α-Tocopherol mediated peroxidation in the copper (II) and met myoglobin induced oxidation of human low density lipoprotein: the influence of lipid hydroperoxides

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Abstract The principal antioxidant in human LDL, α-tocopherol, is converted to the α-tocopheroxyl radical after reaction with peroxyl radicals or Cu^{2+}, and, if it does not terminate with peroxyl radicals, could initiate lipid peroxidation; a phenomenon called 'tocopherol mediated peroxidation'. Only in the presence of Cu^{2+} and low levels of lipid hydroperoxides was an α-tocopherol dependent decrease in the resistance of LDL to oxidation detected. This suggests that tocopherol mediated peroxidation will probably not contribute significantly as a pro-oxidant process in those individuals most at risk of developing atherosclerosis through an oxidative mechanism.

Key words: Lipid peroxidation; Myoglobin; α-Tocopherol; Lipid peroxide; Atherosclerosis; Low density lipoprotein

1. Introduction

The modification of low density lipoprotein (LDL) through an oxidative mechanism may be one of the initial events leading to the development of an atherosclerotic lesion [1–3]. The evidence in favour of this hypothesis has been extensively reviewed [4–6] and its possible relevance to humans emphasised by the findings of recent epidemiological studies that showed that increased intake of antioxidant vitamins may decrease the risk of developing coronary heart disease [7,8]. Of particular interest is the endogenous lipid soluble chain breaking antioxidant α-tocopherol which appears to be the major defence against oxidative damage in the LDL particle [9–14]. In support of this role for α-tocopherol it has been demonstrated that dietary supplementation leads to increased levels in LDL and, for a given individual, is invariably associated with an increased resistance to oxidation promoted ex vivo by pro-oxidants such as copper [10–12]. However, many of these studies have also revealed that the effectiveness of α-tocopherol as an antioxidant in LDL can vary markedly between individuals when Cu^{2+} is used as a pro-oxidant [10–15]. In contrast, this variation is not seen when a transition metal independent process, such as an azo initiator, is used to promote LDL oxidation [13].

Recent studies of the antioxidant action of α-tocopherol in LDL have revealed an intriguing propensity of this antioxidant to promote lipid peroxidation, particularly when the flux of peroxyl radicals, which α-tocopherol scavenges, is low [15–18]. The suggested mechanism for the pro-oxidant reaction of the α-tocopheroxyl radical is the initiation of lipid peroxidation through abstraction of an H atom from an unsaturated fatty acid [16–19]. The rate of this reaction is, however, in the order of 0.12 $M^{-1} \cdot s^{-1}$, which is approximately 1×10^9 times slower than the estimated rate of reaction of the α-tocopheroxyl radical with peroxyl radicals [18–21]. This result suggests that only under conditions where the flux of peroxyl radicals is exceedingly small will tocopherol mediated peroxidation occur. Accordingly, these reactions have been investigated under conditions where the rate of LDL oxidation is either negligible or slow using non-physiological oxidants such as the peroxyl radical generating azo initiators [17]. However, in human atherosclerotic lesions, lipid decomposition products such as 4-hydroxynonenal, malondialdehyde and fragmented LDL protein have been detected, all indicating at least a transitory state where extensive lipid decomposition has occurred [2,3]. Under these conditions the termination reaction of the α-tocopheroxyl radical with peroxyl radicals will negate or diminish its potential for pro-oxidant reactions. An examination of the behaviour of α-tocopherol under different conditions of peroxyl radical flux is required before we can assess the importance of its pro-oxidant reactions during LDL oxidation which, at present, remain unclear.

The copper and haem protein dependent oxidation of both lipids and LDL are thought to be dependent on the presence of pre-formed, or 'seeding', lipid hydroperoxides and may be analogous to the oxidative processes which occur in the atherosclerotic lesion itself [2,3,14,22–27]. Accordingly, we have proposed that variation in the levels of these peroxides between LDL isolated from different individuals may, in part, explain the observed variable efficacy of α-tocopherol as an antioxidant in human LDL; recent studies support this view [13,14,26]. Other possibilities include contributions from ubiquinol, LDL protein and variations in fatty acid composition [5,14,28].

In the present study the interactions between α-tocopherol, lipid hydroperoxides, and transition metals in LDL have been investigated using Cu^{2+} and the haem protein metMb as pro-oxidants. It has been shown that α-tocopherol reacts directly with Cu^{2+} in LDL [19], with the concomitant formation of the α-tocopheroxyl radical and Cu^{+} [18,19]. This could then result in a copper and α-tocopherol dependent pro-oxidant reaction which does not require lipid hydroperoxides. In this respect we have found that metMb is an important control since it does not react with α-tocopherol but shares with Cu^{2+} a requirement

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Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane) 2HCl; LDL, low density lipoprotein; EDTA, ethylenediamine tetraacetic acid; 13-HPODE,(9Z,11E, 13(*S*))-13-hydroperoxy-octadecadienoic acid; metMb, met myoglobin.

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for lipid peroxides for the promotion of oxidation in LDL [24,26]. The protocols chosen have allowed us to explore the pro and antioxidant effects of α-tocopherol under varying conditions of peroxyl radical flux which we have achieved by increasing the peroxide content by supplementation with 13-HPODE and decreasing it with the compound ebselen [24,26,32].

2. Materials and methods

2.1. Isolation and characterisation of LDL

Human LDL was isolated from the plasma of healthy donors by differential centrifugation using the method described previously [26]. In all, five separate LDL preparations were used in this study, with three plasma samples from different donors being supplemented with α-tocopherol using the protocol described in [11,13]. In brief, the plasma was supplemented with α-tocopherol to give a range of concentrations (0–1.2 mM in 1% ethanol v/v) and the LDL then isolated from the plasma as before [26]. Typically, this resulted in α-tocopherol levels of 10–40 nmol/mg LDL protein. After dialysis against Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) containing 10 μM EDTA, the LDL was sterilised by filtration through a 0.2 μM pore size filter and stored at 4°C before use. The LDL protein concentration was determined using bovine serum albumin as a standard and the BCA protein assay reagent (Pierce). The α-tocopherol in LDL was extracted into heptane and the content determined by HPLC [10].

2.2. Measurement of the oxidisability of LDL

Oxidisability of LDL samples initiated by $CuSO_4$ or metMb was determined spectrophotometrically (234 nm) as described previously and the lag phase was measured [30]. The concentrations of metMb and $CuSO_4$ selected for these experiments were determined from prior determination of the concentration dependence of the oxidant in oxidising LDL. In both cases it was a saturable process with 30–50 μM $CuSO_4$ and 10–20 μM metMb yielding the maximum rate of oxidation and minimum period for the lag phase (data not shown). Where possible, saturating levels of these oxidants were used. In those experiments where AAPH was used to initiate lipid peroxidation a concentration of 1 mM was used as described in [17]. All experiments were performed in duplicate and the data presented as the means of these values. Typical variation between experiments was between 3–7% for both α-tocopherol measurements and determination of 'lag phase'. Ebselen (0.1–5 μM) and 13-HPODE (5 μM) were used to modulate the initial concentration of lipid hydroperoxide in LDL and were added as ethanolic solutions (final concentration maintained at 1% v/v). When ebselen was added, LDL was treated for 30 min at room temperature before the addition of other oxidants [23,31,32]. The remaining ebselen was not removed from the LDL.

2.3. Reagents

AAPH was purchased from Polysciences (Warrington, PA, USA) and α-tocopherol and metMb from Sigma. 13-HPODE was obtained from Cascade and used without further purification. Ebselen was the generous gift of the Astra pharmaceutical company, Sweden.

3. Results

3.1. Oxidation of α-tocopherol in homogeneous solution and in ebselen treated LDL by Cu^{2+} and metMb

The addition of Cu^{2+} to α-tocopherol in 1% ethanol/PBS resulted in its rapid oxidation, whereas metMb was completely without effect (Fig. 1a). When either pro-oxidant was added to LDL, depletion of α-tocopherol occurred (Fig. 1b), confirming the results from other laboratories [30]. Interestingly, after treatment with the compound ebselen, which removes lipid hydroperoxides from LDL [25,31,32], the metMb dependent oxidation of α-tocopherol was inhibited whereas the Cu^{2+} dependent oxidation of α-tocopherol was still observed, albeit at a considerably lower rate (Fig. 1c).

3.2. Depletion of α-tocopherol in LDL after treatment with Cu^{2+}, metMb or AAPH

To test the effects of increasing α-tocopherol content on the rate of oxidation with the pro-oxidants Cu^{2+}, metMb or AAPH, samples of plasma from single donors were supplemented with this antioxidant and the LDL isolated [11,13]. The LDL was then exposed to the pro-oxidants Cu^{2+}, metMb and AAPH and the rate of α-tocopherol oxidation measured. Results typical of one of three independent experiments with different LDL preparations are shown in Fig. 2. It is evident that only in the case of Cu^{2+} (Fig. 2b) did the rate of oxidation show any variation with increasing concentration of α-tocopherol. In marked contrast, the rate of α-tocopherol oxidation with either the peroxyl

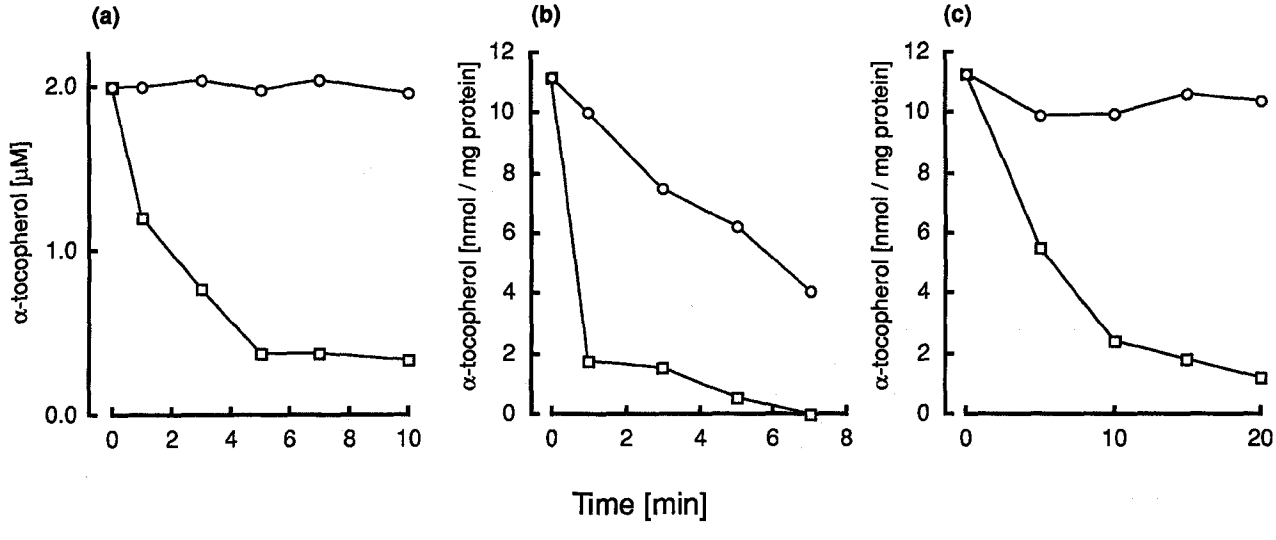

Fig. 1. Reaction of α-tocopherol with metMb and Cu^{2+} in solution and in LDL. MetMb (○) or Cu^{2+} (□) was incubated at 37°C in air equilibrated buffer for the times indicated before analysis by HPLC. (a) α-Tocopherol (2 μM) dissolved in 1% ethanol (v/v PBS) was incubated with either 10 μM Cu^{2+} or 50 μM metMb. (b) LDL (125 μg/ml) was incubated with either 50 μM Cu^{2+} or 50 μM metMb. (c) LDL pre-incubated with ebselen (10 μM) for 30 min was incubated with Cu^{2+} or metMb as described in panel b. Each point represents the mean of experiments performed in duplicate.

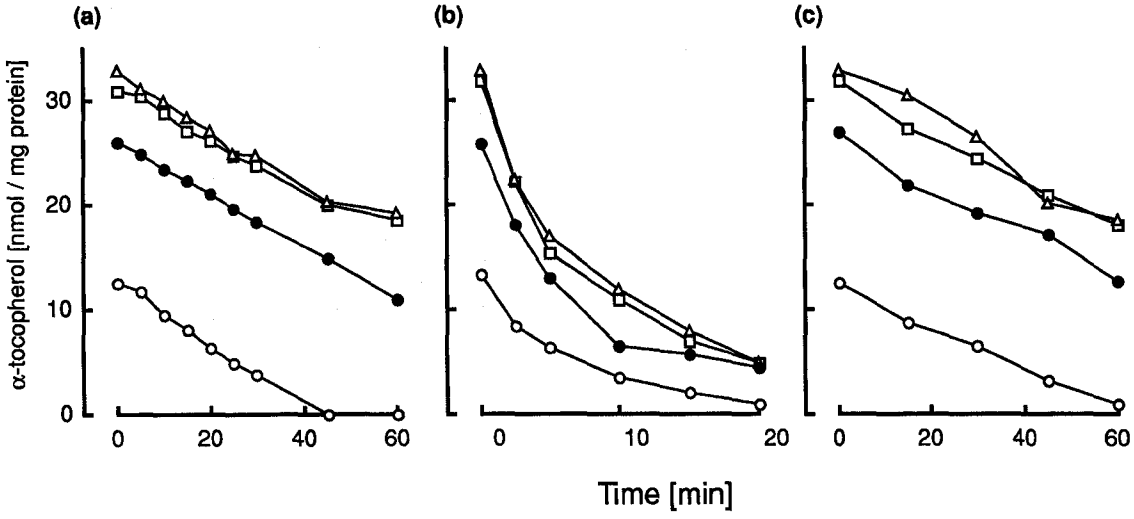

Fig. 2. Depletion of α-tocopherol in LDL with Cu^{2+}, metMb and AAPH. LDL (125 μg/ml) was prepared with 33 (△), 31 (□), 27 (●) or 12 (○) nmol α-tocopherol/mg LDL and incubated with 50 μM metMb (a), 50 μM Cu^{2+} (b) or 1 mM AAPH (c) before samples were taken for α-tocopherol measurement, by HPLC, at the times indicated. Each point represents the mean of experiments performed in duplicate.

radical generator AAPH or the metMb dependent decomposition of lipid hydroperoxides, was constant (Fig. 2a and c, respectively).

We then determined the rate of α-tocopherol depletion for each sequential 5 min period for both Cu^{2+} and metMb as oxidants and the results for both sets of data are shown in Fig. 3. It is clear that while the rate of oxidation of α-tocopherol with Cu^{2+} increases as a direct function of the α-tocopherol concentration during the course of the reaction (Fig. 3b), the rate of oxidation with metMb as a pro-oxidant is constant (Fig. 3a). Furthermore, within experimental error the data derived from all four LDL preparations lies on the same line (Fig. 3b), supporting our underlying assumption that, in all respects other than α-tocopherol content, these LDL preparations were essentially similar.

3.3. *The resistance of LDL to oxidation: the role of lipid hydroperoxides and α-tocopherol*

In the experiments conducted so far we have demonstrated that the rate of oxidation of α-tocopherol in LDL, when exposed to Cu^{2+}, *increases* with increasing antioxidant concentration, implicating a key role for a direct reaction with Cu^{2+} under these conditions. This reaction involves the oxidation of α-tocopherol yielding the α-tocopheroxyl radical [19]. This species is still capable of scavenging one peroxyl radical per mole, which would then decrease by half the total amount of

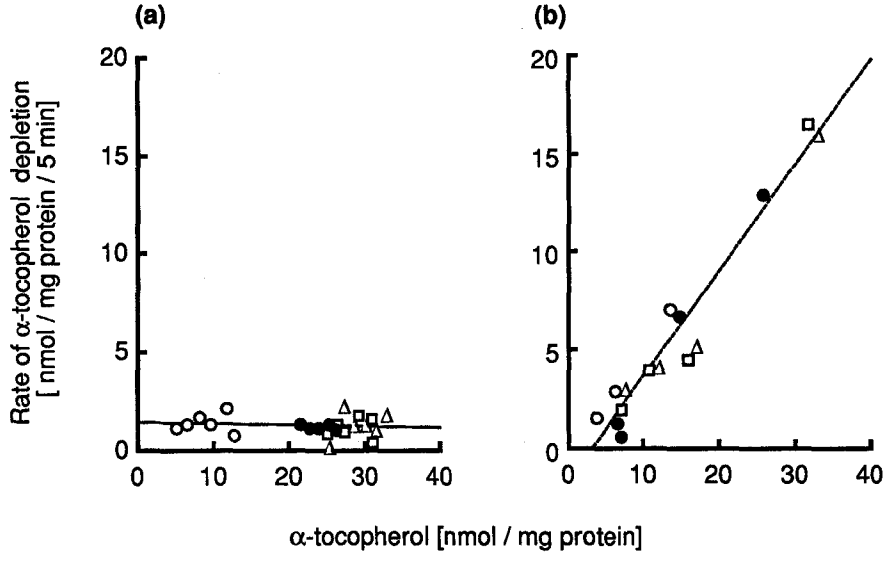

Fig. 3. Rate of loss of α-tocopherol as a function of α-tocopherol concentration with metMb and Cu^{2+} as oxidants. The rate of α-tocopherol depletion over each sequential 5 min period was calculated from the data shown in Fig. 2 for both oxidation by metMb (a) and Cu^{2+} (b), and was plotted as a function of the α-tocopherol concentration at the beginning of each 5 min period of the experiment. LDL (125 μg/ml) was prepared with 33 (△), 31 (□), 27 (●) or 12 (○) nmol α-tocopherol/mg LDL.

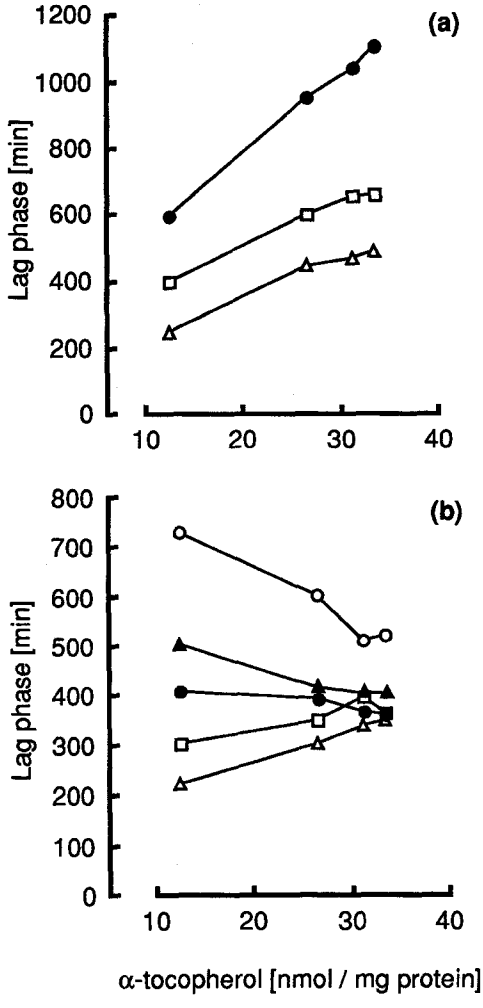

Fig. 4. Oxidative resistance of LDL, supplemented with lipid hydroperoxides or treated with ebselen, to oxidation promoted by metMb or Cu^{2+}. The endogenous lipid hydroperoxide content of normal LDL (□) was manipulated by either the addition of 5 μM 13-HPODE (△) or 0.1 μM (●), 2 μM (▲), 5μM (○) ebselen and either 10 μM metMb (a) or 50 μM Cu^{2+} (b) used as pro-oxidants. Resistance to oxidation was measured by estimating the 'lag phase' as described in [1]. Each point represents the mean of duplicate measurements.

α-tocopherol able to act as an antioxidant. However, if the α-tocopheroxyl radical was also able to initiate lipid peroxidation [16,17] then a pro-oxidant effect may also be observed. To test for this possibility we examined the effect of increasing lipid hydroperoxide levels by supplementation with 13-HPODE [24,26] or decreasing them by pre-treatment with the synthetic glutathione peroxidase mimic ebselen [25,32] and then subjecting LDL supplemented with different levels of α-tocopherol to oxidation with either metMb or Cu^{2+}.

In the case of metMb, which does not oxidise α-tocopherol directly (Fig. 1a), increasing levels of α-tocopherol resulted in greater resistance to oxidation whether the peroxide content of LDL was increased above control levels or decreased by treatment with ebselen (Fig. 4a). With copper as the pro-oxidant, treatment with ebselen increased and supplementation with 13-HPODE decreased the resistance to oxidation [26,32]. However, in marked contrast to the results with metMb, increasing α-tocopherol levels in the presence of ebselen were associated with decreased resistance to oxidation, that is, a shorter lag phase (Fig. 4b). The pro-oxidant effect was apparently dependent upon the lipid hydroperoxide content and reverted to an antioxidant response when the LDL was supplemented with 13-HPODE.

4. Discussion

The copper or haem protein dependent oxidation of LDL is thought to require the presence of pre-formed 'seeding peroxides' in the particle before oxidation will occur [24–26]. Although the origin of these peroxides remains uncertain, it is apparent from recent studies that they are formed in vivo [33]. These results are consistent with the finding that the resistance of LDL oxidation to copper is idiosyncratic with respect to the donor, even when LDL is prepared under identical conditions, and this can be partially ascribed to variation in the content of endogenous lipid hydroperoxides and α-tocopherol [11,13,14]. Indeed, the decreased resistance of LDL to copper dependent oxidation found in groups of subjects at greater risk from developing atherosclerosis supports the further extension of this idea to include the possibility that readily oxidised LDL may be pro-atherogenic [34–36].

It has been shown in a number of studies that dietary supplementation with α-tocopherol results in the incorporation of this lipophilic antioxidant into LDL and an associated increased resistance to oxidation when promoted by Cu^{2+} [11,12,37]. Although this is a plausible approach to suppressing the development of atherosclerosis it must be tempered by the observation that under some circumstances α-tocopherol may initiate lipid peroxidation (16–18).

In the present study we have addressed this issue by examining the resistance of LDL to oxidation promoted by met Mb and Cu^{2+}, both of which require lipid peroxides to promote lipid peroxidation in LDL, and exploited this property to assess the antioxidant or pro-oxidant properties of α-tocopherol. Furthermore, a comparison of the behaviour of these two pro-oxidants may be particularly informative since Cu^{2+} but not met Mb (Fig. 1) oxidises α-tocopherol with the associated formation of Cu^{+} and the α-tocopheroxyl radical [19]. This provides an opportunity to generate α-tocopheroxyl radicals under conditions of high and low lipid hydroperoxide content.

In the first series of experiments we measured the rate of α-tocopherol depletion with both Cu^{2+} and metMb and compared this with the azo-initiator AAPH, which generates a constant flux of peroxyl radicals which enter the lipophilic environment and do not react directly with α-tocopherol under these conditions. The rate of depletion of α-tocopherol with AAPH and metMb is both independent of the initial concentration of antioxidant and linear, again demonstrating the lack of a direct reaction (Fig. 2a,c). This form of progress curve is consistent with a progressively increasing concentration of peroxyl radicals leading to a constant rate of depletion of α-tocopherol, even though its concentration is decreasing throughout the reaction. When Cu^{2+}, a pro-oxidant, is used, the rate of depletion of α-tocopherol increases with the concentration of antioxidant (Figs. 2b and 3b), and the reaction proceeds even in the presence of ebselen (Fig. 1c). We have shown previously that this effect is simply explained by the direct reduction of copper by α-tocopherol, and it follows that, under the conditions we have used, those samples of LDL containing supple-

mented α-tocopherol will contain higher levels of the α-tocopheroxyl radical [19].

In the second series of experiments we examined the effects of these treatments on the resistance of LDL to oxidation with either enhanced or decreased levels of lipid hydroperoxides. To decrease lipid hydroperoxide levels in LDL we used ebselen, which has previously been shown to act in LDL solely by metabolising lipid hydroperoxides to their corresponding alcohols, which are unable to sustain a transition metal dependent oxidation of LDL [26,32]. We did not remove ebselen from LDL since this was not essential for the purpose of testing the role of seeding peroxides, and in so doing we avoided potentially pro-oxidant procedures such as extended dialysis. When metMb was used as the oxidant the lowest concentration (0.1 μM) of ebselen used to treat LDL inhibited lipid peroxidation (Fig. 4a) and with higher concentrations (2–5 μM) it was completely suppressed (result not shown). The endogenous lipid hydroperoxides present in human LDL are probably in the region of 6–12 pmol/mg LDL protein which is at the limit of detection for most analytical procedures including chemiluminescence (33). We were also unable to detect lipid peroxides in untreated LDL and so monitor their decrease on ebselen treatment. Nevertheless, previous studies support both the specificity of action of the compound in metabolising lipid hydroperoxides and its ability to inhibit the increase in lipid hydroperoxides which occurs during the transition metal dependent oxidation of LDL (32,38).

To increase lipid hydroperoxide levels we used supplementation with 13-HPODE which we have previously shown enhances the rate of both metMb and Cu^{2+} oxidation (24,26). The data shown in Fig. 4 suggest that where α-tocopheroxyl radicals are generated as a consequence scavenging peroxyl radicals, in this example through the action of metMb on lipid peroxides, then it behaves as an antioxidant and increasing concentrations predictably increase the resistance of the lipoprotein to oxidation. The efficacy of ebselen in inhibiting LDL oxidation promoted by Cu^{2+} was significantly less when compared to metMb (Fig. 4). For example, after treatment with 2 or 5 μM ebselen, and in contrast to metMb, Cu^{2+} was still capable of promoting lipid peroxidation (Fig. 4b). Since we can assume that the concentration of lipid hydroperoxides is negligible (ebselen is in a 100–5000 molar excess to the probable endogenous levels) under these conditions then it appears that α-tocopheroxyl radicals formed from the reaction with Cu^{2+} are initiating lipid peroxidation resulting in the paradoxical effect of *decreasing* the resistance of LDL to oxidation as the concentration of α-tocopherol increases (Fig. 4b). It is unlikely that Cu^{+}, which is also formed in this reaction with α-tocopherol, is acting as a pro-oxidant since it also requires lipid hydroperoxides to form alkoxyl radicals. Furthermore, recent studies suggest that alkoxyl radicals are similar to peroxyl radicals in their efficiency of propagating lipid peroxidation since they undergo rapid internal rearrangement reactions [39]. An interesting conclusion from this study must be that while metMb may have an absolute requirement for lipid hydroperoxides in promoting LDL oxidation, this is clearly not the case for Cu^{2+}. It would appear that in the absence of lipid hydroperoxides, Cu^{2+} may promote oxidation in an α-tocopherol dependent process which probably involves the α-tocopheroxyl radical.

Whether these reactions are relevant to human atherosclerosis is of course open to argument. The insertion of seeding peroxides into the LDL particle could proceed through a reaction dependent upon 15-lipoxygenase [40,41]. The relevance of copper dependent oxidation to atherosclerosis is also debatable but the recent finding of redox active transition metals in human atherosclerotic lesions and the peroxynitrite dependent release of copper from caeruloplasmin, the major extracellular copper containing protein, provide the evidence for its presence in lesions and a possible mechanism for its release [27,42]. However, it is important to note that tocopherol mediated peroxidation was only detectable in samples of LDL with low lipid hydroperoxide content and a high intrinsic resistance to oxidation (Fig. 4b). This suggests that, at worst, dietary supplementation with α-tocopherol would have little or no effect on the resistance of LDL to oxidation in those individuals probably at least risk of developing atherosclerosis through an oxidative mechanism.

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