Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low-density lipoprotein

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The Cu(II)-promoted oxidation of lipids is a lipid hydroperoxide (LOOH)-dependent process that has been used routinely to assess the oxidizability of low-density lipoprotein (LDL) in human subjects. Metal-dependent redox reactions, including those mediated by copper, have been implicated in the pathogenesis of atherosclerosis. Despite its widespread use and possible biological significance, key elements of the mechanism are not clear. For example, although it is evident that copper acts as a catalyst, which implies a redox cycle between the Cu(II) and Cu(I) redox states, the reductants remain uncertain. In LDL these could include α -tocopherol, amino acid residues on the protein and LOOH. However, both α -tocopherol and amino acid residues are probably consumed before the most rapid phase of lipid peroxidation occurs, suggesting that another reductant must be donating electrons to Cu(II), the most likely candidate being LOOH. This role has been disputed, since LDLs nominally devoid of LOOH are still capable of reducing Cu(II) to Cu(I) and

thermodynamic calculations for this reaction are not favourable. Direct investigation of the role of LOOH as reductant has not been reported and in the present study, using simple lipid systems and LDL, we have re-examined this issue using the Cu(I) chelator bathocuproine. We have shown that Cu(II) may promote lipid peroxidation in liposomes, which do not contain either protein or α -tocopherol, and that this is associated with reduction to Cu(I). The data also indicate that an equilibrium between free Cu(II) and LOOH exists, which only in the presence of an oxidizable substrate, i.e. unsaturated fatty acids, is shifted towards formation of Cu(I) and lipid-derived peroxy radicals. We propose that reduction of Cu(II) by LOOH is a necessary component in sustaining the propagation of lipid peroxidation and that the formation of peroxy radicals and their products in a lipid environment is sufficient to overcome thermodynamic barriers to the reaction.

INTRODUCTION

The modification of low-density lipoprotein (LDL) through an oxidative mechanism is implicated in the development of atherosclerotic lesions [1–3]. The evidence in favour of this hypothesis has been extensively reviewed [4–6] and its possible relevance to humans emphasized by the findings of recent epidemiological studies that show that increased intake of antioxidant vitamins is associated with a decrease in the risk of developing coronary heart disease [7,8].

The nature of the species responsible for initiating LDL lipid oxidation *in vivo* is unknown, but several lines of evidence suggest that metals may be involved, despite the fact that free metal ion concentration in plasma is low. For example, the oxidation of LDL promoted by copper ions *ex vivo* (a common method of evaluating LDL oxidizability) and LDL isolated from atherosclerotic lesions have similar physical and chemical properties [2,9]. Higher serum concentrations of copper ions are associated with accelerated progression of atherosclerosis and with elevated levels of autoantibodies against oxidized LDL [10,11]. Antibodies directed towards antigenic determinants present in atherosclerotic lesions also react with copper-modified lipoprotein [12]. Furthermore analysis of ‘gruel’ samples from advanced atherosclerotic lesions showed them to contain de-

tectable levels of copper and iron capable of promoting pro-oxidant reactions [13]. Recent studies suggest that in some cases copper bound to caeruloplasmin may oxidize LDL and can be released from this protein or activated by pro-oxidant reactions which can occur in the developing atherosclerotic lesion [14–16].

Oxidation of lipids by copper is not unique to LDL as this metal has long been known to promote oxidation in other lipid systems including erythrocyte membranes and liposomes when supplemented with organic lipid peroxides, such as *t*-butyl hydroperoxide [17,18]. The exact mechanistic details for this reaction are unknown but a role for lipid hydroperoxide (LOOH) has been established in lipid-containing systems [19–21]. Since substantially more lipid may be oxidized than the molar equivalent of added copper, then either the kinetic chain length for lipid peroxidation is sufficient to sustain a steady-state level of propagatory peroxy radicals or redox cycling between the Cu(II) and Cu(I) oxidation states occurs. One hypothesis is that the decomposition of LOOH via this cycle generates lipid peroxy and alkoxy radicals, which by further propagating lipid-peroxidation reactions (generating more LOOH) confer a catalytic role on copper. More recent studies suggest that intramolecular rearrangement of the alkoxy radical leads to the formation of an epoxide and peroxy radical which would also be capable of propagating lipid peroxidation [22].

Abbreviations used: PC, phosphatidylcholine; LDL, low-density lipoprotein; DTPA, diethylenetriaminepenta-acetic acid; 13-HPODE, (9Z,11E,13S)-hydroperoxyoctadecadienoic acid; LOOH, lipid hydroperoxide; BC, bathocuproine disulphonate; BC–Cu(I), bathocuproine–Cu⁺ complex; SVD, singular value decomposition; AAPH, 2,2'-azobis-2-amidinopropane hydrochloride; apoB, apolipoprotein B; FOX, ferrous oxidation by Xylenol Orange.

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In the present study we have examined the role of LOOH as reductants for Cu(II) and the concomitant role in establishing the redox cycle between Cu(II) and Cu(I) in the promotion of lipid peroxidation. A role for LOOH-dependent reduction of Cu(II) to Cu(I) can only be demonstrated if the metal exhibits catalytic behaviour in the lipid-peroxidation system and in the absence of alternative reductants such as amino acids or α -tocopherol. We have achieved this by using a protein- and antioxidant-free phosphatidylcholine (PC) liposomal system for Cu(II)-dependent lipid peroxidation and compared these results with those for human LDL. This is important since it has been argued from thermodynamic calculations that LOOH is unable to reduce Cu(II) ions to Cu(I) and that only Cu(I) interacts with LOOH [5]. This has been supported by findings that LDL, which contains very low levels (< 0.005 mol/LDL particle) of LOOH is capable of reducing Cu(II) to Cu(I) [23]. This requires the presence of other reductant(s) endogenous to the LDL particle. Candidates for this include α -tocopherol [24–26] and amino acid residues of apolipoprotein B (apoB) [23]. The presence of apoB was found to be important in Cu(II)-mediated LDL oxidation, and the presence of high- and low-affinity Cu(II)-binding sites on the protein proposed [27]. Recently such specific binding of Cu(II) to apoB was used to explain the saturation kinetics of LDL oxidation by Cu(II) [28], although the kinetic analysis only revealed one type of binding site. Under conditions with low levels of LOOH the reductant of Cu(II) could also be α -tocopherol, since a direct redox reaction between these species has been demonstrated [25]. However, it is during the later stages of LDL oxidation after the concentration of α -tocopherol and other antioxidants has been reduced or even completely depleted that potentially toxic lipid oxidation products are formed, and modification to a form recognized by the scavenger receptor occurs. It is therefore important to gain an insight into the reactions of copper in the propagation phase.

Our results show that LOOH is capable of reducing Cu(II) to Cu(I) and that, like LDL, the parameters of liposome oxidation are also saturable with respect to Cu(II). We propose that interactions between Cu(II) and LOOH do occur and are probably important, particularly in the propagatory phases of lipid peroxidation. Furthermore, although protein or α -tocopherol involvement in LDL oxidation is no doubt important to our understanding of its biological significance, there is no absolute requirement for their involvement in the promotion of LDL oxidation by copper.

EXPERIMENTAL

Materials

PC, type II-S, purified PC (99% pure as estimated by TLC) and linoleic acid were purchased from Sigma Chemical Co., Poole, Dorset, U.K. (9Z,11E,13S)-Hydroperoxyoctadecadienoic acid (13-HPODE) and the corresponding alcohol derivative (13-HODE) were obtained from Cascade Biochemicals Ltd, The Innovation Centre, University of Reading, Whiteknights Campus, Reading, Berks., U.K., and used without further purification. Bathocuproine disulphonate (BC) was purchased from Aldrich Chemical Co., Poole, Dorset, U.K. All other chemicals were obtained from Sigma and used without further purification.

Preparation of PC liposomes

PC was dissolved in 50 mM sodium phosphate buffer/5 μ M diethylenetriaminepenta-acetic acid (DTPA), pH 7.4, to a final concentration of 50 mg/ml using a multitube vortex-mixer. The suspension was stored on ice and purged with N_2 before

sonication. Liposomes were prepared by sonication (12–14 A) for 30 s, and then left to cool for 1 min. This cycle was repeated five times for a 5 ml volume. The resulting liposome solution was diluted before experiments and used at the concentrations indicated in the Figure legends. Liposomes were stored with DTPA (5 μ M) and used within 1 week of preparation.

Isolation and purification of LDL

Human LDL was isolated from plasma of healthy donors by differential centrifugation using the method described previously [29]. The LDL thus obtained was dialysed against Ca^{2+}/Mg^{2+} -free PBS containing 10 μ M EDTA, and then sterilized by filtration through a 0.2 μ m filter and stored at 4 °C before use.

Measurement of protein and α -tocopherol in PC liposomes and LDL

Protein concentrations were determined using both the BCA protein assay reagent (Pierce) and the Bio-Rad protein assay. Concentrations are expressed as mg/ml by comparison with BSA as standard. α -Tocopherol was extracted into heptane and quantified by HPLC using fluorescence detection [30].

Oxidative modification of PC liposomes or LDL

Oxidation of PC liposomes (0.167 mg of lipid/ml) or LDL (75 μ g of protein/ml) was initiated by the addition of various amounts of $CuSO_4$ in either 50 mM sodium phosphate buffer, pH 7.4, for liposomes or PBS (Ca^{2+}/Mg^{2+} -free) for LDL at 37 °C. Lipid peroxidation was assessed by monitoring conjugated diene formation and LOOH formation. Conjugated dienes were measured spectrophotometrically on a Beckman diode array spectrophotometer. Reactions were monitored continuously by measuring increases in A_{234} , attributed to conjugated diene formation [31], using LDL or liposomes at the same concentration as an optical reference and an absorption coefficient $\epsilon_{234} = 24400 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Lag times and maximum rates of propagation were determined graphically as previously described [31]. The rate of initiation is defined by the rate of conjugated diene formation in the lag phase. LOOH was measured by the FOX (ferrous oxidation by Xylenol Orange) assay [32]. Solutions to be analysed for LOOH (100 μ l) were added to the FOX reagent (900 μ l) and left in the dark for 30 min (room temperature) and the A_{560} then measured against an optical reference of buffer (100 μ l) and FOX solution (900 μ l). Concentrations were determined using the calculated absorption coefficient $\epsilon_{560} = 43970 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Singular value decomposition (SVD) analysis

SVD analysis was employed to compare the oxidation process in LDL and PC liposomes. SVD is a mathematical matrix method that provides a way to separate the spectral components of reaction species in a multicomponent system and also allows an objective assessment of the number of components contributing to the spectrum of any reaction system [33]. Time-dependent formation of lipid-oxidation products was monitored by measuring the spectrum between 220 and 550 nm. SVD analysis was carried out using Matlab 4.2 software by Mathworks inc.

Measurement of Cu(I)

Cu(I) formation was monitored using the specific Cu(I) chelator BC. On chelation a characteristic increase in A_{480} is seen [23]. Cu(II) (50 μ M) was added to various amounts of 13-HPODE and BC (500 μ M) in the presence or absence of PC liposomes

(0.167 mg of lipid/ml) and the A_{480} monitored as function of time against the appropriate reference. Cu(I) was quantified using the calculated absorption coefficient, $\epsilon_{480} = 12540 \pm 500$ (mean $\pm$ S.D.) $M^{-1} \cdot cm^{-1}$.

Analysis of products of the reaction between 13-HPODE and Cu(II)

13-HPODE (50 μM) was incubated with Cu(II) (50 μM) in 50 mM sodium phosphate buffer in the presence and absence of PC liposomes (0.167 mg of lipid/ml) and BC (500 μM) at 37 °C. Samples of volume 1 ml were taken at the indicated time intervals and reaction was stopped by the addition of DTPA (100 μM). 13-HPODE was extracted by the addition of 150 μl NaCl (15 %)/1 ml chloroform/ether (1:1, v/v). The solutions were vortex-mixed (1 min) and then centrifuged (2000 g at 10 °C for 5 min). Then 600 μl of the organic (bottom) layer was extracted, dried under nitrogen and redissolved in 0.5 ml of the mobile phase, hexane/propan-2-ol/acetic acid (1000:50:1, by vol.). HPLC was performed using a Beckman Gold HPLC system equipped with a 168 diode array UV-detector set to monitor 200–350 nm, and lipid peroxides were separated on a LiChrospher CN column (Merck) using a flow rate of 1 ml/min.

EPR analysis of the reaction between Cu(II) and LOOH

EPR spectroscopy was used to monitor Cu(II). Reactions were initiated by the addition of Cu(II) (50 μM) to 13-HPODE (50 μM) and/or PC liposomes in the presence or absence of BC. EPR measurements were made at liquid nitrogen temperature using a Bruker quartz Dewar flask containing the frozen samples on a Varian E109 spectrometer equipped with an Archimedes 440 computer. Samples in duplicate were taken for measurement at the stated time intervals by a fast freezing method. Two plastic syringes, taped together, were used to fill simultaneously two plastic tubes (4 mm $\times$ 30 mm) with the reaction mixture. The open ends of the tubes were held in contact with the surface of the liquid nitrogen, thereby sealing them. Once sealed (approx. 5 s) the tubes were completely immersed in liquid nitrogen and the duplicate samples frozen (10–12 s). The frozen tubes were then gradually warmed so that the samples (still frozen) could be carefully pushed out of the plastic tubes into liquid nitrogen with a glass rod. Samples were stored at 77K and measured within 1 week of preparation. Blank samples were made by freezing buffer as described above. EPR spectra of the blanks were subtracted from experimental spectra, thereby excluding all background signals such as those caused by the quartz Dewar machine cavity or by O_2 dissolved in liquid nitrogen. Concentrations were calculated by double integration of EPR signals with reference to a frozen solution of Cu(II) (50 μM).

RESULTS

Oxidation of LDL and PC liposomes

Samples of LDL and liposomes were analysed for protein content using the colorimetric assay based on Coomassie Blue binding to primary amino groups [34]. The concentration of liposomes used for oxidation experiments (0.167 mg of lipid/ml) showed no detectable levels of protein (detection limit of 0.10 μg /ml BSA). In contrast, the concentration of LDL used as substrate for oxidation giving similar levels of material absorbing at 234 nm after oxidation as liposomes, contained protein at a concentration of 75 μg of protein/ml. Similarly, HPLC analysis for α -tocopherol showed that liposomes contained no detectable levels of

Figure 1 Formation of conjugated dienes during Cu(II)-mediated oxidation of LDL and PC liposomes

Time courses of conjugated diene formation as measured by increase in A_{234} . (A) LDL (75 μg of protein/ml) was incubated with increasing concentrations (0, 2, 6, 8 and 10 μM , a–e respectively) of Cu(II). (B) PC liposomes (0.167 mg of lipid/ml) were incubated with various concentrations (0, 6, 10, 25, 50 and 100 μM , a–f respectively) of Cu(II). (C) DTPA (10 μM) was added at $t = 0, 65, 95, 155, 335$ min, or not at all (a–f respectively) during Cu(II) (10 μM)-dependent LDL oxidation. Traces are representative of experiments with three to five LDL and liposome preparations. All experiments were carried out at 37 °C in PBS for LDL and 50 mM phosphate buffer, pH 7.4, for PC liposomes.

this antioxidant, or other tocopherols, in samples 500 times more concentrated than those used in the oxidation experiments, whereas LDL (75 μg of protein/ml) contained between 7 and 9 nmol of α -tocopherol/mg of protein.

Monitoring at 234 nm (the λ_{max} for conjugated dienes [31]) as a function of time provides a relatively simple and useful method for assessing the oxidizability of lipid-based systems. Addition of Cu(II) to LDL and PC liposomes resulted in the formation of conjugated dienes with an absorbance spectrum identical with 13-HPODE, the LOOH generated by the 15-lipoxygenase-dependent oxidation of linoleic acid (results not shown).

Figure 1 shows representative time courses of conjugated diene production after the addition of various amounts of $CuSO_4$ to LDL (Figure 1A) and PC liposomes (Figure 1B). The oxidation of LDL shown here is essentially identical with that reported by a number of investigators and can be divided operationally into the following phases: (1) an initial lag phase in which the rate of conjugated diene formation is relatively low; (2) a propagation phase in which oxidation proceeds at its greatest rate; (3) a period in which absorbance shows first a transitory decrease followed by a slower increase (Figure 1A). No conjugated diene production was seen in the absence of Cu(II), confirming the direct role for this metal ion in promoting lipid-oxidation reactions and that any contaminating transition metal ions were not present at sufficient concentrations to cause peroxidation over the time scale studied. Addition of the metal chelator, DTPA, at all phases of LDL oxidation arrested further increase in A_{234} (Figure 1C). Similar behaviour was seen with liposomes (results not shown).

The length of the lag phase and maximal rates of initiation and propagation were determined from a series of experiments similar to those shown in Figure 1. For both LDL and PC liposomes an increase in Cu(II) concentration decreased the lag time and

Figure 2 Kinetic saturation during Cu(II)-mediated oxidation of LDL or PC liposomes

Effect of Cu(II) concentration on lag times (■), and maximum rates of propagation (◆) in (A) LDL (75 µg of protein/ml) and (B) PC liposomes (0.167 mg of lipid/ml). Rates of initiation (●) are shown for liposomes. Data points are means of two independent experiments or, where shown, means $\pm$ S.D. ($n = 3-5$). CD, conjugated diene.

increased the rates of conjugated diene formation in both the lag and propagation phases in a process that was saturable with respect to Cu(II) concentration (Figure 2). The rates of initiation during LDL oxidation were found to be too low ($< 0.1 \mu\text{M/min}$) for accurate measurement (results not shown). The saturable response with respect to Cu(II) concentration and parameters of oxidation has been reported previously for LDL [28] and clearly occurs with protein- and α -tocopherol-free liposomes (Figure 2B). Liposomes appeared to require approximately five times more copper than LDL before achieving the maximum rate of oxidation. In both lipid systems the concentrations of Cu(II) required to elicit the greatest rate of oxidation also resulted in the shortest lag time.

Spectral deconvolution of oxidized products in liposomes and LDL after exposure to Cu(II)

In these experiments, data from two different preparations of LDL and liposomes were obtained and found to be essentially identical. For the sake of clarity the data from a single preparation are shown. SVD analysis of time-resolved spectra (220–550 nm) obtained during the Cu(II)-dependent oxidation of LDL and protein/ α -tocopherol-free liposomes indicated that the products of the oxidation process consisted of one significant spectrally distinct species. The pseudo-spectrum of this component in LDL

Figure 3 SVD analysis of the reaction between Cu(II) and LDL and PC liposomes

Reconstructed spectra of the most significant spectrally contributing component in the reaction between Cu(II) (10 µM) and LDL (75 µg of protein/ml) or Cu(II) (50 µM) and PC liposomes (0.167 mg of lipid/ml) (A). The corresponding untreated time-resolved spectra are shown in (B) as indicated. Spectra (220–550 nm) were accumulated at 15 min intervals for 600 min with LDL, and 5 min intervals over 180 min for PC liposomes. The spectra shown are representative of data obtained from two different LDL and liposome preparations. Reaction conditions were as stated in the legend to Figure 1.

and liposomes was the same, and similar to the spectrum of 13-HPODE (results not shown). The 's' value for a component in this analysis represents its relative contribution to the overall spectrum of the sample. Within LDL the spectral species corresponding to a conjugated diene (λ_{max} 234 nm) had an 's' value of 39.6, relative to 1.36 for the next significant spectrally distinct component. For liposomes these values were 34.4 and 1.42. This suggests that the major spectrally contributing species (in the range 220–550 nm) formed in the reaction between Cu(II) and LDL or liposomes are conjugated dienes, and that they make the overwhelming spectral contribution. This was further implied by reconstructing the time-resolved spectra for the first component in each lipid system and comparing this with the original data sets (Figure 3). In both LDL and liposomes the reconstructions (Figure 3A) are essentially indistinguishable from the raw data sets (Figure 3B), and are indeed similar to each other.

Reduction of Cu(II) by LOOH

The Cu(I) chelator, BC, was used to test for Cu(II) reduction by LOOH. On Cu(I) chelation, a red–orange colour develops, the intensity of which is proportional to the amount of Cu(I) chelated, thus allowing the reaction to be followed spectrophotometrically. The total concentration of BC–Cu(I) formed at the end of the reaction increased uniformly with respect to the BC concentration, with saturation occurring in the liposomal system at about 500 µM BC (results not shown). Figure 4(A) (progress curve b) shows the time course of BC–Cu(I) formation from the reaction between Cu(II) and 13-HPODE, demonstrating that LOOH can reduce Cu(II) ions in a reaction that was essentially complete after 10 min and with a maximum yield of BC–Cu(I)

Figure 4 Reduction of Cu(II) by LOOH

(A) Time courses of BC-Cu(I) formation on addition of Cu(II) (50 μM) and BC (500 μM) to (curve b) 13-HPODE (50 μM), (curve c) PC liposomes (0.167 mg of lipid/ml), (curve d) PC liposomes (0.167 mg of lipid/ml) + 13-HPODE (50 μM). Curve a, control [i.e. Cu(II) + BC or 13-HPODE + BC]. Traces are representative of two separate experiments with different liposome preparations. (B) Effect of 13-HPODE on initial rate of formation of BC-Cu(I). Initial rates of formation of BC-Cu(I) in both the absence (■) and presence (●) of PC liposomes (0.167 mg of lipid/ml) are shown. Initial rates were determined by measuring the initial slope of progress curves. Data points are means of two separate readings. Reaction conditions were as stated in the legend to Figure 1.

Table 1 Effect of linoleic acid on BC-Cu(I) formation during the reaction between Cu(II) and 13-HPODE

Cu(II) (50 μM) was incubated with 13-HPODE (50 μM) and various concentrations of linoleic acid. The formation of BC-Cu(I) was measured after 20 min as described for Figure 4. Values represent the mean of two separate readings which were within 5% of each other. Reaction conditions were as stated in the legend to Figure 1.

Linoleic acid (μM)	BC-Cu(I) (μM)
0	3.8
50	5.4
100	7.9
500	18.7

complex of $9.2 \pm 0.3 \mu\text{M}$ (mean $\pm$ S.D., $n = 3$). Little or no BC-Cu(I) complex was seen if either of the reaction components was incubated with BC alone (curve a). Liposomes alone, which contained approximately 3–4 μM endogenous LOOH, also showed the formation of BC-Cu(I) to higher levels but over a slower time course with a maximum yield of $36.1 \pm 3 \mu\text{M}$ (mean $\pm$ S.D., $n = 3$; curve c). Supplementation of liposomes with 13-HPODE (50 μM) resulted in an accelerated formation of BC-Cu(I) complex (curve d) relative to unsupplemented liposomes with an unchanged maximum yield of BC-Cu(I) complex of $34 \pm 2.4 \mu\text{M}$ (mean $\pm$ S.D., $n = 3$). The rates of BC-Cu(I) formation were dependent on the LOOH concentration in both the presence and absence of PC liposomes (Figure 4b). The PC preparation used is capable of sustaining lipid peroxidation but is a complex mixture of components. In order to eliminate the possibility that the effects observed are due to reductants other than lipid peroxides, liposomes constructed from highly purified

Figure 5 Effect of BC on the EPR spectrum of Cu(II)

EPR spectra of Cu(II) (50 μM) in 50 mM sodium phosphate buffer in the absence (spectrum A) and presence (spectrum B) of BC (500 μM). Samples for EPR measurement were made after a 10 min equilibration period at 37 °C. Instrument conditions: microwave frequency 9.22 GHz (X-band); microwave power 30 mW; central field 3110 G; scan range 2000 G; scan time 65 s; modulation frequency 100 kHz; modulation amplitude 8 G; time constant 0.064 s; temperature of measurement 77 K. A baseline signal from buffer with no other additions has been subtracted from the spectra. Both spectra shown have the same double integral values.

PC (99%) were used. Under conditions identical with those in Figure 4, purified PC liposomes (0.167 mg of lipid/ml, which also contained approximately 4–5 μM LOOH) resulted in the reduction of a total of $28.6 \pm 1.5 \mu\text{M}$ Cu(II) to Cu(I). Furthermore the reduction of Cu(II) was also found if linoleic acid was incubated with Cu(II) and 13-HPODE (Table 1). An increased yield of the BC-Cu(I) complex was seen as the linoleic acid concentration was increased. The similar behaviour of pure PC liposomes and the effects of adding linoleic acid rule out the involvement of reductants other than lipid peroxides being responsible for the conversion of Cu(II) to Cu(I) under these conditions.

Addition of ascorbate (50 μM) on completion of the reaction under the conditions described in Figure 4 resulted in the maximal yield (50 μM, i.e. the initial Cu(II) concentration) of BC-Cu(I), indicating that the copper was still redox active and in the Cu(II) form before addition of the reducing agent (results not shown).

To ensure that the hydroperoxide group of 13-HPODE was the reducing species, the corresponding alcohol derivative, 13-HODE, was added to Cu(II) and BC in the presence and absence of liposomes. No formation of the BC-Cu(I) complex was seen.

To confirm the data obtained with BC, EPR spectroscopy was used to monitor the decrease in the paramagnetic signal from Cu(II). As Cu(I) is diamagnetic, it is EPR silent. Interestingly, different EPR signal line shapes were obtained when Cu(II) was measured alone in solution and in the presence of BC. In the latter condition the $g_{\perp}$ region of the spectrum is broadened and nearly resolved into two components, indicating that the equatorial ligands are not square planar, as seen without BC (Figure 5, spectrum A), but have a lower symmetry typical of rhombic chelation (Figure 5, spectrum B) [35]. This implies that BC also binds Cu(II). However, Cu(II) in this chelated state is still reducible, as indicated by the loss of EPR signal on addition of ascorbate (results not shown) forming the stable orange-coloured BC-Cu(I) complex.

Using EPR to measure the Cu(II) concentration, it was found that no loss occurred on incubation with BC (Table 2). In the experiments using 13-HPODE and/or liposomes, changes in Cu(II) concentration occurred over 10–20 min, after which the concentration remained constant. In the presence of 13-HPODE or 13-HPODE-supplemented liposomes a small but significant decrease in the Cu(II) signal was observed (Table 2). Interestingly,

Table 2 Effect of BC and PC liposomes on 13-HPODE-mediated loss of Cu(II)

EPR spectroscopy was used to measure the concentration of Cu(II) as described in Figure 5. Concentrations used were; Cu(II), 50 μ M; 13-HPODE, 50 μ M; PC liposomes, 0.167 mg of lipid/ml; BC, 500 μ M. Values represent Cu(II) concentrations (means $\pm$ S.D., $n = 4$) after incubation under the experimental conditions described in the legend to Figure 4.

Experimental condition	[Cu(II)] (μ M)
Cu(II) $\pm$ BC	50 $\pm$ 3
Cu(II) + 13-HPODE	41.7 $\pm$ 4.2
Cu(II) + PC liposomes	53.2 $\pm$ 1.7
Cu(II) + 13-HPODE + PC liposomes	44.5 $\pm$ 0.8
Cu(II) + 13-HPODE + BC	39.8 $\pm$ 2.1
Cu(II) + PC liposomes + BC	19.8 $\pm$ 0.85
Cu(II) + 13-HPODE + PC liposomes + BC	19.1 $\pm$ 3.4

this partial decrease in the Cu(II) signal in the presence of liposomes and 13-HPODE was maintained at the same level during the period (20–75 min, Figure 1B) in which peroxidation occurred. In the redox cycle between Cu(II) and Cu(I), during incubation with 13-HPODE or liposomes, the condition under which the maximum rate of formation of BC–Cu(I) complex occurred should be reflected in the magnitude of the Cu(II) signal when measured by EPR. This is confirmed in Figure 7 where, in the presence of BC, it is evident that the decrease in the Cu(II) signal was significantly enhanced.

Decomposition of 13-HPODE on addition of Cu(II)

The concomitant loss of 13-HPODE that is presumed to occur on its reaction with Cu(II) was monitored by HPLC. No appreciable loss of 13-HPODE was seen when incubated with Cu(II) alone (Figure 6A). In the presence of an oxidizable substrate, in this case liposomes, Cu(II) caused a progressive loss of 13-HPODE (Figure 6B). HPLC and subsequent spectral analysis showed that consumption of 13-HPODE was accompanied by formation of products with different retention times. Two sets of products eluted later than 13-HPODE (retention time 11.9–13 min) between 13.3–14.2 and 14.7–15.9 min had absorption spectra similar to 13-HPODE, with $\lambda_{\max}$ of 230 and 233 nm respectively, indicating that they contained conjugated diene groups. Another set of compounds eluted between 7.3 and 8 min had an absorbance spectrum with a $\lambda_{\max}$ at 268 nm. These latter compounds have previously been attributed to oxodienes [36].

Bathocuproine had interesting effects on the stability of 13-HPODE in the presence of Cu(II). In contrast with the effects of incubating Cu(II) and 13-HPODE alone, in the presence of BC 80 % of the LOOH was consumed within 50 min (Figure 6A). The rate and extent of loss of 13-HPODE in the presence of BC and Cu(II) was not affected by co-incubation with PC liposomes (Figure 6B).

In these experiments formation of the BC–Cu(I) complex was also monitored. In the simple reaction between 1 mol of LOOH and 1 g-atom of Cu(II), it is predicted that 1 molar equivalent of BC–Cu(I) complex should be formed. However, it is clear that in the case where a molar equivalent of 13-HPODE was incubated with Cu(II) in the presence of BC, over 80 % of the 13-HPODE was lost and only 20 % BC–Cu(I) complex formed. The most likely explanation for this finding is the peroxy-radical-dependent destruction of the BC–Cu(I) complex. To test this idea the peroxy radical generator 2,2'-azobis-2-amidinopropane hydrochloride (AAPH) (0.1–1 mM; 37 °C) was added to the

Figure 6 Formation of BC–Cu(I) and loss of LOOH during the reaction between Cu(II) and 13-HPODE

Cu(II) (50 μ M) was added to 13-HPODE (50 μ M) in the absence (A) or presence (B) of PC liposomes (0.167 mg of lipid/ml). Formation of BC–Cu(I) (■) and loss of LOOH [in the presence (●) or absence (▲) of BC] was measured as stated in the Experimental section. All values represent an average from four separate readings. Errors (less than 5%) are not shown for the sake of clarity.

Figure 7 Peroxyl-radical-dependent decay of BC–Cu(I)

Cu(II) (50 μ M) was incubated with 13-HPODE (50 μ M) as described in the legend to Figure 4. At 30 min, when the maximum concentration of BC–Cu(I) was reached, various concentrations of AAPH were added: curve a, no addition; curve b, AAPH (100 μ M); curve c, AAPH (250 μ M); curve d, AAPH (500 μ M); curve e, AAPH (1 mM).

preformed BC–Cu(I) complex and caused a progressive (45–60 min) loss in absorbance (Figure 7). Furthermore, if liposomes were present in the incubation mixture, which could then offer an alternative target for peroxy radicals, the kinetics for the rates of formation of BC–Cu(I) and loss of LOOH were identical for the first 25 min of the reaction (Figure 6B).

DISCUSSION

Oxidation of LDL has been linked to the onset and progression of atherosclerosis. In recent years a number of oxidants have been examined as possible candidates for mediating LDL oxidation *in vivo*. Many investigators have studied the role of transition metal ions in lipid peroxidation, as their presence is essential in cell-mediated LDL oxidation and there is evidence to suggest their involvement in atherogenesis *in vivo* [2,9].

In the interaction of Cu(II) with LDL it has been reported that the removal of endogenous or seeding LOOH by the compound *eb*selen both inhibits peroxidation and results in an α -tocopherol-dependent reduction of Cu(II) and initiation by the α -tocopheroxyl radical [26]. It was concluded that this latter reaction would not play a significant role in the propagation phase of lipid peroxidation or samples of LDL with significant endogenous concentrations of LOOH. In native LDL supplemented with LOOH or treated by the enzyme 15-lipoxygenase, the rate of oxidation was accelerated [20,37]. These data are consistent with the view that LOOH reduces Cu(II) to Cu(I), although other reductive pathways including those involving α -tocopherol and amino acid residues may also be occurring [23–28]. The oxidative arm of the redox cycle with copper is not disputed and involves the formation of lipid alkoxyl radicals ($\text{LO}^\bullet$), from the reaction of LOOH and Cu(I), which rapidly rearrange to the epoxyallylic radical and ultimately form peroxy radicals which may in turn initiate peroxidation or undergo β -scission with the formation of aldehydes [22,38].

In the present study we have assessed the role of LOOH in Cu(II)-mediated lipid oxidation by using PC liposomes, a model membrane system which contained no protein or α -tocopherol, and compared these data with those obtained with human LDL. As with LDL, addition of Cu(II) to PC liposomes caused lipid peroxidation reactions to occur, as evidenced by formation of conjugated dienes (Figure 1). Under the conditions used for isolation, the liposomal preparations contained approximately 3–4 μM lipid peroxides which is less than 1 % of the total lipid and is comparable with the 1–3 μM lipid peroxide groups present in the LDL preparations at the concentration used in the experiments. These values were determined using the FOX assay and may be an overestimate if components in the liposomal mixture other than lipid peroxides are capable of oxidizing the added Fe^{2+} to Fe^{3+} . Using more sensitive chemiluminescence techniques the concentration of lipid peroxides in LDL is typically estimated to be 250 pmol/mg of protein which is approximately 80-fold lower than that measured by either the FOX or iodometric assays [32,39–41]. However, the chemiluminescence methods are likely to underestimate lipid peroxide groups if they are (a) less stable during extraction with organic solvents, (b) not present in the specific fraction resolved by the HPLC method selected and (iii) the heterogeneous lipid peroxide groups react at different rates with the peroxidase used to generate the chemiluminescent signal [42].

The reaction of Cu(II) with both liposomes and LDL is inhibited in the propagation phase by metal chelation and chain-breaking antioxidants. This suggests that Cu(II) is necessary to sustain lipid peroxidation over the propagation phase and that the kinetic chain length for peroxy-radical-dependent propagation is short. Taken together we can conclude that copper is acting catalytically in these reactions. In order to confirm that monitoring at the single wavelength (234 nm) in both liposomes and LDL truly reflected the generation of conjugated dienes, we applied the analytical procedure SVD to a series of time-resolved spectra collected from the Cu(II)-dependent oxidation in both systems. The results of this procedure were striking in that during

both initiation and propagation the dominant species formed in both LDL and liposomes was spectrally identical with the authentic conjugated diene LOOH derived from linoleic acid, 13-HPODE, suggesting that the Cu(II)-dependent oxidation mechanisms are the same in the two different reaction systems and lead to the formation of similar products.

Furthermore the indices of lipid peroxidation measured in this study show that kinetic saturation occurs in both the absence and presence of protein (Figure 2). This does not exclude the involvement of protein and/or α -tocopherol in Cu(II)-dependent LDL oxidation, but shows that these potential reductants are not required for Cu(II) to show pro-oxidant behaviour. It is probable therefore that some of the characteristics of the Cu(II)–LDL interaction that have been ascribed to protein, such as kinetic saturation [28], may be properties of the interaction of the metal with LOOH.

In PC liposomes, which lack potential reducing agents such as amino acid residues or α -tocopherol, we have demonstrated that the formation of Cu(I) and loss of LOOH is consistent with the reduction of Cu(II) to Cu(I) by the peroxide. However, the contemporaneous increase in BC–Cu(I) and decrease in LOOH was only seen when liposomes were present in the reaction system. The presence of liposomes affected significantly the concentration of BC–Cu(I) formed, whereas when BC was present, liposomes did not affect the loss of LOOH (Figure 6). As peroxy radicals are produced from the reaction between Cu(II) and LOOH, we postulate that a lower yield of BC–Cu(I) was seen because the complex is destroyed by the radicals and this was confirmed experimentally. The presence of liposomes or linoleic acid presents an alternative substrate for peroxy radicals. The efficient scavenging of peroxy radicals prevents its attack on BC–Cu(I), thereby increasing the apparent yield of BC–Cu(I). This conclusion is supported by the close agreement in both the rate and amount of BC–Cu(I) complex and LOOH formed during co-incubation with liposomes.

The expected decomposition products of the oxidation of 13-HPODE to a peroxy radical were detected by spectral analysis after HPLC, which also provided evidence for the formation of oxodienes. These are similar to the reported products of the reaction between myoglobin and LOOH, and the peroxidase reaction of 15-lipoxygenase, which are both processes in which one-electron redox cycling of LOOH occurs [36,43].

We propose that Cu(II) interacts with LOOH forming Cu(I) and $\text{LOO}^\bullet$, via a reversible intermediate complex, denoted as [Cu(II)–LOOH] (Scheme 1, reaction 1). We assume that this intermediate has some of the character of an $\text{LOO}^\bullet$ bound to Cu(I) (reaction 2). Cu(I) can then react either with another LOOH forming Cu(II) and $\text{LO}^\bullet$ (reaction 6), or be tightly (but reversibly) chelated by BC thus perturbing the redox equilibrium strongly in favour of Cu(I) and $\text{LOO}^\bullet$ (reaction 3). The $\text{LOO}^\bullet$ will either attack the BC–Cu(I) complex (reaction 4) or, when liposomes are present, abstract a hydrogen atom from a polyunsaturated fatty acid (reaction 5) forming $\text{L}^\bullet$ and LOOH. It should be noted that Cu(I) will react with LOOH (reaction 6), forming, via an epoxyallylic radical ($\text{L}_{\text{epox}}^\bullet$), a peroxy radical (reaction 8) [22]. Lipid alkoxyl radicals can also undergo β -scission reactions forming aldehydes and lipid alkyl radicals [38] (reaction 7) and self-termination of the peroxy radicals will also occur.

We have shown that when Cu(II) is incubated with LOOH alone, little or no LOOH decomposition occurs. In the framework of Scheme 1, this implies that the redox equilibrium between LOOH and Cu(II) lies to the left. The depletion of $\text{LOO}^\bullet$ by liposomes will also perturb the equilibrium between Cu(II) and LOOH in favour of Cu(I) and $\text{LOO}^\bullet$, in agreement with the data

Scheme 1 Proposed reaction between Cu(II) and LOOH in the presence of BC and/or oxidizable lipid substrate

$L_{\text{epoxy}}^{\bullet}$, epoxyallylic radical; $L^{\bullet}$, carbon centred lipid radical; LH, liposomes; 'Y', product of reaction between peroxyl radical and BC-Cu(I).

Scheme 2

provided in Figure 6. Thus the formation of peroxyl radicals in a lipid environment is likely to overcome the proposed thermodynamic barrier for the reaction between Cu(II) and LOOH.

It has been reported that BC also binds Cu(II) [44], and our EPR data confirmed this (Figure 5). The BC-Cu(II) complex, owing to steric constraints, is relatively unstable compared with the BC-Cu(I) complex, and, as a result, the chelated Cu(II) is easily reduced to the more stable Cu(I) chelate. Thus the LOOH-mediated reduction of Cu(II) in the presence of BC could also occur via the process shown in Scheme 2.

The redox reaction between Cu(II) and LOOH must, however, occur, as in the presence of liposomes only (i.e. no BC), LOOH loss still occurs, albeit at a lower rate than seen with BC. From the EPR experiment it is clear that during the time scale of LOOH loss, a small (approx. 6–10%) decrease in Cu(II) is seen. This we propose represents the approach to a steady state in Cu(II), with the Cu(II)/Cu(I) redox cycle slowly decomposing the LOOH. This confirms that Cu(I) reacts with LOOH much faster than Cu(II), and that the Cu(II) reaction with LOOH is the rate-limiting step in this process. Furthermore the observation that Cu(II) promoted extensive oxidation of liposomes (Figure 1B), and that during this period no significant decrease in Cu(II) concentration occurred (Table 2), can be accounted for by such a redox cycling process.

All stages of LDL or liposome oxidation were inhibited by metal chelation, indicating that, without reduction of Cu(II) to Cu(I), catalytic oxidation of lipid by copper cannot occur. At the inception of the oxidative process, α -tocopherol or protein may act as reductants, but their contribution in the latter stages of oxidation are likely to be minimal as they are also consumed in radical termination reactions. They are clearly not essential for the Cu(II)-dependent oxidation of lipids. We propose therefore that Cu(II)/LOOH redox reactions do occur and that such reactions are likely to be particularly important in sustaining the propagatory phase of lipid peroxidation. This is especially so when potentially cytotoxic lipid-derived products responsible for

apoB modification are formed, and when the concentration of LOOH is high relative to other potential reductants of Cu(II).

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