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Prooxidant and Antioxidant Properties of Trolox C, Analogue of Vitamin E, in Oxidation of Low-Density Lipoprotein

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Trolox C (Trolox), a water-soluble analogue of vitamin E lacking the phytyl chain, was investigated with respect to its effect on the oxidation of low-density lipoprotein (LDL). Trolox was added at different time points of LDL oxidation induced by Cu^{2+} and aqueous peroxyl radicals. In the case of Cu^{2+}-induced LDL oxidation, the effect of Trolox changed from antioxidant to prooxidant when added at later time points during oxidation; this transition occurred whenever α-tocopherol was just consumed in oxidizing LDL. Thus, in the case of Cu^{2+}-dependent LDL oxidation, the presence of lipophilic antioxidants in the LDL particle is likely to be a prerequisite for the antioxidant activity of Trolox.

When oxidation was induced by peroxyl radicals, as a model of metal-independent oxidation, the effect of Trolox was always antioxidant, suggesting the importance of Cu^{2+}/Cu^{+} redox-cycling in the prooxidant mechanism of Trolox. Our data suggest that, in the absence of significant amounts of lipophilic antioxidants, LDL becomes highly susceptible to oxidation induced by transition metals in the presence of aqueous reductants.

Keywords: Low-density lipoprotein, lipid peroxidation, antioxidants, Trolox, prooxidant effect

Abbreviations: LDL, low-density lipoprotein; CD, conjugated dienes; BHT, butylated hydroxytoluene; AAPH, 2,2′-azobis (2-amidinopropane hydrochloride); PBS, phosphate buffered saline; LL-CL, low-level chemiluminescence; TocOH, α-tocopherol; Trolox C, Trolox

INTRODUCTION

There is strong experimental evidence supporting the oxidative hypothesis of atherosclerosis, which assumes that low-density lipoprotein (LDL), after oxidation, contributes to lesion formation.[1] A wide array of biological effects of oxidized LDL was demonstrated up to now; among the most important are: the uncontrolled uptake by macrophages (leading to foam cell formation), the increased synthesis of growth factors, chemoattractants and cell adhesion molecules by cells of arterial wall, cytotoxicity, and several prothrombotic mechanisms.[1–3] LDL, due to its content of polyunsaturated fatty acids, is highly

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susceptible to oxidation,[3] which is prevented in physiological fluids by high concentrations of water-soluble antioxidants,[4–6] as well as by the endogenous liposoluble antioxidants of LDL.

According to a general model, oxidation of isolated LDL starts after consumption of its antioxidants, among which α-tocopherol (TocOH) is the most abundant, at least on molar basis per LDL particle.[3] This view was challenged when evidence appeared that, under experimental conditions characterized by low radical flux, noticeable peroxidation occurs also in the presence of TocOH.[7–9] At present, there is ongoing debate about the overall role of TocOH during LDL oxidation, which was described to be antioxidant,[3] prooxidant[8] or neither.[10]

Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Trolox) is a water-soluble analogue of TocOH which lacks the phytyl side chain.[11] The antioxidant activity of Trolox has been documented in several different experimental models; the mechanism of this effect has been ascribed to a peroxyl-radical scavenging reaction and to synergism with other antioxidants, such as ascorbate.[12–14] Despite the structural difference, the redox potentials of Trolox and TocOH are quite similar.[15] Therefore, the chemical reactivity of Trolox and TocOH are comparable as regards the phenolic OH-group, the function involved in radical scavenging.

The experimental approach of the present study consists of adding Trolox at different time points during LDL oxidation mediated by either Cu^{2+} ions or peroxyl radicals. As the physical and chemical properties of LDL differ considerably from native to oxidized LDL, it is conceivable that Trolox induces different effects, depending on the time of addition. Our study gives insights into the anti- and pro-oxidant effect of Trolox, which is of potential interest as a pharmacological agent and was recently considered as a useful substitute of TocOH for determination of kinetic parameters of LDL oxidizability.[16]

We show that the presence of a certain amount of endogenous antioxidants within the LDL particle is a prerequisite for the antioxidant effect of Trolox, in case of Cu^{2+}-induced LDL oxidation. Moreover, by showing the anti- and pro-oxidant potentials of the 6-OH-chroman ring under the same experimental conditions, our study contributes to reconciliating apparently conflicting findings regarding the role of TocOH in LDL oxidation.

MATERIALS AND METHODS

Materials

2,2′-Azobis (2-amidinopropane hydrochloride) (AAPH) was purchased from Polysciences (Warrington, PA, USA). Trolox C was purchased from Aldrich (Milwaukee, WI, USA). Other reagents were of AR grade, obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

Preparation of LDL

Ethylenediaminetetraacetate (EDTA)-plasma (1 mg/ml) was prepared from venous blood of normolipidemic, healthy volunteers of both sexes (age 25–37) after overnight fast. Plasma samples were pooled and frozen at −80°C in 0.6% sucrose for up to four weeks. LDL was isolated by ultracentrifugation (Beckman L70) in a single step discontinuous gradient using a NVT 65 rotor (Beckman).[17] LDL was stored in a vial under argon at 4°C in the dark and used within one week after preparation. Chemical composition of LDL was normal according to Esterbauer and Ramos.[3]

LDL Oxidation

Before oxidation, LDL was freed of EDTA by gel filtration with an Econo-Pac 10 DG column (Biorad, Hercules, CA, USA) using PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) as eluent. LDL concentration was determined by total cholesterol assay (CHOD-PAP kit, Boehringer Mannheim, Germany). Assuming

a molecular mass of 2.5 MDa for LDL and a cholesterol content of 31%, a concentration of 0.1 μM LDL corresponded to 0.25 mg/ml total mass and 79 μg/ml total cholesterol.[18]

EDTA-free LDL was oxidized with either 1.6 μM $CuSO_4$ or 1 mM AAPH in PBS at 37°C. Oxidation was followed by monitoring the increase in absorbance at 234 nm (A234 nm), due to the formation of conjugated diene hydroperoxides from polyunsaturated fatty acids.[3] When LDL oxidation was induced by AAPH, which thermally decomposes to UV absorbing products, the absorbance at 234 nm of a PBS solution containing AAPH alone was subtracted. Lag-time and rate of CD formation were considered as indexes of LDL oxidation and were determined according to Puhl *et al.* and Esterbauer *et al.*[19,20] The coefficient of variation for lag-time, calculated from all the oxidation profiles described in this study, was 5%. The concentration of CD was calculated from A234 nm using a molar absorbance of 29500 l mol^{-1} cm^{-1}.

Determination of α-tocopherol

TocOH concentration was determined as described.[21] Briefly, 1 ml of sample (containing 0.1 nmol LDL) was withdrawn from the reaction mixture and immediately mixed with the same volume of ice-cold ethanol containing 2 mg/ml butylated hydroxytoluene and 50 μl of an aqueous EDTA-solution (100 mg/ml). For extraction, 2 ml of hexane were used, 1.4 ml of which were dried *in vacuo*. The residue was dissolved in ethanol/ethyl acetate (10/1, v/v) and analyzed by HPLC on a LichroSpher 100 RP-18 column (eluent methanol/acetonitrile/ethanol/water, v/v, 0.01% ammonium sulfate), using fluorescence detection (292/335, excitation/emission wavelength) and external standardization.

Chemiluminescence Measurements

Low-level chemiluminescence (LL-CL) was measured in a Lucy 1 luminometer (Anthos Labtech Instruments, Salzburg, Austria) equipped with a photon counting photomultiplier (sensitivity 300–700 nm). Integration time for each data point was set to 90 s. LDL (0.3 μM) was incubated in PBS in the absence and in the presence of Trolox at the concentrations indicated; oxidation was induced by addition of 1.6 μM Cu^{2+}. The assays were performed at 37°C in a white microplate.

The LL-CL results from recombination of peroxyl radicals which, according to Russell's mechanism, yield triplet carbonyl compounds and singlet oxygen.[22] According to previous results, the main chemiluminescent species detected under our experimental conditions are triplet carbonyls.[23]

RESULTS

The oxidation of LDL by Cu^{2+} as assayed by CD exhibited a lag phase, a propagation phase characterized by the rapid increase of CD content, and a decomposition phase, consistent with previous reports (Figure 1A)[18,20] under the experimental conditions used. Trolox exhibited a protective effect against LDL oxidation, when added just before Cu^{2+}. Lag-time, a commonly used index of resistance to oxidation, increased proportionally with Trolox concentration (Figure 1B). Maximum rate of propagation and maximum CD concentration were not affected by Trolox, and were 0.7 ± 0.08 μM/min, and 32 ± 4 μM, respectively.

When Trolox, at the final concentration of 2.5 μM, was added at different time points during the time-course of Cu^{2+}-induced LDL oxidation, the progress of oxidation showed remarkable differences. When added at 15 and 25 min, Trolox was still capable of delaying the onset of propagation, i.e. increased lag-time, compared to the control and even to Trolox added at the beginning of incubation (Figure 2A). Trolox added at 35, 50 and 65 min stimulated the oxidation process, as indicated by the immediate increase of CD absorbance (Figure 2B). Thus, the effect of Trolox on Cu^{2+}-induced LDL oxidation changed from

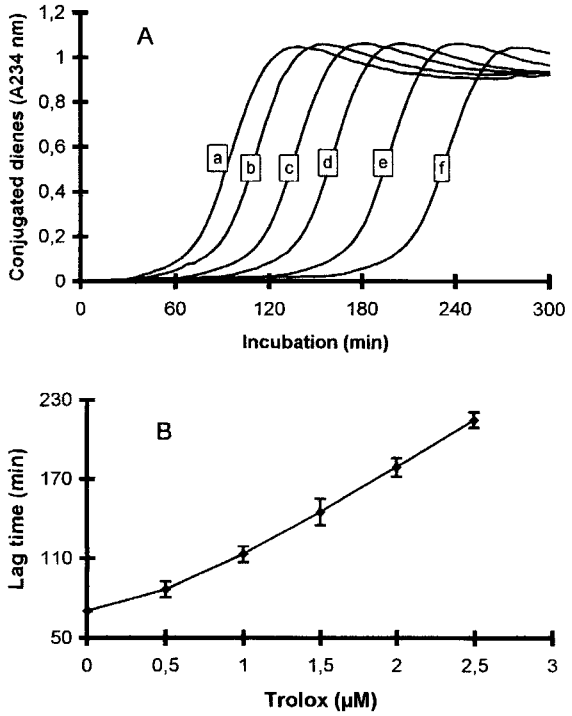

FIGURE 1 Kinetics of LDL oxidation induced by Cu^{2+} in the presence of Trolox. Panel A: CD vs time profiles. LDL (0.25 mg/ml, total mass, equivalent to 0.1 μM) was incubated in PBS at 37°C with 1.6 μM Cu^{2+} in the absence (a) and in the presence of.0.5 μM (b), 1 μM (c), 1.5 μM (d), 2 μM (e), 2.5 μM (f) Trolox, which was added just before Cu^{2+}. Oxidation was followed by monitoring CD formation. The results are representative of four independent experiments. Panel B: Modification of lag-time of LDL oxidation by Trolox. LDL was incubated with Trolox as described above. Lag-time, index of resistance to oxidation, was determined graphically as the intercept of the tangents to the slow and fast increase of CD vs time profiles. Values are the mean ± SD of four independent experiments.

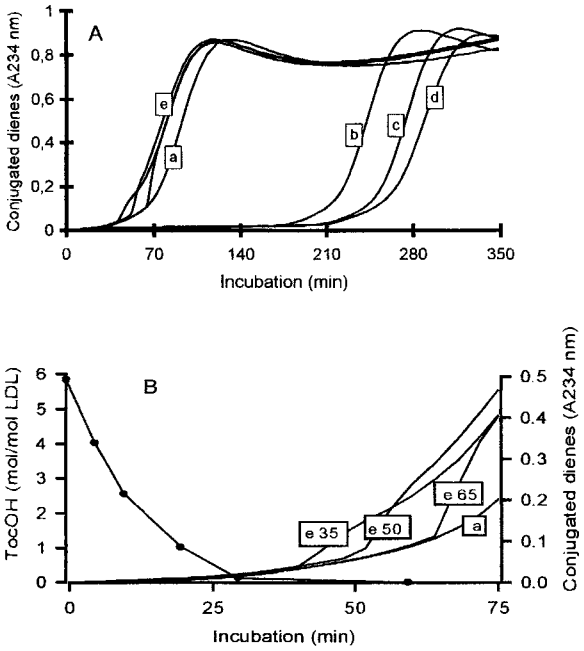

FIGURE 2 Antioxidant and prooxidant effect of Trolox added at different time points on LDL oxidation induced by Cu^{2+}, as assessed by CD formation. Panel A: LDL was incubated as described in the legend to Figure 1. Trolox either was absent (a) or was added, at the final concentration of 2.5 μM, at 0 min (b), at 15 min (c), at 25 min (d) and at 35, 50 and 65 min (e). The results are representative of three independent experiments. Panel B: a magnified view of Panel A, showing that Trolox addition at 35 min (e 35), 50 min (e 50), 65 min (e 65) of incubation resulted in a fast increase of CD formation. Moreover, the time-course of TocOH consumption during LDL oxidation in the absence of Trolox is shown.

antioxidant to prooxidant between 25 and 35 min of incubation. The absorbance of 2.5 μM Trolox at 234 nm is 0.01 a.u., thus its contribution to the observed increase of absorbance is negligible.

Consistent with previous reports,[3,20] the concentration of TocOH was decreasing during lag-time (Figure 2B); it was 5.87 ± 0.16 mol TocOH/mol LDL ($n = 4$) at the beginning of incubation and after 30 min it was below 0.1 mol TocOH/mol LDL. Thus, the switch between anti- and pro-oxidant effect of Trolox coincided with the consumption of TocOH.

The addition of Trolox at later time points during incubation increased immediately the rate of CD formation (Figure 3).

When 2.5 μM BHT, a hydrophobic phenolic antioxidant, scavenger of peroxyl radicals, was added at different time points, from 0 to 65 min of incubation, Cu^{2+}-mediated LDL oxidation was stopped (Figure 4).

Similar results were obtained when LDL oxidation was monitored by LL-CL. As expected from the CD assay, Trolox (5 μM) added immediately before Cu^{2+} acted as an antioxidant, delaying the onset of LL-CL as compared to the control (Figure 5). Trolox added at 65 min of incubation increased the level of LL-CL, a measure of lipid

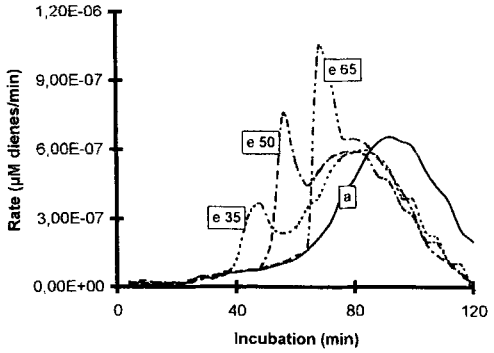

FIGURE 3 Rate of CD formation after Trolox addition. LDL was incubated as described in the legend to Figure 1. The profiles of the rate of CD formation vs time result from the first derivative of CD vs time profiles. Trolox either was absent (a) or was added, at the final concentration of 2.5 μM, at 35 min (e 35), at 50 min (e 50), and at 65 min (e 65) of incubation. The results are representative of three independent experiments.

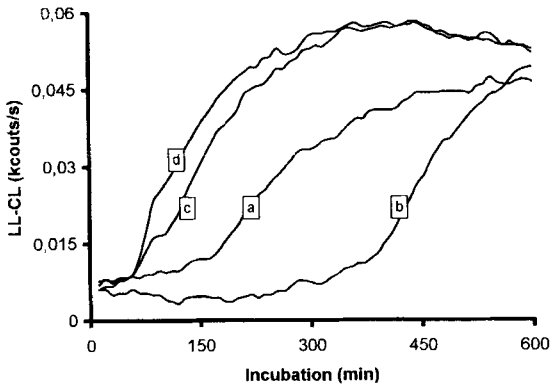

FIGURE 5 Variable effect of Trolox added at different time points on LDL oxidation induced by Cu^{2+}, as assessed by low-level chemiluminescence. LDL (0.3 μM) was oxidized with 1.6 μM Cu^{2+} in PBS at 37°C. Trolox was either absent (a), or added at 0 (b) and at 65 min (c) of oxidation, at the final concentration of 5 μM; the addition of 16 μM Trolox, at 65 min, corresponds to (d). The results are representative of four independent experiments.

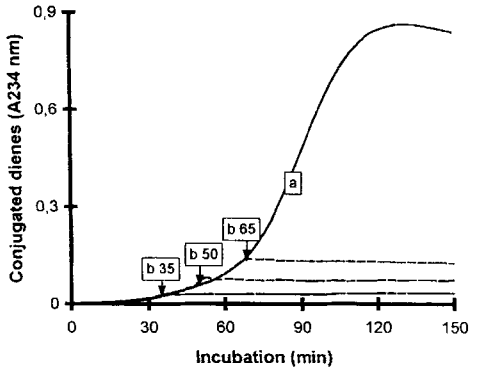

FIGURE 4 Effect of BHT addition at different time points on LDL oxidation induced by Cu^{2+}. LDL was incubated as described in the legend to Figure 1. BHT, at the final concentration of 2.5 μM, was added at 35, 50, and 65 min of incubation, as indicated by the arrows. When BHT was added at 0 and 25 min of incubation, the resulting CD profiles coincided with the *x*-axis; therefore, they are not shown. LDL oxidation in the absence of BHT is shown in (a). The results are representative of three independent experiments.

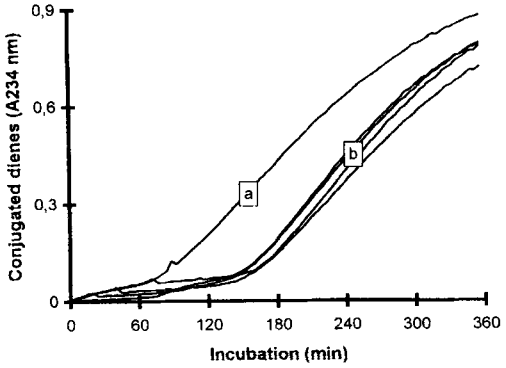

FIGURE 6 Effect of Trolox on LDL oxidation induced by peroxyl radicals. LDL (0.1 μM) was incubated in PBS and oxidation was triggered by 1 mM AAPH, at 37°C. Trolox either was not present (a), or was added, at the final concentration of 2.5 μM, at 0, 25, 45 and 75 min of incubation (lines are grouped as (b)). The results are representative of three independent experiments.

peroxidation.[23] Whenever a higher concentration of Trolox (16 μM) was added at the same time point, the increase of LL-CL was more pronounced (Figure 5).

When LDL oxidation was mediated by aqueous peroxyl radicals (i.e. a metal independent agent), Trolox increased lag-time, indicating efficient antioxidant activity, *irrespective of the time points of addition* (Figure 6); no evidence for a prooxidative effect of Trolox was found during peroxyl-radical-induced LDL oxidation.

DISCUSSION

Our study explores the effect of Trolox, analogue of vitamin E, on Cu^{2+}- and peroxyl-radical-induced LDL oxidation; two different methods for detection of lipid peroxidation, the CD assay and the emission of LL-CL, led to similar results, supporting our conclusions.

The observation that Trolox can act both as anti- and pro-oxidant, depending on time of addition is in line with the evidence that other antioxidants, including ascorbate and dehydroascorbate,[24,25] flavonoids[25,26] and caffeic acid,[27] have a similar effect when LDL oxidation was induced by transition metals.

Regarding the mechanism of the antioxidant effect of Trolox, the simple interpretation that it scavenges lipid oxidizing species in LDL lipid phase conflicts with its water solubility and does not account for the prooxidant activity at later stages of Cu^{2+}-induced oxidation. We propose that Trolox may act in synergism with TocOH; in particular, Trolox may be able to reduce tocopheroxyl radical back to TocOH, regenerating TocOH. This is consistent with the similar value of their redox potentials[15] and with previous studies showing the rapid regeneration of β-, γ-, and δ-TocOH from their parent radicals by α-TocOH.[28] From a structural point of view, TocOH is positioned at the LDL–water interface[28,29] and is therefore promptly accessible to react with the water-soluble Trolox.

A particular point is that the antioxidant effect of Trolox is higher (as deduced from duration of lag-times) when added at 15 and 25 min of incubation than at time zero. Recently, we showed that the rate of initiation during LDL oxidation induced by Cu^{2+} is not constant, but is decreasing during lag phase;[21] on this basis, it is conceivable that the efficiency of antioxidants appears to be higher at lower rate of initiation during advanced lag-phase, as long as TocOH is present.

Under our experimental conditions, TocOH was consumed at about 30 min of incubation (in the absence of Trolox), before the onset of the propagation phase of LDL oxidation. Consistent with the proposed mechanism, Trolox added at 35 and 65 min did not inhibit oxidation, rather it increased LDL oxidation, conceivably by accelerating the reduction of Cu^{2+} to Cu^{+}. The Cu^{2+} reducing ability of Trolox was shown in,[30,31] in accordance with our results (data not shown); in addition, Burkitt and Milne proposed that this reaction in absence of LDL is accompanied by the release of $OH^{\bullet}$ radicals.[30] It is uncertain whether this mechanism is involved in the prooxidant effect of Trolox, because various mixtures including H_2O_2, Fe^{2+}, EDTA and ascorbate are inefficient in triggering oxidation of LDL, in contrast to other lipid substrates.[32] It is likely that Cu^{+} may be the 'prooxidant' agent under our experimental conditions, acting by decomposing rapidly minute amounts of lipid peroxides formed during lag phase to propagating alkoxyl radicals. Moreover, formation of $OH^{\bullet}$, could not explain the particular 'switching' from antioxidant to prooxidant mode of the action of Trolox, triggered by the consumption of TocOH.

Under our experimental conditions, the prooxidant effect of Trolox is observed under the following conditions: (1) LDL is depleted from endogenous lipophilic antioxidants, (2) oxidation is induced by Cu^{2+}, suggesting that redox-cycling of the metal has a pivotal role, (3) the reductant, Trolox, is water-soluble and does not enter the lipid phase.

The behavior of BHT is fully compatible with our model. Due to its hydrophobicity, BHT enters LDL and scavenges free radicals directly; on this basis, it is unimportant whether BHT is added at early or advanced oxidation.

When AAPH was used as oxidant, as a model of metal-independent oxidation, the effect of Trolox was invariably antioxidant; it is well conceivable that Trolox can intercept aqueous peroxyl radical generated by thermal decomposition of AAPH. Moreover, the antioxidant effect of Trolox at early and later stage of AAPH-induced oxidation stresses the bearing of Cu^{2+}/Cu^{+} redox cycles in its prooxidant effect.

By showing the ambiguous anti- and pro-oxidant effect of chromanols on LDL oxidation, our results may contribute to explain why, in different *in vitro* studies, partially conflicting conclusions about the role of TocOH in lipoprotein oxidation have been drawn. The extrapolation of our results from this experimental model to the *in vivo* situation suggests that the depletion of lipophilic antioxidants from biological membranes is a major prerequisite for lipid peroxidation, which may not be overcome by water-soluble antioxidants alone, at least in the case of transition-metal-induced oxidative stress.

Acknowledgments

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