, coenzyme Q_{10}; Q_{ox}, ubiquinone; Q_{red}, ubiquinol; ROS, reactive oxygen species; SIN-1, morpholino sydnonimine.

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