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Prooxidant role of vitamin E in copper induced lipid peroxidation

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When exposed to Cu^{2+}, α-tocopherol, in detergent dispersion, is rapidly oxidised. Moreover, if phospholipids and traces of their hydroperoxide derivatives are included in these dispersions, Cu^{2+} initiates lipid peroxidation, the rate of which is dramatically stimulated by α-tocopherol. The observation that the rate of α-tocopherol consumption is identical in the absence and in the presence of lipids undergoing peroxidation, apparently rules out any antioxidant effect. These results are consistent with a prooxidant effect of vitamin E, mediated by its capability to reduce Cu^{2+} to Cu^{+} which, in turn, produces, from lipid hydroperoxides, the highly reactive alkoxyl radicals. Present data highlight the risk of misleading results in interpreting the significance of lags in peroxidation of LDL challenged with Cu^{2+}.

Vitamin E; α-Tocopherol; Copper; Phospholipid hydroperoxide; Low density lipoprotein; Atherosclerosis

1. INTRODUCTION

Peroxidation of low density lipoproteins (LDL) has been suggested to be a pathophysiologically relevant mechanism leading to the increased lipoprotein uptake by macrophages which eventually are transformed into foam cells. This abnormal LDL metabolism, occurring through scavenger receptors, has been involved in the pathogenesis of atherosclerosis [1–4]. Several studies have been published therefore, dealing with LDL peroxidation, their oxidative resistance and the biological effects of oxidatively modified LDL on cells present in the arterial wall during atherogenesis. Different oxidative challenges have been used, to characterise the factors affecting LDL oxidative resistance.

LDL undergo an oxidative degradation when incubated with: (i) cells, endothelial cells, smooth muscle cells, macrophages [1,5–8]; (ii) specific enzymes, lipoxygenases [9]; (iii) free radical generators, diazocompounds [10]; and (iv) hemoproteins [11] and transition metal ions [12,13]. The peroxidation of LDL with Cu^{2+} is simple and quite reproducible and became the most widely used challenge to asses the overall oxidative resistance of these lipoproteins. Although the molecular mechanism of Cu^{2+} induced LDL peroxidation has not been completely elucidated, from its kinetics several conclusions on the oxidative resistance of LDL have been drawn ([14] and literature herein cited). When LDL are incubated with Cu^{2+}, after a period of resistance to oxidation, the lipid peroxidation rate, measured as absorbance of conjugated dienes, production of lipid hydroperoxides or their decomposition products, progressively increases. During this induction period or lag phase, when the formation of oxidation products is minimal, antioxidants are consumed. Particularly, α-tocopherol consumption is very fast, being first consumed among other antioxidants present in LDL particles.

α-Tocopherol, the most active form of vitamin E, has been suggested to be a major antioxidant in LDL and its activity as chain-breaking antioxidant has been conclusively shown in several peroxidation models [15,16]. It has been argued, therefore, that α-tocopherol must play a key role in modulating oxidative resistance of LDL challenged with Cu^{2+} as well.

However, recent reports indicate that the length of the induction period is only partially accounted for by vitamin E content in LDL [17–19].

In this paper we show that Cu^{2+} is able per se to rapidly oxidise α-tocopherol, thus preventing any relevant antioxidant effect of the vitamin. Instead, a faster rate of peroxidation was observed when α-tocopherol was added to phospholipid mixed micelles undergoing Cu^{2+}-induced peroxidation. This indicates that, upon the reaction between α-tocopherol and Cu^{2+}, prooxidant species are formed.

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Abbreviations: DOC, sodium deoxycholate; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; PLPC, palmitoyl linoleoyl phosphatydilcoline; PBS, 10 mM Na-Phosphate buffer, 0.16 M NaCl, pH 7.4; PLPCOOH, palmitoyl linoleoyl phosphatydilcoline 13-hydroperoxide.

2. MATERIALS AND METHODS

2.1. *Phospholipid and vitamin E purification*

Commercially available PLPC and vitamin E were purified by HPLC as follows.

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(i) PLPC (Sigma Chemical Co.) was loaded onto a 4.6 × 250 mm Ultrasphere ODS column. Elution was carried out isocratically with 100% methanol, at a flow rate of 1 ml/min, with simultaneous detection at 210 and 233 nm (Beckman 168 diode array detector module). PLPC eluted as main peak absorbing at 210 nm after 13 min. This peak, collected from several chromatographic runs, was concentrated under argon and quantified by determination of phosphorous content [20].

(ii) DL-α-Tocopherol (Merck) was purified by HPLC using chromatographic conditions identical to those reported above for PLPC, and the detection was carried out at 292 nm. Under these conditions, α-tocopherol eluted after 11 min. The peak, collected from several runs, was concentrated under argon and quantified spectrophotometrically using the extinction coefficient $E = 3144\ M^{-1} \cdot cm^{-1}$.

2.2. *Preparation of micellar solutions*

An appropriate amount of PLPC and/or α-tocopherol was dried under argon and re-suspended with 10 mM Na_2HPO_4, 0.16 M NaCl, pH 7.4, containing 0.1% sodium deoxycholate (PBS/DOC). Phospholipid and α-tocopherol concentrations in the micellar solutions were 1 mg/ml and 10 nmol/ml, respectively.

Micellar solutions prepared as above were incubated with $CuSO_4$ at room temperature.

2.3. *Analysis of vitamin E and phospholipid*

Analysis of phospholipid (PLPC and PLPCOOH) and α-tocopherol was carried out on 50 μl aliquots withdrawn from the incubation mixture and directly injected to HPLC (mobile phase 100% methanol, 4.6 × 250 mm. Ultrasphere ODS column, flow rate 1 ml/min).

The serial alignment of UV (Beckman 168 diode array detector module, recording simultaneously at 210 and 233 nm) and fluorescence (Shimadzu RF. 530 detector module, recording at ex. 286–em. 330) detectors, allowed the simultaneous monitoring of PLPCOOH, α-tocopherol and PLPC, whose retention times were 7, 10 and 13 min, respectively. Quantification of PLPCOOH was accomplished on the basis of a standard curve obtained with standard PLPCOOH prepared by enzymatic hydroperoxidation of HPLC purified PLPC as described [21], and quantified enzymatically as described [22].

Although care was taken to avoid peroxidation of PLPC, after phospholipid purification and/or micellar solution preparation, a minute amount of PLPCOOH (approx. 0.7 nmol/mg) was always detected.

3. RESULTS AND DISCUSSION

Vitamin E, dispersed in deoxycholate micelles, was consumed in the presence of Cu^{2+} (Fig. 1).

Furthermore, the rate of α-tocopherol consumption was the same also when phospholipids were included in micelles (Table I). Moreover, phospholipids, under these conditions, became peroxidized and, surprisingly, in the presence of α-tocopherol, the peroxidation rate was dramatically faster (Fig. 2).

These data indicate that Cu^{2+} reacts with α-tocopherol, apparently leading to its oxidation (Fig. 1) or with phospholipid hydroperoxides, sparking lipid peroxidation (Fig. 2).

The thermodynamically favourable electron transfer reaction between Cu^{2+} and α-tocopherol, producing Ca^{+} and the tocopheroxyl radical, is in agreement with the observed fast rate of α-tocopherol consumption.

The reaction between phospholipid hydroperoxides and Cu^{2+} is the peroxidation sparking reaction, since Cu^{2+} oxidatively decomposes phospholipid hydroperoxides, present in mixed micelles, to the corresponding peroxyl radical, in turn able to propagate peroxidation chain reactions [23].

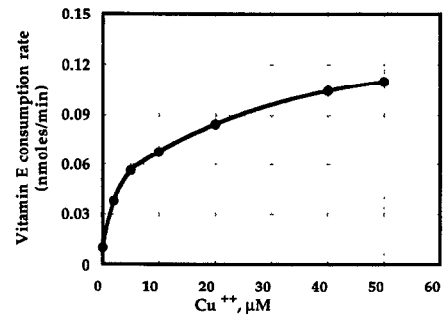

Fig. 1. Dependence of the vitamin E consumption rate on Cu^{2+} concentration. α-Tocopherol (10 μM), dispersed in PBS/DOC, was incubated with different amounts of Cu^{2+}. On aliquots withdrawn at different times, remaining α-tocopherol was measured by HPLC as described in section 2. Initial rates of α-tocopherol exponential decay are reported in the figure. Results represent the mean of three separate experiments.

Furthermore, the reaction of Cu^{2+} with either α-tocopherol or lipid hydroperoxides, produce Cu^{+}, which is also known to promote lipid peroxidation by reductively decomposing lipid hydroperoxides [23] to highly reactive [24] alkoxyl radicals. For this reason we might expect a prooxidant effect of α-tocopherol, counteracting its well known antioxidant effect, related to quenching of free radicals.

Present data, indeed, indicate that, in these conditions, the prooxidant effect of vitamin E by far prevails over the antioxidant effect, peroxidation rate being faster in the presence than in the absence of α-tocopherol (Fig. 2). The progressive increase of lipid peroxidation rate in the presence of vitamin E reported in Fig. 2, is accounted for by the progressively increasing concentration of lipid hydroperoxides, but apparently not by an antioxidant effect of α-tocopherol in the system (see below). A faster peroxidation rate, not increasing, was observed, in fact, if the amount of PLPCOOH present in micelles with PLPC and vitamin E was increased to more than 5 nmol (not shown).

Table I

Vitamin E consumption rate in the presence of Cu^{2+}: effect of phospholipids

	α-Tocopherol consumption (nmol/min)
+ Cu^{2+}	0.109 ± 0.06
+ Cu^{2+} +PLPC	0.114 ± 0.04

α-Tocopherol (10 nmol/ml) was dispersed in PBS/DOC and incubated with 50 μM Cu^{2+}. When present, PLPC was 1 mg/ml. The initial rate of α-tocopherol consumption was measured as reported in legend to Fig. 1. Under these condition PLPC is peroxidised (see Fig. 2).

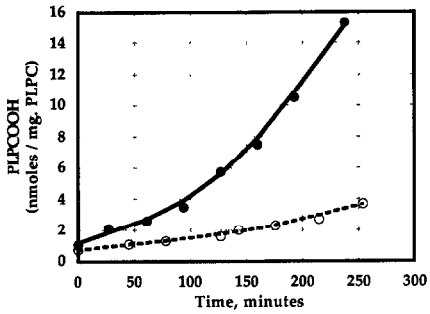

Fig. 2. Time course of phospholipid hydroperoxide formation in the presence of Cu^{2+}: effect of vitamin E. Micelles of PLPC (1 mg/ml), prepared in PBS/DOC with (-●-) or without (-○-) α-tocopherol (10 nmol/ml), were incubated with 50 μM Cu^{2+}. PLPCOOH formation was measured by HPLC on aliquots withdrawn, at different times, as described in section 2. A minute amount of PLPCOOH (0.7 nmol/mg phospholipid) was present at time 0. Formation of PLPCOOH, taking place in the absence of Cu^{2+}, was subtracted. Results represent the mean of three separate experiments.

The observed prooxidant effect of vitamin E, could also be due to the production of the chromanoxyl radical. Indeed, this has been suggested to be reactive enough to propagate peroxidation of lipids in LDL, but not in organic solvent solution, when challenged with radicals produced by thermal decomposition of a diazocompound [25].

The observation that the rate of α-tocopherol consumption in micelles during incubation with Cu^{2+}, is not affected by the presence of phospholipid undergoing peroxidation, apparently rules out the involvement of chromanoxyl radical as a prooxidant species in this system. If this would be the case, indeed, the rate of vitamin E consumption is expected to be decreasing, due to reduction to α-tocopherol of the chromanoxyl radical by phospholipids being oxidised.

Data reported in Table I seem to rule out an antioxidant effect of vitamin E as well. In fact, an antioxidant effect of α-tocopherol would result in a faster consumption rate of the vitamin in the presence of phospholipids undergoing peroxidation.

We may therefore conclude that the observed prooxidant effect of vitamin E is simply due to the reduction of Cu^{2+}.

This is obviously relevant to studies where susceptibility of LDL to lipid peroxidation is tested by challenging these particles with copper.

The measurement of the resistance to an oxidative challenge brought by copper is, therefore, apparently misleading in micelles and, by analogy, in LDL, since α-tocopherol, the claimed key antioxidant, apparently does play a prooxidant effect.

The observations reported in this paper contribute to understanding the reported poor correlation between vitamin E content and lag phase duration when LDL are challenged with copper [17–19].

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