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DOI:

[10.1016/j.abb.2007.07.005](https://doi.org/10.1016/j.abb.2007.07.005)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Horsley, E. T. M., Burkitt, M. J., Jones, C. M., Patterson, R. A., Harris, L. K., Moss, N. J., del Rio, J. D., & Leake, D. S. (2007). Mechanism of the antioxidant to pro-oxidant switch in the behavior of dehydroascorbate during LDL oxidation by copper(II) ions. *Archives of Biochemistry and Biophysics*, 465(2), 303-314.
<https://doi.org/10.1016/j.abb.2007.07.005>

Published in:

Archives of Biochemistry and Biophysics

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Mechanism of the antioxidant to pro-oxidant switch in the behavior of dehydroascorbate during LDL oxidation by copper(II) ions

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Running title: Dehydroascorbic acid and LDL oxidation

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Subject area: Systems biochemistry

Abbreviations: BCDS, bathocuproine disulphonic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; 13-HPODE, 13(*S*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid

ABSTRACT

Oxidised low density lipoprotein (LDL) may be involved in the pathogenesis of atherosclerosis. We have therefore investigated the mechanisms underlying the antioxidant/pro-oxidant behavior of dehydroascorbate, the oxidation product of ascorbic acid, toward LDL incubated with Cu^{2+} ions. By monitoring lipid peroxidation through the formation of conjugated dienes and lipid hydroperoxides, we show that the pro-oxidant activity of dehydroascorbate is critically dependent on the presence of lipid hydroperoxides, which accumulate during the early stages of oxidation. Using electron paramagnetic resonance spectroscopy, we show that dehydroascorbate amplifies the generation of alkoxyl radicals during the interaction of copper ions with the model alkyl hydroperoxide, *tert*-butylhydroperoxide. Under continuous-flow conditions, a prominent doublet signal was detected, which we attribute to both the erythroascorbate and ascorbate free radicals. On this basis, we propose that the pro-oxidant activity of dehydroascorbate toward LDL is due to its known spontaneous interconversion to erythroascorbate and ascorbate, which reduce Cu^{2+} to Cu^+ and thereby promote the decomposition of lipid hydroperoxides. Various mechanisms, including copper chelation and Cu^+ oxidation, are suggested to underlie the antioxidant behavior of dehydroascorbate in LDL that is essentially free of lipid hydroperoxides.

Key words: ascorbic acid; atherosclerosis; copper; dehydroascorbate; EPR spectroscopy; lipid hydroperoxide; lipid peroxidation; lipoprotein; low density lipoprotein; low density lipoprotein oxidation.

INTRODUCTION

The oxidative modification of low density lipoprotein (LDL) is believed widely to be involved in the pathogenesis of human atherosclerosis [1], the underlying cause of coronary heart disease and most strokes. A number of large clinical trials have shown no decrease in cardiovascular disease after antioxidant supplementation, however, and the importance of oxidised LDL in atherosclerosis is therefore an unresolved issue [2]. Several studies have demonstrated that LDL can be oxidised *in vitro* by cells associated with atherosclerotic lesions [3-7]. The mechanisms of LDL oxidation *in vivo* are controversial [1]. Possibilities to explain how LDL is oxidised in atherosclerotic lesions include lipoxygenase, myeloperoxidase/nitrite, peroxynitrite, NADPH oxidase, xanthine oxidase, mitochondrial respiration, iron or copper [8]. LDL oxidation by cells *in vitro* appears to be dependent usually on the presence of iron or copper ions in a redox-active form, which are believed to catalyse the free-radical reactions responsible for LDL oxidation [3-5, 9-11]. Metal ions can also oxidise LDL directly when incubated in cell-free medium [3, 4, 9, 12]. Ceruloplasmin, which contains most of the plasma copper, has also been shown to oxidise LDL by cells *in vitro* [13-15], which depends on superoxide generated by these cells [15]. The presence in human atherosclerotic lesions of redox-active metal ions [16] and products indicative of free-radical attack upon proteins [17, 18] is consistent with oxidation catalyzed by metal ions. This is also supported by the findings of a study, employing techniques that minimise the release of metal ions from proteins, showing elevated levels of copper and nonhaem iron in lesions [19], although it is uncertain if these increases are merely the result of tissue damage due to inflammation or are actively involved in the disease process.

Due to the epidemiological evidence indicating an inverse relationship between dietary fruit and vegetable intake and the incidence of cardiovascular disease [20, 21], numerous studies have been undertaken to show that micronutrients provided by such diets, including α -tocopherol [22, 23], ascorbic acid [24-27] and various flavonoids [28-31], can protect LDL from oxidative

modification [32]. Under certain circumstances, however, they have been shown to display pro-oxidant activity. Indeed, it has been suggested the α -tocoperoxyl radical, generated by the oxidation of α -tocopherol within LDL, is the species responsible for the initiation of lipid peroxidation in the particle [22, 33, 34].

Although ascorbic acid can be effective in the protection of LDL from oxidation by Cu^{2+} ions, it was first noted over a decade ago that this antioxidant acts as a pro-oxidant in LDL that has already undergone partial oxidation [25, 35]. Subsequent studies have revealed similar properties in other common antioxidants, including flavonoids [30], nonflavonoid phenols [36], cysteine [37, 38], and urate [39-41]. The effects of ascorbate are further complicated by the fact that dehydroascorbate, the product of its two-electron oxidation, can itself act as both a pro-oxidant and an antioxidant toward LDL [24, 26, 35, 42]. Indeed in one study it was concluded that ascorbate *per se* offers LDL no protection from copper-dependent oxidation [24]. Instead, it was proposed that the protection observed with ascorbate is due solely to its oxidation to dehydroascorbate and, perhaps, subsequent degradation to other products. As dehydroascorbate is extremely unstable in aqueous solution, undergoing facile delactonization to 2,3-diketo-L-gulonic acid, followed by transformation into a large number of products, which include ascorbic acid and erythroascorbic acid [43-46], it is possible that the effects of dehydroascorbate on LDL oxidation are indirect and are caused by its transformation products.

The concentrations of dehydroascorbate reported in normal human plasma vary greatly, with values of undetectable levels [47], 3 μM [48], 2-5 μM [49], 6 μM [50], 12 μM [51] and 29 μM [52] being reported. The concentrations of dehydroascorbate can sometimes increase in states of inflammation or oxidative stress, for instance, in wounded skin in guinea-pigs [53] or in the plasma of smokers [54]. The levels of ascorbate were about ten times greater in human atherosclerotic lesions than in the normal arterial intima and the percentage contribution of dehydroascorbate to total vitamin C was 22% and 11%, respectively [55].

In the present study, we have investigated in detail the mechanisms by which dehydroascorbate can act as both an antioxidant and a pro-oxidant toward Cu^{2+} -challenged LDL. In particular, we have sought to identify the factors responsible for this switch in behavior. Our electron paramagnetic resonance (EPR) experiments show very clearly that dehydroascorbate solutions bring about the rapid reduction of Cu^{2+} , which is suggested to be caused by reaction with erythroascorbate, formed spontaneously from dehydroascorbate. The decomposition of lipid hydroperoxides by the Cu^+ formed in this reaction, which we have investigated by EPR spectroscopy using the model compound *tert*-butyl hydroperoxide, is proposed to be responsible for the pro-oxidant activity of dehydroascorbate toward partially oxidised LDL. The possible mechanisms by which dehydroascorbate may inhibit the initiation of lipid peroxidation in native LDL by Cu^{2+} are also discussed.

Materials and methods

PBS (without calcium or magnesium) was from Gibco Life Technologies, Paisley, Scotland. Ascorbic acid, bathocuproine disulphonic acid (BCDS), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, dehydroascorbate (M_r 174.1; stated by the supplier to be at least 80% pure), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), *tert*-butyl hydroperoxide (*t*BuOOH), α -tocopherol, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich, Poole, Dorset, UK. α -Tocopheryl acetate was from ICN Biomedicals Inc, Aurora, OH, USA; and 13(*S*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid (13-HPODE) was from Affiniti, Exeter, Devon, UK. Dehydroascorbate was dissolved immediately before use. The DMPO was purified by vacuum distillation (Kugelrohr) and stored at -80°C .

Isolation of LDL

LDL (density 1.019-1.063 g/ml) was isolated from the pooled plasma of healthy volunteers by sequential density ultracentrifugation in KBr solutions at 4 °C in the presence of EDTA (100 µM), as described previously [56]. The LDL was dialysed against 3 × 4 litres 140 mM NaCl, 8.1 mM Na₂HPO₄, 1.9 mM NaH₂PO₄, pH 7.4, containing 100 µM EDTA, sterilised by membrane filtration and assayed for protein by a modified Lowry assay [57]. Separate LDL preparations were used to repeat the experiments.

Preparation of lipid hydroperoxide-rich LDL

LDL (150 µg protein/ml) was oxidised at 37°C by adding CuSO₄ (5 µM net above the calculated final EDTA concentration) in PBS, as described previously [58]. Briefly, the initial absorbance at 234 nm was read and further readings were taken every 15-20 min. The reaction was stopped by the addition of 1 mM EDTA once the absorbance had peaked. The lipid hydroperoxide-rich LDL was then concentrated by ultracentrifugation at 1.2 g/ml and dialysed against 1 l of the above phosphate buffer containing 100 µM EDTA. It was then filter-sterilized and assayed for protein [57].

Measurement of conjugated dienes

LDL (50 µg protein/ml) was incubated at 37 °C in PBS (without calcium or magnesium) in quartz cuvettes (1 cm light path). Additions of CuSO₄ (5 µM above the final calculated concentration of EDTA carried over from the LDL preparation, which was about 1 µM) were made to initiate the oxidation of LDL, in the presence or absence of dehydroascorbate, as indicated. The absorbance at 234 nm was recorded using a Perkin-Elmer Lambda 2 split beam UV/visible spectrophotometer at regular intervals [59].

Lipid hydroperoxide assay

The tri-iodide method of El-Saadani *et al.* [60], modified slightly as described previously [56], was used to determine lipid hydroperoxides in oxidised LDL (100 µg protein/ml). This concentration of oxidised LDL was used, rather than 50 µg protein/ml, because the lipid hydroperoxide assay is less sensitive than the conjugated diene assay.

Determination of α -tocopherol levels in stored LDL

Triplicate samples (200 µg protein) of LDL, stored in 140 mM NaCl, 8.1 mM Na₂HPO₄, 1.9 mM NaH₂PO₄, pH 7.4, containing 100 µM EDTA at 4°C for 4 days, were taken daily and frozen. The samples were then thawed and made up to 100 µl with PBS. The internal standard α -tocopheryl acetate (200 µl of 25 µM dissolved in acetonitrile containing 20 µM butylated hydroxytoluene) was then added to each of the vials followed by 0.8 ml ethanol, 1.6 ml iso-octane and 1ml water. Each vial was vortex mixed and centrifuged at $1,500 \times g$ for 5 min. The upper organic layer was then removed and placed in a glass vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 110 µl acetonitrile and 100 µl injected into a HPLC for analysis. A Hypersil ODS 3×100 mm, particle size 5 µm, Chrompack column was used. The mobile phase consisted of acetonitrile:water:tetrahydrofuran (80:6:14, by vol) at a flow rate of 1 ml/min. Absorbance at 280 nm was used to detect α -tocopherol.

Determination of copper binding to LDL

Copper binding to LDL was determined by equilibrium dialysis for 18 h in 3-(*N*-morpholino)propanesulphonic acid buffer, pH 7.4, containing 10 µM CuSO₄, as described previously [61].

EPR spin-trapping studies

The basic reaction mixture contained 0.1 M DMPO, 10 mM ^tBuOOH and 1 mM CuCl₂ in 0.1 M Tris-HCl, pH 7.4. Where indicated, 5 mM ascorbate or 5 mM dehydroascorbate was included, and particular reactants were omitted. After thorough mixing, reactions were transferred to a quartz flat-cell and spectra (X-band) recorded using a Bruker EMX spectrometer (Bruker UK Ltd, Coventry, UK) equipped with a cylindrical cavity (HS model) and operating with the following instrument settings: modulation frequency, 100 kHz; sweep width, 80 G; microwave power, 20 mW; modulation amplitude 0.5 G; sweep time 42 s; time constant, 10.24 ms; receiver gain, 2×10^5 . Four spectra were accumulated and added.

Continuous-flow EPR studies

To observe directly the radicals generated during the mixing of Cu²⁺ with dehydroascorbate (or its degradation products), solutions containing 5 mM CuCl₂ and 50 mM dehydroascorbate (both in 0.1 M Tris-HCl, pH 7.4) were flowed continuously through a dielectric mixing-resonator (model ER 4117 D-MVT, Bruker UK Ltd) housed in a Bruker EMX spectrometer, operating at the following instrument settings: modulation frequency, 100 kHz; sweep width, 150 G; microwave power, 20 mW; modulation amplitude, 1 G; sweep time, 10.5 s; time constant, 41 ms; and receiver gain, 4×10^5 . Where indicated, additional experiments were performed using ascorbate in place of dehydroascorbate. Flow through the cavity was maintained using a two-stream syringe infusion pump (model 22, Harvard Apparatus Ltd, Edenbridge, Kent, UK). The combined flow-rate was varied to achieve the range of mixing (reaction) times shown in Fig. 7. The calibration of this system (*i.e.*, conversion of pump speeds to reaction times) has been described previously [62]. Complementary studies were also performed using ascorbate in place of dehydroascorbate.

Copper reduction assay

The reduction of Cu^{2+} was assayed using BCDS, an indicator molecule whose absorbance at 480 nm increases with Cu^+ but not with Cu^{2+} [63]. The reaction was started by adding CuSO_4 (5 μM) to cuvettes containing PBS and increasing concentrations of dehydroascorbate. Ascorbate was also added to one cuvette to reduce rapidly Cu^{2+} to Cu^+ and act as a standard. BCDS (360 μM) was added immediately to the cuvettes and the formation of the $(\text{BCDS})_2\text{Cu}^+$ complex followed at 480 nm.

Statistics

One-way ANOVA was used to test for the significance of differences observed between various incubation conditions, as indicated.

RESULTS

Dehydroascorbate protects native LDL from oxidation by Cu^{2+} , but promotes the oxidation of partially oxidised LDL

The incubation of native human LDL (50 μg protein/ml) with 5 μM CuSO_4 resulted in oxidation of the constituent polyunsaturated fatty acids, as indicated by the formation of conjugated dienes (Fig. 1A). Consistent with previous studies [59, 64], the oxidation profile was characterized by an initial lag phase followed by a propagation phase, where the rate of conjugated diene formation was maximal, and then a decomposition phase. The addition of dehydroascorbate at concentrations of 10, 20, or 100 μM , before the addition of Cu^{2+} (*i.e.*, at time zero), completely inhibited the formation of conjugated dienes for the duration of the experiment (Fig. 1A). In other experiments, dehydroascorbate did not inhibit the oxidation of LDL at a concentration of 0.1 μM , it sometimes inhibited at 1 μM and it increased the lag phase by about 5-9 times at 3 μM . There was little effect on the rate of the propagation phase (data not shown). To investigate the

possibility that dehydroascorbate protects LDL from oxidation by decreasing copper binding to the particle, an equilibrium dialysis technique was used. Consistent with previous studies, native LDL was found to bind 42.7 ± 3.1 copper ions per particle [61]. This was decreased to 28.6 ± 1.4 , 22.2 ± 0.9 , and 18.1 ± 0.7 copper ions in the presence of 10, 25, and 50 μM dehydroascorbate, respectively (mean $\pm$ S. E. M. of 3 independent experiments each carried out in triplicate; $P < 0.001$ by one-way ANOVA).

When 10 μM dehydroascorbate was added to LDL that had been stored for 2 weeks at 4 $^{\circ}\text{C}$ in the presence of 100 μM EDTA (to allow partial oxidation), it promoted oxidation of the particle upon exposure to Cu^{2+} ions (Fig 1B). In contrast, at higher concentrations (20-100 μM), dehydroascorbate inhibited oxidation of the particle (Fig. 1B). We also measured the hydroperoxides generated in this LDL upon incubation with Cu^{2+} , using a tri-iodide assay (Fig. 1C). A maximum of about 500-600 nmol of hydroperoxides/mg protein were produced in the LDL, compared with about 400-500 nmol of conjugated dienes (calculated from their molar absorption coefficient of $29,500 \text{ M}^{-1} \text{ cm}^{-1}$ [59]). These values agree with previous estimates [32]. Similarly to the results when conjugated dienes were measured, a low concentration of dehydroascorbate (10 μM) increased the rate of lipid hydroperoxide formation, whereas a higher concentration (50 μM) completely inhibited the process (Fig. 1C).

We next examined the effects of adding dehydroascorbate at different stages during LDL oxidation by copper. Dehydroascorbate (10 μM) added at zero time, before the addition of Cu^{2+} (5 μM), or during the early lag phase completely inhibited the formation of conjugated dienes for the duration of the experiment (Fig. 1 A and 2A). However, when dehydroascorbate was added during the mid lag phase, when the conjugated diene content of the LDL had reached only $\sim 2\%$ of its maximum level, the propagation rate was increased by some 60 %. This switch from antioxidant to pro-oxidant behavior was remarkably sharp. For instance, in the experiment shown in Fig. 2A, dehydroascorbate behaved as a pro-oxidant when added after 26 min, but displayed very effective

antioxidant activity when added after 21 min. A similar switch in behavior was also seen when using the lipid hydroperoxide assay. The addition of dehydroascorbate (10 μ M) to native LDL at time zero, before the addition of Cu^{2+} , almost completely inhibited the formation of lipid hydroperoxides, but caused a marked enhancement in their formation when added during the mid lag-phase (Fig. 2B).

Depletion of endogenous α -tocopherol is not a prerequisite for the switch in behaviour of dehydroascorbate

To investigate the possibility that the α -tocopherol content of LDL determines whether dehydroascorbate behaves as an antioxidant or a pro-oxidant, freshly isolated LDL was stored at 4°C in the presence of 100 μ M EDTA and tested daily for susceptibility to Cu^{2+} -induced oxidation in the presence or absence of dehydroascorbate. This was continued until the compound switched from being an antioxidant to a pro-oxidant. Samples were also assayed for α -tocopherol content. In the representative example shown in Fig. 3, dehydroascorbate (10 μ M) strongly inhibited the formation of conjugated dienes when added prior to Cu^{2+} on days 1 - 3 following the isolation of the LDL (Fig. 3A). On day 4, however, dehydroascorbate switched to being a pro-oxidant for LDL (Fig. 3B). HPLC analysis of LDL samples taken before and after the antioxidant to pro-oxidant switch revealed that more than 80 % of the original α -tocopherol was still present in LDL when the switch occurred (Fig. 3C). These results show that the disappearance of endogenous α -tocopherol is not a requirement for the switch to the pro-oxidant activity of dehydroascorbate.

The pro-oxidant activity of dehydroascorbate is dependent on the presence of lipid hydroperoxides

We next investigated whether lipid hydroperoxides are required for dehydroascorbate to act as a pro-oxidant. As expected from the findings described above, the addition of dehydroascorbate

(10 μ M) to native LDL (50 μ g protein/ml) at time zero completely prevented the induction of conjugated diene formation by Cu^{2+} (Fig. 4). The inclusion of a small amount of lipid hydroperoxide-rich LDL (5 μ g protein/ml) in the incubation without dehydroascorbate decreased the lag phase before rapid oxidation (Fig. 4). Interestingly, the addition of dehydroascorbate (10 μ M) to incubations containing native LDL supplemented with lipid hydroperoxide-rich LDL resulted in a further shortening (~25 %) of the lag phase and increased the propagation rate by almost 40 % (Fig. 4).

Similar experiments were performed in which native LDL was spiked with the lipid hydroperoxide 13-HPODE instead of lipid hydroperoxide-rich LDL. Whereas dehydroascorbate (10 μ M) added at time zero completely inhibited the Cu^{2+} -induced formation of conjugated dienes in the absence of 13-HPODE (Fig. 5A), a progressive reduction in the lag phase was seen in the presence of increasing amounts of the hydroperoxide. In the presence of 13-HPODE at a concentration of 10 nmol/mg LDL protein, 10 μ M dehydroascorbate completely inhibited the formation of conjugated dienes. However, as the concentration of the lipid hydroperoxide was increased to 20 and then 30 nmol/mg LDL protein, dehydroascorbate switched from antioxidant to pro-oxidant behavior. The effects of dehydroascorbate were again concentration-dependent. In the presence of 13-HPOPE at 20 nmol/mg LDL protein, 10 μ M dehydroascorbate had a pro-oxidant effect, whereas 30 or 100 μ M dehydroascorbate had a highly effective antioxidant effect (Fig. 5B).

Dehydroascorbate serves as a pro-oxidant by reducing Cu^{2+} ions following its spontaneous conversion to ascorbate and erythroascorbate

Having established that the pro-oxidant activity of dehydroascorbate toward Cu^{2+} -challenged LDL particles requires the initial accumulation (or direct addition) of lipid hydroperoxides, we next employed a model alkyl hydroperoxide, $t\text{BuOOH}$, to investigate in mechanistic detail the interactions between Cu^{2+} ions, dehydroascorbate and alkyl hydroperoxides under relatively well

defined, biomimetic conditions. In reactions analogous to those of the higher M_r lipid hydroperoxides, $t\text{BuOOH}$ undergoes catalytic decomposition to free radicals by redox-active metal ions in reactions that are amenable to study by EPR spectroscopy [62, 65]. Since the resultant radicals are extremely unstable, this usually entails the use of a spin trap compound, such as the nitron DMPO. Unstable free radicals add to the double bond in DMPO to form relatively stable nitroxide radical adducts, the EPR spectra of which provide information on the identity of trapped species [66].

The addition of Cu^{2+} to $t\text{BuOOH}$ in the presence of DMPO resulted in the detection of an EPR spectrum containing a prominent signal from the *tert*-butoxyl radical ($t\text{BuO}^\bullet$) adduct of the spin trap, together with weaker signals from the methyl ($^\bullet\text{Me}$) and methoxyl ($^\bullet\text{OMe}$) radical adducts (Fig. 6A). Although it has been widely assumed for many years that lipid hydroperoxides undergo a direct (albeit slow) oxidation by Cu^{2+} ions, to the corresponding peroxy radicals, more recent EPR spin-trapping studies using $t\text{BuOOH}$ have shown that alkyl hydroperoxides are in fact reduced by Cu^{2+} ions, giving alkoxyl radicals and Cu^{3+} [65]. Thus, generation of the three radicals trapped and detected in the above reaction between Cu^{2+} and $t\text{BuOOH}$ has been shown to result from the initial one-electron reduction of the peroxide (Reaction 1), followed by the β -scission (Reaction 2) and oxygenation (Reactions 3 - 5) of untrapped $t\text{BuO}^\bullet$ and $^\bullet\text{Me}$ radicals, respectively.

The inclusion of ascorbic acid in the reaction system resulted in an increase in the intensity of the signals from the *tert*-butoxyl and methyl radical-adducts of DMPO (DMPO/ $\cdot$ O $\cdot$ Bu and DMPO/ $\cdot$ Me, respectively). If affected at all, the signal from the methoxyl adduct (DMPO/ $\cdot$ OMe) was slightly reduced (Fig. 6B). These effects can be attributed to the rapid reduction of Cu²⁺ by ascorbate, giving Cu⁺, which reacts very rapidly with $\cdot$ BuOOH to initiate generation of the same radical species as when the reduction is brought about by Cu²⁺ (Reactions 6 - 8, in which AscH⁻, Asc²⁻ and Asc $\cdot^-$ are the ascorbate monoanion, dianion and radical, respectively, and DHA is dehydroascorbate).

The slight reduction in intensity of the DMPO/ $\cdot$ OMe signal, noted above, may reflect the reduction of $\cdot$ OMe by Cu⁺ or ascorbate before reaction with the spin trap. Alternatively, this may reflect oxidation of the species by Cu^{2/3+} after undergoing a 1,2-H shift to the highly reducing $\cdot$ CH₂OH radical [65]. Somewhat surprisingly, dehydroascorbate, despite being the two-electron oxidation product of ascorbate, was almost equally as effective as ascorbate in stimulating production of the DMPO/ $\cdot$ O $\cdot$ Bu and DMPO/ $\cdot$ Me radical adducts (Fig. 6C). These findings suggest that dehydroascorbate brings about the reduction of Cu²⁺ to Cu⁺, which in turn reduces $\cdot$ BuOOH to the $\cdot$ BuO $\cdot$ radical very rapidly. If this were the case, then we would expect to detect a dehydroascorbate-derived radical following electron transfer to the metal. Although very weak signals were detected when Cu²⁺ and dehydroascorbate were mixed in the presence of DMPO, without the peroxide (Fig. 6 D), subsequent experiments indicated that these were caused largely by Cu²⁺-catalyzed nucleophilic addition reactions, rather than the authentic generation of free

radicals from dehydroascorbate (data not shown). Indeed, even in the absence of dehydroascorbate, Cu^{2+} catalyzed the nucleophilic addition of water to the spin trap, giving the DMPO hydroxyl radical-adduct (Fig. 6E), which is a well known reaction [67]. Interestingly, a weak doublet signal, characteristic of the ascorbate radical (a resonance-stabilized radical that fails to react with spin traps) [68-70], was observed in the dehydroascorbate solution in the absence of both Cu^{2+} and DMPO (Fig. 6F), suggesting formation of the radical by a comproportionation reaction between dehydroascorbate and ascorbate, which is known to arise spontaneously from dehydroascorbate (Reaction -8)[45].

Although the rate constant for the spontaneous disproportionation of ascorbate radicals is high ($k_8 \sim 10^5 - 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in the physiological pH range) [68, 71], at very low concentrations the reaction rate will be slow (being proportional to $[\text{Asc}^{\bullet-}]^2$) and in equilibrium with the reverse reaction (Reaction -8).

In order to detect directly any short-lived radicals generated in the reduction of Cu^{2+} by dehydroascorbate, continuous-flow EPR spectroscopy was employed. This involves the mixing of the reactant solutions just as they enter the EPR cavity, thereby allowing the direct observation of any radicals formed over the millisecond time scale without the use of a spin trap: although each individual radical will decay rapidly, it is replaced immediately as fresh reagent enters the cavity, thereby achieving a detectable steady-state concentration of radicals [62, 72]. The continuous mixing of Cu^{2+} and dehydroascorbate in the EPR cavity resulted in the detection of a prominent doublet signal, which was seen to decrease in intensity as the rate at which the reactants were flowed through the cavity was lowered (Fig. 7A). This signal is essentially identical to that of the

ascorbate radical [68-70], which for comparison was generated in the reaction between Cu^{2+} and ascorbate (Reaction 6) (Fig. 7B).

Czapski has proposed mathematical models for the generation and decay of radicals in EPR continuous-flow systems [72]. In one extreme case, when the rate of radical generation reaction is low, it is assumed that a true steady state concentration of radicals exists at the point in the flow cell at which the spectrum is recorded, *i.e.* radical generation is still underway when the mixed reagents have reached the observation point. In the other extreme case, the initial reaction is very fast and assumed to be completed immediately after the reagents have been mixed, as expected for the reaction between Cu^{2+} and ascorbate. In this case, the concentration of radicals detected is determined only by their rate of decay and would be described, in the system studied here, by the equation,

$$1/[\text{Asc}^{\bullet-}]_t = 2k_8t + 1/[\text{Asc}^{\bullet-}]_0$$

where $[\text{Asc}^{\bullet-}]_t$ is the concentration of ascorbate radicals t seconds after the reagents have been mixed (*i.e.*, the delay time between reactant mixing and the point of EPR observation in the flow system) and $[\text{Asc}^{\bullet-}]_0$ is the initial ascorbate radical concentration ($t = \text{zero}$). The validity of this equation was tested by plotting the reciprocal of the EPR signal height (the EPR signal height is proportional to $[\text{Asc}^{\bullet-}]$) against t for the data points obtained during the initial, rapid phase of ascorbate radical decay (within the first 45 ms), which gave the expected straight-line fit (Fig. 7C). Having established that the reaction between Cu^{2+} and ascorbate is essentially ‘instantaneous’ under the pseudo first-order conditions employed, it was assumed that the concentration of ascorbate radicals at $t = 0$ is equal to the initial, post-mixing concentration of Cu^{2+} ions, which was 2.5 mM. Therefore, using the value from the y-axis intercept of the reciprocal plot (Fig. 7C), EPR signal heights (in arbitrary units) were converted to ascorbate radical concentrations, as given on the y-axis of Fig. 7B. From the gradient of the plot given in Figure 7C (after conversion of signal heights to concentration units), $2k_8$ was estimated to be $\approx 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which is in reasonable

agreement with the values reported in the literature [68, 71], thereby supporting the kinetics approach used above to obtain ascorbate radical concentrations from EPR signal intensities.

Although the EPR signal observed upon the reaction of Cu^{2+} and dehydroascorbate appears to be identical to that of the ascorbate radical, we suggest that this is in fact the erythroascorbate radical (Fig. 7A). Jung and Wells have demonstrated that in aqueous solution dehydroascorbate undergoes spontaneous conversion to at least three products, which include ascorbate and erythroascorbate [45]; other studies have also demonstrated such reactions [43, 44]. The radicals generated upon the one-electron oxidation of ascorbate and erythroascorbate would be expected to give essentially identical EPR spectra under the conditions employed in

this study¹, therefore it was considered acceptable to assume the same signal height-to-radical concentration relationship for erythroascorbate radicals as determined for ascorbate radicals, giving the signal calibration indicated in Fig. 7A. Since the amount of ascorbate generated spontaneously from dehydroascorbate is much smaller than the amount of erythroascorbate formed [45], it is concluded on kinetics grounds that the erythroascorbate radical is the major species detected in the Cu^{2+} -dehydroascorbate reaction system (Fig. 7A). Making the reasonable assumption that Cu^{2+} reacts with ascorbate and erythroascorbate with similar rate constants, then the latter would be the major species oxidised when present in large excess over the former. This suggests that erythroascorbate, generated by the spontaneous interconversion mechanism identified by Jung and Wells [45], is responsible for the ability of dehydroascorbate solutions to reduce Cu^{2+} ions. Complementary experiments using BCDS, which forms a stable, coloured complex with Cu^+ ions, showed that dehydroascorbate solutions bring about the reduction of Cu^{2+} to Cu^+ (data not shown). However, since chelation by BCDS increases the reduction potential of the $\text{Cu}^{2+}/\text{Cu}^+$ couple by approximately 0.5 V [73], this finding is of limited relevance to situations in which Cu^{2+} is less oxidizing (e.g., when bound to amino acids, peptides and biological buffers).

DISCUSSION

The mechanisms by which Cu^{2+} ions initiate and propagate the oxidation of LDL remain a matter of contention [12]. Both α -tocopheroxyl ($\alpha\text{-Toc-O}^\bullet$) and hydroxyl ($^\bullet\text{OH}$) radicals have been suggested to be responsible for the initiation of polyunsaturated fatty acid oxidation within LDL. The former species, generated upon the oxidation of α -tocopherol by Cu^{2+} (Reaction 9), is

¹ The predominant doublet hyperfine structure of the signal from the ascorbate radical [70] I. Yamazaki, in *Foundations of Modern EPR* G. R. Eaton, S. S. Eaton, K. M. Salikho, Eds. (World Scientific Publishing, London, 1998) pp. 436-450. arises from coupling to the hydrogen at C4 ($a^{\text{H-4}} = 1.75$ G). Further splitting (though rarely resolved) arises through interaction with the two equivalent hydrogens at C6 ($a^{2\text{H-6}} = 0.19$ G) in the hemiketal form. Both of these couplings are possible in the corresponding erythroascorbate radical, where the two equivalent hydrogens would be on C5 instead of C6. The only difference expected would result from the absence of interaction with a third hydrogen (C5 in the ascorbate radical), which results in only an extremely small splitting ($a^{\text{H-5}} = 0.06$ G), the absence of which would be barely detectable in the spectrum of the erythroascorbate radical.

believed to be capable of H-atom abstraction from the bisallylic methylene groups (LH) of polyunsaturated fatty acids (Reaction 10) [33].

Since this reaction is extremely slow ($k_{10} \approx 0.1 \pm 0.05 \text{ M}^{-1} \text{ s}^{-1}$ at 37 °C), it has been suggested that the much more reactive $\bullet\text{OH}$ radical, generated *via* the sequential reduction of molecular oxygen by Cu^+ , is the species responsible for the initiation of LDL oxidation (Reactions 11-15, showing both the initial one- and two-electron oxygen reduction pathways) [12, 74].

The $\bullet\text{OH}$ radical is expected to initiate fatty acid oxidation *via* addition to the bisallylic-methylene double bonds and *via* H-atom abstraction, both of which yield carbon-centred radicals (Reactions 16a and 16b, respectively) [75].

Both $L^\bullet$ and $(HO)HL^\bullet$ will add oxygen to give the corresponding peroxy radicals and, *via* subsequent H-atom abstraction, their hydroperoxides (Reactions 17-18 and 19-20, respectively).

We postulate that:

- (i) the pro-oxidant activity of dehydroascorbate is related to its ability to reduce Cu^{2+} to Cu^+ (involving spontaneous conversion to ascorbate and erythroascorbate) and
- (ii) the pro-oxidant behavior of dehydroascorbate is dependent on the presence of lipid hydroperoxides.

It could be argued that, through their rapid reduction of Cu^{2+} , ascorbate and erythroascorbate prevent generation of the initiating α -Toc $O^\bullet$ radical from endogenous α -tocopherol *via* Reaction 9 (Reactions 6 and 21, respectively, in which $E-AscH^-$, $E-Asc^{2-}$ and $E-Asc^{\bullet-}$ are the erythroascorbate monoanion, dianion and radical, respectively).

Since the rate constant for the reaction of Cu^{2+} with ascorbate ($k_6 \sim 880 \text{ M}^{-1} \text{ s}^{-1}$) [76] is some 1500 times higher than that for the corresponding reaction with α -tocopherol ($k_9 \sim 0.56 \text{ M}^{-1} \text{ s}^{-1}$) [77], it is reasonable to assume that ascorbate, and the chemically-similar erythroascorbate, will out-compete α -tocopherol for reaction with the metal. In arguing that the protection afforded by dehydroascorbate is due to its diversion of Cu^{2+} away from reaction with α -tocopherol, it would

be necessary to assume that $\cdot\text{OH}$ radicals, generated from oxygen upon reduction by the Cu^+ generated in Reactions 6 and 21, do not play a significant role in the initiation of LDL oxidation. However, following the accumulation of lipid hydroperoxides, Cu^+ would assume a pro-oxidant role in the generation of reactive, chain-propagating alkoxyl radicals (Reaction 22, analogous to Reaction 7).

Although the above interpretation takes account of postulates (i) and (ii), a further postulate must be made to account for the fact that *dehydroascorbate behaves as a pro-oxidant only at low concentrations* (Fig. 1B, C and 5B). At high concentrations, any pro-oxidant activity resulting from the ability of dehydroascorbate to generate Cu^+ for reaction with lipid hydroperoxides must be overwhelmed by an antioxidant action. One possibility is that the ascorbate and erythroascorbate derived from dehydroascorbate act as radical-scavenging antioxidants. Indeed, the finding that around 80 % of the original α -tocopherol was still present in the LDL when it contained sufficient lipid hydroperoxide to allow dehydroascorbate to behave as a pro-oxidant (Fig. 3) allows for the possibility of lipid-radical scavenging by α -tocopherol, with its regeneration from the α -tocopheroxyl radical by ascorbate and erythroascorbate across the phase boundary [78].

An additional mechanism by which dehydroascorbate could serve as an antioxidant involves its interference with copper binding to LDL, which has been reported to be necessary for oxidation of the particle [27]. This view is supported by our finding that dehydroascorbate decreased the binding of copper to LDL. This could entail either the chelation of copper ions by dehydroascorbate (or its degradation products) or the modification of LDL such that it has reduced ability to bind the metal ion. The latter possibility has been raised already by Retsky *et al.*, who

suggested that apo B100 may undergo a stable modification by dehydroascorbate or its degradation products [24].

It is also necessary to account for the ability of dehydroascorbate at all concentrations tested to prevent the *initiation* of fatty acid oxidation in LDL not already containing significant lipid hydroperoxide (Fig. 1A). The protection afforded to native LDL by dehydroascorbate is absolute: there is not even a gradual increase in conjugated diene formation over the 120 min incubation period, even with only 10 μ M dehydroascorbate (Fig. 1A). This argues against radical scavenging by dehydroascorbate, which would simply extend the lag phase in the same way as other antioxidants of this type [32, 64]. Instead, we suggest that dehydroascorbate prevents the actual *formation* of the species responsible for the initiation of LDL oxidation. Whilst we have described how ascorbate and erythroascorbate derived from dehydroascorbate may achieve this by competing with α -tocopherol for oxidation by Cu^{2+} , thereby preventing formation of the $\alpha\text{-Toc-O}^\bullet$ radical, it seems unlikely that LDL would not begin to oxidise eventually because dehydroascorbate would be depleted through this process (quickly at the Cu^{2+} concentrations employed).

Taking, instead, the alternative view, that the $\alpha\text{-Toc-O}^\bullet$ radical is not responsible for the initiation of lipid peroxidation in native LDL, it has to be assumed that it is the Cu^+ formed upon reaction of the added Cu^{2+} with endogenous α -tocopherol that is responsible for the generation of a powerful oxidant. In native LDL that is low in lipid hydroperoxides, oxidation will be initiated by the $^\bullet\text{OH}$ radical, formed *via* the reduction of oxygen by Cu^+ according to Reactions 11-15 [12, 74]. Dehydroascorbate may compete with oxygen as the electron acceptor from Cu^+ (Reaction 23).

The equilibrium position of this reaction would be shifted to the right by the disproportionation of ascorbate radicals (Reaction 8). Thus dehydroascorbate may prevent the reduction of oxygen to the hydroxyl radical (Reactions 11-16). Given the similarity between the reduction potentials of the DHA/Asc^{•-} couple ($E^\circ = -0.174$ V) [79], which prevails at physiological pH (the pK_a of the ascorbate radical is -0.45 [79]), and that of the $O_2(1M)/O_2^{\bullet-}$ couple ($E^{\circ'} = -0.179$ V at pH 7) [80], it is quite feasible that *excess* dehydroascorbate may prevent oxygen reduction by competing for the electron from Cu⁺ (Reaction 23). The spontaneous conversion of dehydroascorbate to ascorbate and erythroascorbate would also protect by scavenging [•]OH radicals.

Dehydroascorbate would not, however, be expected to compete with lipid hydroperoxides for reduction by Cu⁺, simply because the LOOH/LO[•],OH⁻ couple is far more oxidizing ($E^\circ \sim 1.0$ V) [81]. As a result, the pro-oxidant, reducing properties of dehydroascorbate will prevail in the presence of lipid hydroperoxides.

In summary, we have shown that dehydroascorbate displays dual, antioxidant/pro-oxidant behavior in LDL challenged with Cu²⁺. The switch from antioxidant to pro-oxidant behavior occurs only in the presence of preformed lipid hydroperoxides. If added to native LDL, dehydroascorbate at all concentrations is protective because it prevents the initiation of lipid peroxidation by the mechanisms proposed above. As a result, the lipid hydroperoxides needed for dehydroascorbate to act as a pro-oxidant are not generated. The pro-oxidant action of dehydroascorbate can be best explained in terms of its provision of Cu⁺ ions for the rapid decomposition of lipid hydroperoxides to chain-propagating alkoxyl radicals. This provision of Cu⁺ most likely involves the spontaneous conversion of DHA to the reductants erythroascorbate and ascorbate. These reactions are shown schematically in Scheme 1.

Our findings suggest that dehydroascorbate has the potential to increase the oxidation of mildly-oxidised LDL in atherosclerotic lesions. Further studies will be needed, however, to determine if this applies *in vivo*.

This research was funded by grants from the Ministry of Agriculture, Fisheries and Food (United Kingdom) and Cancer Research UK.

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FIGURE LEGENDS

Figure 1. Effects of dehydroascorbate on the oxidation of native and mildly oxidised LDL. A. Native LDL (50 µg protein/ml) in PBS was incubated at 37°C with CuSO₄ (5 µM net) (○). Dehydroascorbate (DHA) was added at zero time at concentrations of 10µM (●), 20 µM (×) or 100 µM (■). B. The same LDL preparation as that shown in A was left to become partially oxidised by storage at 4°C in the presence of 100 µM EDTA for 2 weeks. LDL (50 µg protein/ml) in PBS was oxidised by the addition of CuSO₄ (5 µM net) at 37 °C (○). Dehydroascorbate was added at zero time at concentrations of 10 µM (●), 20 µM (×), 40 µM (■), 80 µM (□) or 100 µM (▲). C. The same batch of LDL as that shown in A was stored at 4°C in the presence of 100 µM EDTA for 2 weeks and incubated in triplicate at 37°C at 100 µg protein/ml (rather than 50 µg protein/ml, as the lipid hydroperoxide assay is less sensitive than the conjugated diene assay) with CuSO₄ (10 µM net) (○). Dehydroascorbate was added at zero time at concentrations of 10 µM (●) or 50 µM (×) and samples (25 µg) were removed every 15 minutes and the oxidation halted with butylated hydroxytoluene (20 µM, final) and EDTA (1 mM, final). The samples were assayed for lipid hydroperoxides. The means ± S.E.M. for the triplicate samples are shown, except where the error bar is smaller than the symbol. These results were confirmed by two other experiments.

Figure 2. Effect of time of addition of dehydroascorbate on LDL oxidation. A. Native LDL (50 µg protein/ml) in PBS was incubated with CuSO₄ (5µM net) (○). Dehydroascorbate (DHA; 10 µM) was added at zero time (×), 4 min (■), 8 min (□), 12 min (△), 16 min (▲) or 21 min (⊕) or during the mid lag phase at 26 min (●), and the formation of conjugated dienes monitored. The results are expressed as the change in A₂₃₄ and were confirmed by two other experiments. B. Native LDL (100 µg protein/ml) in PBS was incubated in triplicate at 37°C with CuSO₄ (10 µM net) (○). Dehydroascorbate (DHA; 10 µM) was added at zero time (●) or during the mid lag

phase at 27 min (×). Samples (25 µg) were removed every 15 minutes, the oxidation halted with butylated hydroxytoluene (20 µM) and EDTA (1 mM) and a lipid hydroperoxide assay carried out. The means ± S.E.M. for the triplicate samples are shown, except where the error bars are smaller than the symbols. These results were confirmed by two other experiments.

Figure 3. α-Tocopherol depletion does not determine the antioxidant to pro-oxidant switch for dehydroascorbate. LDL was stored at 4°C in the presence of 100 µM EDTA. Each day a sample of LDL (50 µg protein/ml) in PBS was incubated at 37°C with CuSO₄ (5 µM net) (◇). Dehydroascorbate (DHA; 10 µM) was added at time zero (□) and the formation of conjugated dienes monitored. Dehydroascorbate displayed antioxidant activity toward LDL stored for up to three days (A), but behaved as a pro-oxidant after LDL storage for four days (B). The results are expressed as the change in A₂₃₄. The α-tocopherol in LDL was extracted in triplicate and measured by HPLC (C). The results are means ± S.E.M. of triplicates. Similar results were observed in two other experiments, except that in one experiment the dehydroascorbate switched to pro-oxidant behavior on day 8 following the isolation of LDL.

Figure 4. The effect of lipid hydroperoxide-rich LDL on the antioxidant to pro-oxidant switch for dehydroascorbate. Native LDL (50 µg protein/ml) in PBS was incubated at 37°C with CuSO₄ (5 µM net) (○). Dehydroascorbate (DHA; 10 µM) (●) or lipid hydroperoxide-rich LDL (LOOH-LDL), containing 206 nmol lipid hydroperoxides/mg protein (5 µg protein/ml) (×), or both together (■) were added to native LDL at zero time. The results are expressed as the change in A₂₃₄ and were confirmed by two other experiments.

Figure 5. The effect of the addition of a fatty acid hydroperoxide to native LDL on the antioxidant to pro-oxidant switch for dehydroascorbate. (A) Native LDL (50 µg protein/ml) in

PBS was incubated at 37°C with CuSO₄ (5 μM net) (○). Dehydroascorbate (DHA; 10 μM) was added to native LDL at zero time (●). The lipid hydroperoxide 13-HPODE was added to native LDL at zero time at concentrations of 10 nmol (×), 20 nmol (◇) or 30 nmol/mg LDL protein (□). Lipid hydroperoxides were also added to native LDL in the presence of dehydroascorbic acid (10 μM) at concentrations of 10 nmol (▲), 20 nmol (◆) or 30 nmol/mg LDL protein (■). The results are expressed as the change in A₂₃₄ and were confirmed by two other experiments. (B) Native LDL (50 μg protein/ml) in PBS was incubated at 37°C with CuSO₄ (5 μM net) in the presence of 13-HPODE (20 nmol/mg LDL protein), to induce the switch in the antioxidant to pro-oxidant activity of dehydroascorbate (duplicates shown by ○ and ×). Dehydroascorbate at a concentration of 10 μM (duplicates shown by ▲ and □), 30 μM (duplicates shown by + and *) or 100 μM (duplicates shown by △ and ◇) was added at zero time. The results are expressed as the change in A₂₃₄ and were confirmed by another experiment.

Figure 6. EPR spectra of DMPO radical adducts detected following the addition of CuCl₂ to *tert*-BuOOH in the presence of ascorbate or dehydroascorbate. Spectrum D is shown expanded 10-fold in the y-axis relative to the others.

Figure 7. Decay kinetics of the erythroascorbate (A) and ascorbate (B) radicals observed by EPR spectroscopy following the reaction of 25 mM dehydroascorbate and 25 mM ascorbate, respectively, with 2.5 mM Cu²⁺ under continuous-flow conditions (post-mixing concentrations). The insets in A and B show the spectra of the erythroascorbate and ascorbate radicals, respectively, and the measurements taken as peak heights. The reciprocal plot (C) of the ascorbate data was used to convert peak heights to radical concentrations (see text for details).

Scheme 1. Summary of reactions proposed to be responsible for the behavior of dehydroascorbate (DHA) toward LDL undergoing oxidation catalyzed by Cu²⁺ ions. See text for details.