Contribution of copper binding to the inhibition of lipid oxidation by plasmalogen phospholipids

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The role of plasmalogen phospholipids for copper-induced lipid oxidation was evaluated. Using ^{1}H-NMR we observed that the copper ($CuSO_4$)-promoted oxidative degradation of polyunsaturated fatty acids in micellar solution was dose-dependently attenuated by the plasmalogen lysoplasmenylethanolamine from bovine brain (lysoBP-PtdEtn). This was due to a direct interaction of copper ions with the plasmalogen-specific enol ether double bond. The enol ether methine ^{1}H signal decreased on the addition of copper, saturation being reached at a molar ratio of lysoBP-PtdEtn to copper of 1:1. The original ^{1}H signal was recovered almost completely after the addition of EDTA. Enrichment of micelles and low-density lipoproteins (LDLs) with plasmalogen phospholipids led to a decrease in the Cu(II) concentration in the aqueous media. After loading of LDLs *in vitro* with BP-PtdEtn, the LDL-dependent formation of Cu(I) was decreased, in particular in particles experimentally supplemented with α-tocopherol. The suppression of copper-promoted lipid oxidation that was observed in the presence of plasmalogen phospholipids plus α-tocopherol was greater than the sum of the protective effects elicited by the two substances alone. In conclusion, the formation of a complex between copper ions and the plasmalogens accounts partly for their inhibition of copper-induced lipid oxidation.

Key words: bathocuproine, conjugated diene formation, lysophosphatidylcholine, plasmenylcholine, Triton X-100.

INTRODUCTION

Lipid peroxidation has been implicated as an essential factor in the pathogenesis of a variety of common diseases such as atherosclerosis [1,2] and Alzheimer's disease [3,4]. The most convincing evidence for a pathogenetic role of lipid peroxidation has probably been obtained for atherosclerosis. Oxidation of the lipid components of low-density lipoproteins (LDLs) is generally thought to favour the development of atherosclerosis. Products of LDL oxidation such as lysophosphatidylcholine (lysoPtdCho) have been shown to mimic several pathological changes in the vascular wall that are typical of the atherosclerotic process. These include enhanced adhesion of monocytes to the endothelium [5], increased production of vascular growth factors [6] and impaired endothelium-dependent vasodilation [7].

In recent years considerable attention has been devoted to lipid-soluble antioxidants that potentially prevent the development of diseases for which lipid oxidation has been demonstrated as a pathogenetic factor. The most abundant lipid-soluble antioxidant of cellular membranes and lipoproteins is thought to be α-tocopherol. The concentrations of other lipophilic antioxidants such as β-carotene and ubiquinol-10 are in general considerably lower. Major targets of pro-oxidants (transition-metal ions, peroxyl radicals) in lipidic compartments are polyunsaturated fatty acids. The molar ratio of polyunsaturated fatty acids to α-tocopherol in LDL particles is approx. 200:1 [2]. As the oxidative resistance of LDLs cannot be explained entirely by the presence of α-tocopherol and the other known antioxidants, additional, as yet unknown, structural components have been proposed to be relevant for the oxidative resistance of LDLs [8].

We have recently reported that plasmalogen phospholipids, a class of phospholipids containing a characteristic enol ether double bond at sn_1 of the glycerol backbone, are able to scavenge peroxyl radicals, thereby inhibiting the oxidative degradation of polyunsaturated fatty acids [9]; 1 mol of the plasmalogen phospholipids interacted on average with 2 mol of peroxyl radicals. Products of enol ether degradation did not propagate oxidation, in agreement with the hypothesis that plasmalogen phospholipids act as endogenous antioxidants [10].

Copper ions are supposed to be of relevance for the induction of LDL oxidation and for the development of atherosclerosis *in vivo* [11–13]. This view is supported by recent results indicating that atherosclerotic plaques and the human plasma compartment contain autoantibodies against copper-oxidized LDLs [14–16]. Nevertheless oxidative modifications of lipoproteins catalysed by enzymes such as myeloperoxidase and lipoxygenase are most probably also important oxidants for lipoproteins *in vivo* [17]. Previous work has indicated that the copper-mediated oxidation of LDL-associated polyunsaturated fatty acids is decreased by the plasmalogen phospholipids [18,19]. Importantly, the molar ratio of plasmalogen phospholipids to α-tocopherol is approx. 4:1 in lipoproteins [20] and more than 100:1 in cellular membranes (as, for example, in platelets [21,22] and erythrocytes [23,24]). Thus plasmalogen phospholipids could have a decisive role in the defence systems of lipoproteins and cellular membranes

Abbreviations used: PtdCho, phosphatidylcholine; $C_{16:0}/C_{18:2}$-PtdCho, 1-palmitoyl-2-linoleoyl PtdCho; $C_{16:0}/C_{18:2}$-PtdEtn, 1-palmitoyl-2-linoleoyl phosphatidylethanolamine; AAPH, 2,2′-azobis-(2-amidinopropane hydrochloride); BC, bathocuproinedisulphonic acid; BP-PtdEtn, 1-alkenyl-2-acyl phosphatidylethanolamine from bovine brain; LDLs, low-density lipoproteins; lysoBP-PtdEtn, 1-alkenyl 2-lysophosphatidylethanolamine from bovine brain; lysoPtdCho, 1-palmitoyl 2-lysoPtdCho; lysoPtdEtn, 1-palmitoyl 2-lysophosphatidylethanolamine.

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against lipid oxidation. In the present study we attempted to gain insight into the mechanisms of the interactions of plasmalogens with copper.

MATERIALS AND METHODS

Materials

1-Hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphocholine [1-palmitoyl-2-linoleoyl phosphatidylcholine ($C_{16:0}/C_{18:2}$-PtdCho)], 1-hexadecanoyl-2-[*cis*-9,12-octadecadienoyl]-*sn*-glycero-3-phosphoethanolamine [1-palmitoyl-2-linoleoyl phosphatidylethanolamine ($C_{16:0}/C_{18:2}$-PtdEtn)], 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine from bovine brain (bovine brain phosphatidylethanolamine), 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk [egg phosphatidylcholine (PtdCho)], 1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine [1-palmitoyl-2-lysophosphatidylethanolamine (lysoPtdEtn)], 2,9-dimethyl-4,-7-diphenyl-1,10-phenanthrolinedisulphonic acid [bathocuproinedisulphonic acid (BC)] and α-tocopherol were obtained from Sigma (Deisenhofen, Germany). The purity of all commercially obtained phospholipids was assessed by one-dimensional TLC with chloroform/methanol/ammonia/water (90:54:5.5:5.5, by vol.) as solvent. 1-Alkenyl 2-lysophosphatidylethanolamine (lysoBP-PtdEtn) and 1-alkenyl 2-acyl phosphatidylethanolamine (BP-PtdEtn) were prepared from bovine brain phosphatidylethanolamine by alkaline methanolysis as described previously [9]. The purity of the products as determined by exposure to HCl fumes [20] was between 93 % and 95 %. All isolation procedures were performed under nitrogen. Tubes were opened only while pipetting was being performed. For storage of the phospholipids, the solutions were kept at −20 °C under argon in closed tubes. Triton X-100 was from Boehringer (Mannheim). Deuterium oxide (^2H_2O, 99.8 % pure) was obtained from Merck (Darmstadt, Germany). 2,2′-Azobis-(2-amidinopropane hydrochloride) (AAPH) was from Polysciences (Eppelheim, Germany).

^{1}H-NMR analysis of oxidative modifications of alkenyl and acyl chains

The early phase of oxidative modifications of fatty acid and enol ether double bonds was analysed by determining the peak amplitudes of intra-chain methine and bisallylic methylene ^{1}H signals (acyl chains) as well as of α-vinyl methine ^{1}H signals (alkenyl chains) by using ^{1}H-NMR [9]. The chemical shifts of the intra-chain and enol ether methine ^{1}H signals were identified at 5.4 and 6.0 p.p.m. respectively. In the Results section, the data on the oxidative degradation of the intra-chain double bonds are based exclusively on the determinations of the amplitudes of the methine proton signals. The measurements of the peak amplitudes of the bisallylic methylene signals were always performed in parallel and gave similar results under all experimental conditions. In brief, micelles were prepared in ^2H_2O from Triton X-100 and different phospholipids in a molar ratio of 5:1. The supensions were incubated at 37 °C in the presence of oxidants; NMR spectra were recorded with a Varian 400S spectrometer operating at a ^{1}H frequency of 400 MHz.

Analysis of oxidative degradation of LDL-associated phospholipids by TLC

Oxidative degradation of LDL-associated phospholipids was monitored by measuring the decrease in phospholipid substrates and by the formation of oxidation products. In brief, after the end of the incubations with oxidants, the lipids from LDLs were extracted [25] and phospholipids separated by different TLC procedures as follows: Procedure 1, one-dimensional, solvent chloroform/methanol/ammonia/water (90:54:5.5:5.5, by vol.); Procedure 2; two-dimensional, solvents chloroform/methanol/acetic acid (65:25:10, by vol.) in the first direction and chloroform/methanol/formic acid/water (65:25:8.9:1.1, by vol.) in the second direction. The phospholipids were detected with iodine vapour, the spots scraped off and their phosphate contents determined. For the estimation of the amounts of plasmalogen phospholipids, the spots were detected with diphenylhexatriene spray, and total phosphatidylethanolamine as well as total PtdCho were eluted by extracting the silica three times with chloroform/methanol (1:4, v/v). The plasmalogen contents of the phospholipids were determined as described [20].

Enrichment of LDLs with plasmalogen phospholipids and α-tocopherol

Fresh venous blood obtained from healthy male and female donors was drawn into tubes containing EDTA; plasma was recovered by centrifugation. For the enrichment of LDLs with BP-PtdEtn and α-tocopherol, phospholipid vesicles were prepared by the ethanol-injection method. Usually, 0.5 μmol of BP-PtdEtn and 1.5 μmol of egg PtdCho were dissolved in 100 μl of ethanol without or with 0.5 μmol of α-tocopherol. Other samples contained 0.5 μmol of $C_{16:0}/C_{18:2}$-PtdEtn and 1.5 μmol of egg PtdCho alone or in combination with 0.5 μmol of α-tocopherol. The solutions were added very slowly, with stirring, to 10 ml of plasma and the suspensions were incubated at 37 °C under argon for 6 h. Subsequently, LDLs were prepared by ultracentrifugation [26]. LDLs were dialysed against a buffer containing 0.3 mM EDTA and stored under argon at 4 °C. The amount of LDL-associated protein was determined by using the Bradford procedure [27]. Before the start of the oxidation, LDLs were freshly dialysed against PBS.

Determination of the lag time of conjugated diene formation

Samples of freshly dialysed LDLs (0.07 mg/ml, suspended in PBS) were incubated at 37 °C for a total of 240 min together with 2.5 μM $CuSO_4$. The A_{234} value, representing the quantity of conjugated dienes, was registered every 5 min. The lag time of conjugated diene formation was determined as the intercept of the baseline and the slope of the absorbance curve in the propagation phase [18].

Determination of the α-tocopherol contents of LDLs

The α-tocopherol contents of LDL particles were measured fluorimetrically. Usually, 50 μl aliquots taken from the suspensions were mixed with 100 μl of ethanol containing butylated hydroxytoluene (1 g/l) and 1 mg/ml δ-tocopherol as internal standard. The tocopherols were extracted with 400 μl of n-hexane, the extract was resuspended in methanol and 10 μl of the solution was injected onto a C_{18} reverse-phase column (Merck LiChrospher) coupled to an HPLC pump. Methanol was used as the mobile phase at a flow rate of 1 ml/min. Fluorimetric detection was performed at excitation and emission wavelengths of 295 and 330 nm respectively.

Measurement of copper ion concentrations

The concentration of Cu(I) was quantified by using the Cu(I) chelator BC [28]. To suspensions containing different amounts of $CuSO_4$, BC was added at the indicated concentrations and the A_{480} value was registered. For the estimation of the Cu(II) contents of the micellar suspensions, their lipids were extracted

[25] and the remaining upper phase was recovered. To this phase ascorbic acid (300 μM) was added [to reduce Cu(II) to Cu(I)] together with BC (360 μM). The absorbance of the Cu(I)–BC complex was determined. For LDL suspensions the lipoproteins were first precipitated with heparin acetate buffer [0.3 M sodium acetate/100 i.u./ml sodium heparinate (pH 4.85)] and the supernatant was isolated. For determination of the Cu(II) concentrations, ascorbate and BC were added to the supernatant as described above.

Values with errors are given as means $\pm$ S.D.

RESULTS

Interaction of micellar plasmalogen phospholipids with copper ions

The effect of the brain plasmalogen lysoplasmenylethanolamine (lysoBP-PtdEtn) on the copper-catalysed oxidation of the polyunsaturated $C_{16:0}/C_{18:2}$-PtdCho was analysed in micelles by using ^{1}H-NMR. $CuSO_4$ (10 μM) caused a time-dependent decrease in the amplitude of the intra-chain methine ^{1}H signal of micellar $C_{16:0}/C_{18:2}$-PtdCho (double bonds at sn_2). The decrease of the amplitude proceeded almost linearly at a rate of 15.7 % per h (Figure 1A). Increasing amounts of lysoBP-PtdEtn were added to the micelles enriched with the polyunsaturated diacyl phospholipid. At molar ratios of $C_{16:0}/C_{18:2}$-PtdCho to lysoBP-PtdEtn of 80:1, 13:1 and 4:1, the degradation rate of the intra-chain methine ^{1}H signal was decreased by 21 %, 53 % and 60 % respectively (Figure 1A). At the same time, the peak amplitude of the enol ether methine ^{1}H of lysoBP-PtdEtn (80 nmol) decreased rapidly by 24 % within the first 12 min of incubation with $CuSO_4$ (Figure 1B). No further decrease in this peak amplitude was observed in the remaining incubation period up to 1.5 h. Therefore the plasmalogen dose-dependently prevented the copper-induced decrease in the intra-chain methine ^{1}H signal of the polyunsaturated ester phospholipid.

To gain more insight into the mechanism of the interaction of plasmalogens with copper, lysoBP-PtdEtn was titrated with $CuSO_4$. The addition of increasing amounts of $CuSO_4$ to 100 nmol of lysoBP-PtdEtn led to a sudden broadening of the enol ether methine ^{1}H signal (Figure 2, left panels). The values for the peak amplitudes of the enol ether methine proton of lysoBP-PtdEtn as determined in the presence of different quantities of $CuSO_4$ are summarized in Figure 2 (right panel). A constant signal amplitude was attained with 100 nmol of copper, suggesting that copper binding to the plasmalogen saturated at an equimolar ratio.

The enol ether double bond of the plasmalogens was previously shown to bind mercury ions [29]. It can be assumed that the decrease in the amplitude of the enol ether methine ^{1}H peak induced by $CuSO_4$ is due to the formation of a complex between Cu(II) and the plasmalogen-specific enol ether. This would imply a reversible interaction of the plasmalogens with copper. To test this hypothesis, 100 nmol of lysoBP-PtdEtn was first incubated for 120 min with 150 nmol of $CuSO_4$; 0.3 mM EDTA was then added. This led to the reappearance of a narrow ^{1}H signal (Figure 2, left panels), the peak amplitude of which amounted to 84 % of the original value (Figure 2, right panel). Micellar lysoBP-PtdEtn (100 nmol) was treated for 2 h at 37 °C with 100 μM $CuSO_4$. The lipids were extracted and then separated by TLC (procedure 1); the spots corresponding to lysoPtdEtn were subjected to phosphate determinations. The values measured after 0 and 2 h were comparable, indicating no substantial degradation of the lysoBP-PtdEtn by copper (results not shown).

In separate experiments, BP-PtdEtn (100 nmol) solubilized in Triton X-100 (500 nmol) was incubated with $CuSO_4$ (100 μmol). After a 2 h incubation, the peak amplitudes of the enol ether and of the intra-chain methine ^{1}H of BP-PtdEtn were decreased by 41.4 % and 15.6 % respectively (means for two independent experiments). After adding 0.3 mM EDTA, 92 % of the original enol ether methine ^{1}H signal was recovered. In contrast, the original peak amplitude of the intra-chain methine ^{1}H could not be restored after the addition of EDTA. Thus the interaction of copper with the enol ether double bond is reversible, but not that with the intra-chain double bonds.

We next tested whether increasing the micellar concentration of the plasmalogen phospholipids affected the free Cu(II) con-

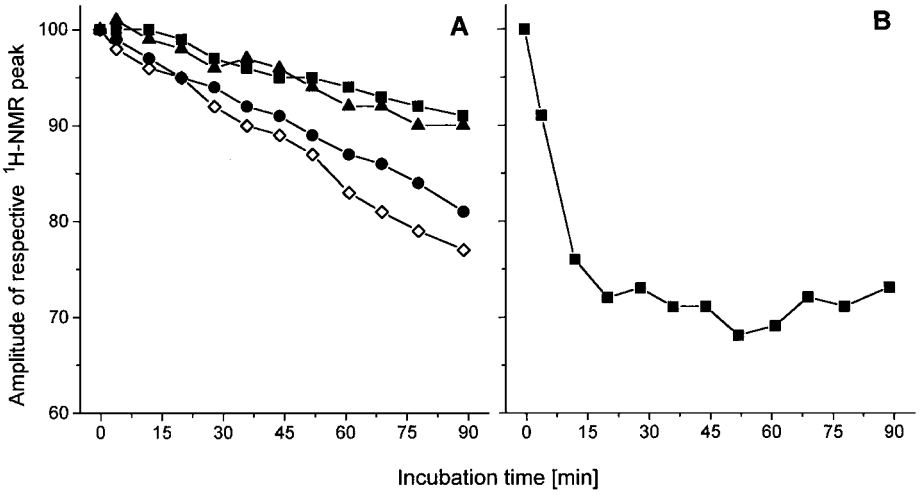

Figure 1 Effects of lysoBP-PtdEtn on the copper-mediated degradation of $C_{16:0}/C_{18:2}$-PtdCho

$C_{16:0}/C_{18:2}$-PtdCho (320 nmol) was solubilized in Triton X-100 micelles (1600 nmol) in the absence or the presence of increasing amounts of lysoBP-PtdEtn. The micelles were dispersed in 0.75 ml of ^2H_2O and incubated at 37 °C with 10 μM $CuSO_4$. (**A**) Decrease in the amplitude of the intra-chain methine ^{1}H peak of $C_{16:0}/C_{18:2}$-PtdCho. Symbols: ◇, $C_{16:0}/C_{18:2}$-PtdCho; ●, $C_{16:0}/C_{18:2}$-PtdCho + 4 nmol of lysoBP-PtdEtn; ▲, $C_{16:0}/C_{18:2}$-PtdCho + 25 nmol of lysoBP-PtdEtn; ■, $C_{16:0}/C_{18:2}$-PtdCho + 80 nmol of lysoBP-PtdEtn. (**B**) Effect of $CuSO_4$ on the enol ether methine ^{1}H peak of lysoBP-PtdEtn (80 nmol). A representative experiment from a total of four separate experiments is shown.

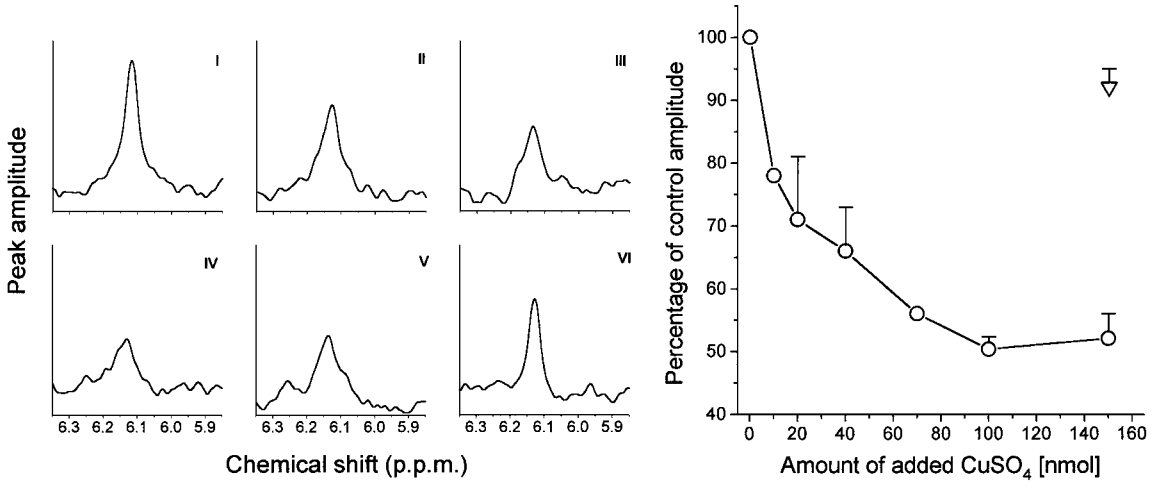

Figure 2 Decrease in the peak amplitude of the enol ether methine ^{1}H peak of lysoBP-PtdEtn as induced by increasing amounts of $CuSO_4$

Left panels: enol ether methine ^{1}H peak of lysoBP-PtdEtn (100 nmol) enriched in Triton X-100 micelles (500 nmol, dispersed in 0.75 ml of ^2H_2O) and incubated for 120 min at 37 °C with 0 (I), 20 (II), 40 (III), 100 (IV) and 150 nmol $CuSO_4$ (V). In (VI), 0.3 mM EDTA was added after the 2 h incubation with 150 nmol of $CuSO_4$. Right panel: summarized data obtained from three to five separate experiments [or from two experiments where no S.D. bar is given] under the same conditions as in the left panels. The point ▽ shows a sample with added EDTA.

Table 1 Cu(II) contents of the aqueous media in suspensions of micelles with plasmalogen phospholipids

TX-100 (500 nmol) and 100 nmol of the indicated phospholipids were dispersed in 0.5 ml of water and incubated for 5 min at room temperature with 100 μM $CuSO_4$. Thereafter, the aqueous phase of the suspensions was extracted [21]. BC (360 μM) and ascorbate (300 μM) were added and the absorbance of the Cu(I)–BC complex at 480 nm was determined. Mean values from three to five separate experiments are shown (or from one experiment where no S.D. is given).

Phospholipids	A_{480}
No addition	0.90 ± 0.13
LysoPtdEtn	0.82 ± 0.07
$C_{16:0}/C_{18:2}$-PtdEtn	0.79 ± 0.17
$C_{16:0}/C_{18:2}$-PtdCho	0.81
LysoBP-PtdEtn	0.35 ± 0.13
BP-PtdEtn	0.32 ± 0.10

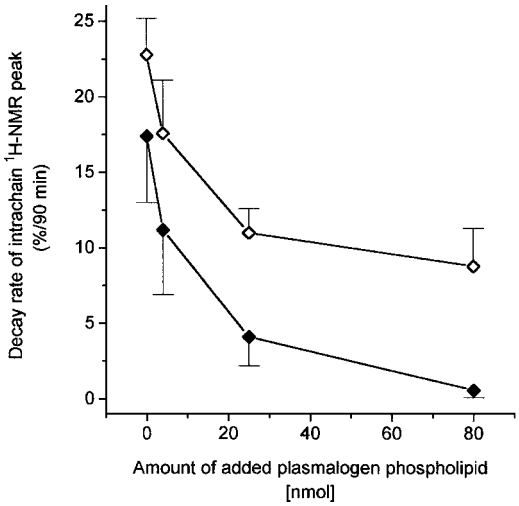

Figure 3 Effects of plasmalogen phospholipids plus α-tocopherol on the copper-mediated degradation of $C_{16:0}/C_{18:2}$-PtdCho

$C_{16:0}/C_{18:2}$-PtdCho (320 nmol) was enriched in micelles (1600 nmol) in the absence or the presence of the indicated amounts of lysoBP-PtdEtn, without (◇) or with (◆) α-tocopherol (1 nmol). The micelles were dispersed in 0.75 ml of ^2H_2O and incubated at 37 °C with 10 μM $CuSO_4$. The decrease in the amplitude of the intra-chain methine ^{1}H peak was determined and the decay rates were calculated. Values are means ± S.D. from three separate experiments.

centration of the aqueous phase. After a 5 min incubation of micellar suspensions with $CuSO_4$ (100 μM), the aqueous phase was extracted and the Cu(II) concentration determined by measuring the absorbance of the Cu(I)–BC complex in the presence of ascorbate [which converts Cu(II) to Cu(I)]. In the presence of 100 nmol of the ester phospholipids lysoPtdEtn, $C_{16:0}/C_{18:2}$-PtdEtn or $C_{16:0}/C_{18:2}$-PtdCho, the absorbance of the Cu(I)–BC complex was decreased by 9%, 13% or 10% respectively, compared with the control value without the ester phospholipids (Table 1). When the micelles contained either lysoBP-PtdEtn or BP-PtdEtn, the absorbance of the Cu(I)–BC complex was decreased by 61% and 64% respectively (Table 1). The Cu(I) concentration determined in the absence of ascorbate in the aqueous media was less than 2% of the absorbances measured in the presence of ascorbate under all experimental conditions shown in Table 1. The results thus confirm that plasmalogens bind Cu(II) atoms selectively.

In further experiments we studied the influence of lysoBP-PtdEtn on the copper-catalysed degradation of $C_{16:0}/C_{18:2}$-PtdCho (320 nmol) in micelles containing α-tocopherol. After the addition of $CuSO_4$ (10 μM), the peak amplitude of the intra-chain methine ^{1}H of the polyunsaturated diacyl phospholipid decreased by 22.8% within 90 min (Figure 3). At a molar ratio of $C_{16:0}/C_{18:2}$-PtdCho to α-tocopherol of 320:1, α-tocopherol decreased the decay rate of the intra-chain methine ^{1}H signal of $C_{16:0}/C_{18:2}$-PtdCho to 17.1% in 90 min. When the micellar amount of lysoBP-PtdEtn was increased from 4 to 80 nmol, the inhibitory effect of α-tocopherol (1 nmol) on the degradation of the intra-chain methine ^{1}H signal tended to be more effective, the

Table 2 Oxidative degradation of LDL phospholipids in plasmalogen- and α-tocopherol-enriched LDL

LDL particles were loaded *in vitro* with diacyl PtdEtn (control), BP-PtdEtn, α-tocopherol or BP-PtdEtn plus α-tocopherol, as described in the Materials and methods section. The total α-tocopherol contents of the particles thus obtained were (in nmol/mg of LDL protein): 10.5 ± 2.1 (control), 11.6 ± 0.9 (BP-PtdEtn), 17.3 ± 3.8 (α-tocopherol) and 18.7 ± 3.4 (BP-PtdEtn plus α-tocopherol). The lipoproteins (0.2 mg/ml LDL protein) were incubated for 2 h at 37 °C with either 2.5 μM $CuSO_4$ or 4 mM AAPH. At times 0 and 120 min, LDL lipids were extracted and separated by TLC with procedures 1 and 2. The contents of diacyl PtdCho, lysoPtdCho, sphingomyelin, plasmenylethanolamine and plasmenylcholine were determined by quantification of the phosphate contents of the phospholipid spots. The increase in the degradation products of PtdCho oxidation was calculated as the sum of the differences in phosphate contents of the spots corresponding to lysoPtdCho and sphingomyelin at times 120 and 0 min. The results are expressed as nmol/mg of LDL protein. The lag time of conjugated diene formation was determined as described in the Materials and methods section. Values are means ± S.D. for three or four independent experiments.

	Copper oxidation				AAPH oxidation	
LDL enriched with ...	Diacyl PtdEtn	BP-PtdEtn	α-Tocopherol	BP-PtdEtn + α-tocopherol	Diacyl PtdEtn	BP-PtdEtn
Plasmalogen contents, 0 h (nmol/mg of LDL protein)	57.3 ± 12.4	75.2 ± 6.5	55.0 ± 10.7	74.3 ± 13.8	52.6 ± 4.7	70.2 ± 10.6
Plasmalogen contents, 2 h (nmol/mg of LDL protein)	40.2 ± 8.8	50.8 ± 7.3	45.4 ± 7.2	60.9 ± 11.1	33.5 ± 9.4	49.0 ± 3.1
Decrease in diacyl PtdCho (nmol/mg of LDL protein)	77.8 ± 7.2	51.1 ± 5.4	57.6 ± 7.3	25.4 ± 4.7	97.5 ± 16.2	65.4 ± 12.8
Increase in PtdCho degradation products (nmol/mg of LDL protein)	72.5 ± 10.1	49.4 ± 3.6	55.9 ± 7.5	22.7 ± 4.8	88.6 ± 14.0	54.3 ± 8.9
Lag time (min)	65.4 ± 6.3	74.6 ± 3.1	76.7 ± 8.2	98.5 ± 10.1	–	–

inhibition curve being slightly steeper in the presence of α-tocopherol than in its absence. The copper-catalysed degradation of $C_{16:0}/C_{18:2}$-PtdCho was completely prevented by lysoBP-PtdEtn (80 nmol) plus α-tocopherol. The decrease in the peak amplitude of the enol ether methine ^1H signal of 80 nmol of lysoBP-PtdEtn was quite similar in the absence and in the presence of α-tocopherol (results not shown).

Interactions of copper with the plasmalogen phospholipids of LDLs

To evaluate whether the mechanisms governing the inhibition of copper-promoted oxidation by plasmalogens in micelles were also of relevance for the oxidation of LDLs, the particles were enriched with BP-PtdEtn or diacyl PtdEtn (as a control) and then treated with $CuSO_4$. In further experiments, control and BP-PtdEtn-loaded LDLs were oxidized with the peroxyl radical generator AAPH. After being loaded *in vitro* with BP-PtdEtn, the LDL contents of plasmalogen phospholipids (plasmenylethanolamine plus plasmenylcholine) in the LDLs were augmented by 31–35 % (Table 2). After a 2 h incubation of LDLs (0.2 mg/ml) with $CuSO_4$ (2.5 μM), the plasmalogen concentrations of LDLs were decreased by 30 % and 32 % in control and BP-PtdEtn-enriched LDLs respectively.

Oxidation of LDLs is accompanied by a time-dependent decrease in the contents of PtdCho. In this way lysoPtdCho and oxidation products that migrate with sphingomyelin on TLC plates are generated [30,31]. The diacyl PtdCho contents of control LDLs were decreased by 78 nmol/mg of LDL protein, whereas the increase in the concentrations of PtdCho degradation products amounted to 73 nmol/mg of LDL protein (Table 2). In plasmalogen-enriched LDLs, the oxidative changes in choline phospholipid contents were mitigated by 32–34 %. Control and plasmalogen-enriched particles were loaded *in vitro* with α-tocopherol, resulting in a 61–65 % increase in the LDL contents of α-tocopherol (see the legend to Table 2). In these particles, the plasmalogen concentrations were decreased by 17–18 % on copper oxidation. Enrichment with α-tocopherol alone attenuated the decrease in diacyl PtdCho as well as the formation of degradation products by 23–26 % compared with control particles. In LDLs loaded with BP-PtdEtn plus α-tocopherol, the copper-induced oxidative changes in the choline phospholipid contents were mitigated by 67–69 % (Table 2). The lag time of conjugated diene formation was measured in the same experimentally modified LDL particles. The duration of the lag time was increased by 14 % (BP-PtdEtn-loaded LDLs) and by 17 % (α-tocopherol-loaded LDLs) compared with the control LDLs (Table 2). In LDLs loaded *in vitro* with BP-PtdEtn plus α-tocopherol, the lag time was elevated by 51 %.

Table 3 Formation of Cu(I) in suspensions of LDL enriched with plasmalogen and α-tocopherol

LDL particles were loaded *in vitro* with diacyl PtdEtn, BP-PtdEtn, α-tocopherol or BP-PtdEtn plus α-tocopherol. The lipoproteins were suspended at 0.2 mg of LDL protein in 1 ml of PBS. BC (360 μM) and $CuSO_4$ (100 μM) were added and the changes in absorbance of the Cu(I)–BC complex at 480 nm was registered after a 20 min incubation at 37 °C. The differences were statistically significant ($P < 0.05$; one-way analysis of variance for multiple comparisons), except for those between BP-PtdEtn and BP-PtdEtn plus α-tocopherol and between diacyl PtdEtn and BP-PtdEtn plus α-tocopherol. Values are means ± S.D. for three separate experiments.

Additive to LDL	Cu(I) concentration (nmol/ml)
Diacyl PtdEtn	63.9 ± 2.5
BP-PtdEtn	57.4 ± 2.4
α-Tocopherol	73.8 ± 1.6
BP-PtdEtn plus α-tocopherol	59.8 ± 2.9

After the 2 h incubation of control and BP-PtdEtn-enriched LDLs (0.2 mg/ml) with AAPH (4 mM), their plasmalogen contents were decreased by 36 % and 30 % respectively (Table 2). The presence of AAPH decreased the contents of LDL PtdCho by 98 nmol/mg of LDL protein and led to an increase in the concentrations of PtdCho degradation products by 89 nmol/mg of LDL protein. In LDLs with experimentally increased plasmalogens, these changes were attenuated by 33 % (PtdCho decrease) and by 39 % (increase in degradation products).

Recent studies indicate that LDL promotes the reduction of Cu(II) to Cu(I) [28,32]. BC was used to monitor the formation of Cu(I) in suspensions of control (diacyl PtdEtn) and BP-PtdEtn-loaded LDLs. Cu(I) generation occurred rapidly within the first 10 min after addition of BC to the suspensions, the Cu(I) formation not being further increased up to 60 min of incubation (results not shown). After a 20 min incubation, the Cu(I) concentration was 10 % lower in BP-PtdEtn-enriched LDLs than in the control particles (Table 3). Within the same time

interval, the generation of Cu(I) was enhanced by 15% in α-tocopherol-enriched particles compared with the control LDLs. In LDLs loaded with BP-PtdEtn, additional enrichment with α-tocopherol was not accompanied by an increase in the formation of Cu(I) (Table 3).

To investigate whether the enrichment of LDLs with BP-PtdEtn (and/or α-tocopherol) affected the Cu(II) contents of the aqueous medium, the particles were enriched with diacyl PtdEtn (control), BP-PtdEtn, α-tocopherol or BP-PtdEtn plus α-tocopherol as described in the legend to Table 2. The particles (0.2 mg/ml) were incubated for 10 min at room temperature with $CuSO_4$ (50 μM). Subsequently, the LDLs were precipitated (see the Materials and methods section) and the supernatants were recovered. BC (360 μM) and ascorbate (300 μM) were added and the absorbance of the Cu(I)–BC complex was measured. In supernatants recovered from suspensions of BP-PtdEtn-enriched LDLs previously treated with $CuSO_4$ (50 μM), the Cu(II) contents were 15% lower than in those isolated from suspensions of LDLs enriched with diacyl PtdEtn [20.9 ± 2.7 nmol/ml (BP-PtdEtn-LDLs) compared with 24.6 ± 1.9 nmol/ml (control); $P < 0.05$, paired t test, $n = 4$]. The Cu(II) concentrations of the supernatants obtained from suspensions of LDLs with experimentally increased contents of plasmalogen phospholipids plus α-tocopherol were decreased by 23% compared with the media recovered from the particles with α-tocopherol alone [20.7 ± 4.8 nmol/ml (BP-PtdEtn-LDLs) compared with 26.8 ± 4.2 nmol/ml (control); $P < 0.05$, paired t test, $n = 4$]. The supernatants obtained from the different suspensions of experimentally modified LDLs were also analysed for their Cu(I) contents (with the use of 360 μM BC in the absence of ascorbate). The Cu(I) concentrations of the media were less than 1 nmol/ml.

DISCUSSION

Oxidized LDLs are thought to be an essential element in the development of atherosclerosis [1,2]. Considerable evidence has been accumulated indicating that copper ions might have a major role in the oxidation of LDLs under conditions *in vivo* [11–16]. Among the antioxidants that efficiently protect LDLs against oxidation by copper, most attention has been paid to α-tocopherol [33]. As outlined in the Introduction section, plasmalogen phospholipids, in particular because of their high concentrations, could also be an important component of the defence systems protecting lipidic compartments against copper-induced lipid oxidation. In plasmalogen-deficient pyrene-enriched Chinese hamster ovary cells, irradiation with UV was associated with an increased cytotoxicity compared with the wild-type cells [10]. Supplementation of the cells with a plasmalogen precursor restored the normal plasmalogen levels and also the oxidative resistance towards UV treatment. In LDLs [18,19] as well as in liposomes [34] oxidized with copper, plasmalogens were found to delay the oxidative degradation of polyunsaturated fatty acids.

To learn more about the mechanisms determining the interaction between copper and the plasmalogens, the influence of the metal ion on the oxidative degradation of the plasmalogen-specific enol ether was analysed. The very early phase of the interaction of copper with the enol ether as well as with the fatty acid double bonds was registered with the use of ^{1}H-NMR by determining the peak amplitudes of the signals of the enol ether and intra-chain methine ^{1}H respectively. Using this method we observed that the plasmalogen phospholipid lysoBP-PtdEtn efficiently prevented the copper-induced degradation of the intra-chain methine ^{1}H signal of the micellar polyunsaturated diacyl phospholipid $C_{16:0}/C_{18:2}$-PtdCho (Figure 1). Concomitantly, the amplitude of the enol ether methine ^{1}H was decreased, with saturation apparently being reached rapidly.

The addition of EDTA to a micellar suspension of plasmalogen phospholipids previously incubated with $CuSO_4$ restored the original peak of the enol ether methine ^{1}H by 80–90% (Figure 2). These results indicated that the interaction of copper with the plasmalogen-specific enol ether was mostly reversible. It is therefore unlikely that pro-oxidant degradation products are formed by the interaction of copper with the enol ether double bond. At a molar ratio of lysoBP-PtdEtn to $CuSO_4$ of 1:1, the peak amplitude of the enol ether methine ^{1}H signal was decreased maximally (Figure 2), indicating that Cu(II) forms a 1:1 complex with the enol ether double bond.

The Cu(II) concentration of the aqueous medium was decreased when the micelles contained plasmalogen phospholipids (Table 1). The ability of plasmalogens to extract Cu(II) atoms from the aqueous phase was confirmed in the experiments with BP-PtdEtn-enriched LDLs (Table 2). Complexation of Cu(II) by the enol ether is thus responsible for the inhibition of the copper-induced oxidation of micellar polyunsaturated fatty acids by the plasmalogens. It most probably also contributes to the decrease in lipid oxidation in plasmalogen-enriched LDLs. The enhanced binding of Cu(II) to the LDL particles loaded with BP-PtdEtn *in vitro* was indeed accompanied by a decrease in the degradation of LDL-associated diacyl PtdCho and an increased lag time of conjugated diene formation (Table 2). Approx. 80% of the total binding sites for copper within the LDL particles is provided by their protein components [35,36]. The remaining binding sites were proposed to reside within the phospholipid portion of LDLs [36]. Our results suggest that the plasmalogen phospholipids are part of these non-proteinaceous sites.

It is generally assumed that the initiation of LDL oxidation by copper requires the prior reduction of Cu(II) to Cu(I) [2,28]. Several LDL-associated factors have been proposed as Cu(II) reductants, including α-tocopherol [32,37,38] and hydroperoxides [39]. In the present study we confirmed that α-tocopherol enrichment enhances the formation of Cu(I) from Cu(II) (Table 3). The increase in Cu(I) formation elicited by LDL particles loaded with α-tocopherol *in vitro* was almost completely prevented by the additional enrichment with BP-PtdEtn. This suggests that plasmalogen phospholipids attenuate the capacity of α-tocopherol to reduce Cu(II) in LDLs. As a consequence, the inhibitory effects of the plasmalogen phospholipids plus α-tocopherol on the oxidation of micellar polyunsaturated fatty acids and of LDL particles were overadditive (Figure 3 and Table 2). Increased binding of copper to plasmalogen-enriched LDLs was associated with a diminished capacity of α-tocopherol to reduce Cu(II) (Table 3). Accordingly, these results suggest that Cu(II) is less efficiently reduced to Cu(I) when complexed to the enol ether double bond.

Chemical analyses of the LDL-associated plasmalogen phospholipids indicated that oxidation with $CuSO_4$ led to a decrease in the contents of this phospholipid fraction (Table 2). The complex of the enol ether with copper is reversible in micelles (Figure 2). The decrease in LDL plasmalogen mass indicates that, apart from the formation of Cu(II)–plasmalogen complexes, other mechanisms are also involved in the inhibitory effect of the plasmalogens on copper-mediated LDL oxidation. The results in Table 2 show that there is also degradation of polyunsaturated fatty acids within the period of copper oxidation employed. The oxidation of polyunsaturated fatty acids with copper results in the formation of peroxyl, alkoxyl and other radicals. Peroxyl-radical-mediated oxidation of LDLs substantially decreased the LDL plasmalogen contents (Table 2). We therefore assume that the decrease in LDL plasmalogen concentrations in copper-

oxidized particles is due to reactions of plasmalogens with products of the oxidation of polyunsaturated fatty acids. On the basis of these considerations we suggest that the prevention of copper-induced LDL oxidation by the plasmalogen phospholipids is related not only to a direct interaction of the enol ether with copper but also to reactions with oxidation products of polyunsaturated fatty acids.

In conclusion, the results indicate that the enol ether double bond of plasmalogens complexes Cu(II) with a 1 : 1 stoichiometry. In this way plasmalogen phospholipids provide binding sites for copper within the lipid phase of the LDL particles. The ability of the plasmalogens to protect the LDL polyunsaturated fatty acids against copper-mediated degradation is in part due to the formation of copper–plasmalogen complexes. In addition, reactions of the plasmalogens with oxidized species of polyunsaturated fatty acids (previously generated by copper oxidation) are most probably also involved.

We thank Dr. Joachim Thiery and Gudrun Haas for the determinations of the α-tocopherol contents. This study was supported by grants of the Wilhelm Sander-Stiftung and of the Friedrich-Baur-Stiftung to B. E.

REFERENCES

1 Steinberg, D. (1989) New Engl. J. Med. **320**, 915–924
2 Esterbauer, H. and Ramos, P. (1996) Rev. Physiol. Biochem. Pharmacol. **127**, 31–64
3 Markesbury, W. R. (1997) Free Radicals Biol. Med. **23**, 134–147
4 Kienzl, E., Puchinger, L., Jellinger, K., Linert, W., Stachelberger, H. and Jameson, R. F. (1995) J. Neurol. Sci. **134** (suppl.), 69–78
5 Quinn, M. T., Parthasarathy, S. and Steinberg, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2805–2809
6 Kume, N. and Gimbrone, Jr., M. A. (1994) J. Clin. Invest. **93**, 907–911
7 Kugiyama, K., Kerns, S. A., Morrisett, J. D., Roberts, R. and Henry, P. (1990) Nature (London) **344**, 160–162
8 Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Puhl, H. and Tatzber, F. (1990) Biochem. Soc. Trans. **18**, 1059–1061
9 Reiss, D., Beyer, K. and Engelmann, B. (1997) Biochem. J. **323**, 807–814
10 Zoeller, R. A., Morand, O. H. and Raetz, C. R. H. (1988) J. Biol. Chem. **263**, 11590–11596
11 Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3883–3887
12 Jürgens, G., Ashy, A. and Esterbauer, H. (1990) Biochem. J. **265**, 605–608
13 Salonen, J. T., Salonen, R., Seppanen, K., Kantola, M., Suntioinen, S. and Korpela, H. (1991) Br. Med. J. **302**, 756–760
14 Holvoet, P., Theilmeier, G., Shivalkar, B., Flameng, W. and Collen, D. (1998) Arterioscler. Thromb. Vasc. Biol. **18**, 415–422
15 Hörkkö, S., Bird, D. A., Miller, E., Itabe, H., Leitinger, N., Subbanagounder, G., Berliner, J. A., Friedman, P., Dennis, E. A., Curtiss, L. K. et al. (1999) J. Clin. Invest. **103**, 117–128
16 Lehtimaki, T., Lehtinen, S., Solakivi, T., Nikkila, M., Jaakola, O., Jokela, H., Ylä-Herttuala, S., Luoma, J. S., Koivula, T. and Nikkari, T. (1999) Arterioscler. Thromb. Vasc. Biol. **19**, 23–27
17 Heinecke, J. W. (1998) Atherosclerosis **141**, 1–15
18 Engelmann, B., Bräutigam, C. and Thiery, J. (1994) Biochem. Biophys. Res. Commun. **204**, 1235–1242
19 Jürgens, G., Fell, A., Kledinski, G., Chen, Q. and Paltauf, F. (1995) Chem. Phys. Lipids **77**, 25–31
20 Bräutigam, C., Engelmann., B., Reiss, D., Reinhardt, U., Thiery, J., Richter, W. O. and Brosche, T. (1996) Atherosclerosis **119**, 77–88
21 Broekman, M. J., Handin, R. J., Derksen, A. and Cohen, P. (1976) Blood **47**, 963–971
22 Calzada, C., Bruckdorfer, K. R. and Rice-Evans, C. A. (1997) Atherosclerosis **128**, 97–105
23 Dacremont, G. and Vincent, G. (1995) J. Inherit. Metab. Dis. **18** (suppl. 1), 84–89
24 Vatassery, G. T. (1994) Methods Enzymol. **234**, 327–331
25 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 912–917
26 Havel, R. J., Eder, H. A. and Bragdon, J. H. (1955) J. Clin. Invest. **34**, 1345–1353
27 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
28 Lynch, S. M. and Frei, B. (1995) J. Biol. Chem. **270**, 5158–5163
29 Carey, E. M. (1982) Lipids **17**, 656–661
30 Meyer, D. F., Nealis, A. S., Macphee, C. H., Groot, P. H., Suckling, K. E., Bruckdorfer, K. R. and Perkins, S. J. (1996) Biochem. J. **319**, 217–227
31 Goyal, J., Wang, K., Liu, M. and Subbaiah, P. V. (1997) J. Biol. Chem. **272**, 16231–16239
32 Kontush, A., Meyer, S., Finckh, B., Kohlschütter, A. and Beisiegel, U. (1996) J. Biol. Chem. **271**, 11106–11112
33 Ziouzenkova, O., Gieseg, S. P., Ramos, P. and Esterbauer, H. (1996) Lipids **31**, S71–S76
34 Zommara, M., Tachibana, N., Mitsui, K., Nakatani, N., Sakono, M., Ikeda, J. and Imaizumi, K. (1995) Free Radicals Biol. Med. **18**, 599–602
35 Kuzuya, M., Yamada, K., Hayashi, T., Funaki, C., Naito, M., Asai, K. and Kuzuya, F. (1992) Biochim. Biophys. Acta **1123**, 334–341
36 Wagner, P. and Heinecke, J. W. (1997) Arterioscler. Thromb. Vasc. Biol. **17**, 3338–3346
37 Maiorino, M., Zamburlini, A., Roveri, A. and Ursini, F. (1993) FEBS Lett. **330**, 174–176
38 Yoshida, Y., Tsuchiya, J. and Niki, E. (1994) Biochim. Biophys. Acta **1200**, 85–92
39 Patel, R. P., Svistunenko, D., Wilson, M. T. and Darley-Usmar, V. M. (1997) Biochem. J. **322**, 425–433

Received 4 December 1998/26 February 1999; accepted 22 March 1999