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Lipid Peroxidation in the Presence of Albumin, Inhibitory and Prooxidative Effects

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Oxidative modifications of LDL are involved in atherogenesis. Previously we have developed a simple assay to evaluate the susceptibility of lipids to copper-induced peroxidation in the relatively natural milieu of unfractionated serum in the presence of excess citrate. Based on our previous results we have proposed that the inducer of peroxidation in our optimized assay is a copper–citrate complex. Recent investigations indicate that under certain conditions a copper–albumin complex may induce peroxidation of ascorbate. Two different complexes may be formed in albumin-containing systems (e.g. serum) namely 1:1 and 2:1 copper–albumin complexes. The aim of the present work was to evaluate the possibility that at least one of these complexes may be responsible for the induction of peroxidation of lipids in lipidic systems containing copper and albumin, including our optimized assay. Towards this end, we have investigated the dependence of copper-induced peroxidation on the concentration of added albumin in lipidic systems in the absence and presence of citrate. In all the systems investigated in this study (PLPC liposomes, LDL, HDL and mixtures of HDL and LDL) we found that at low concentrations of free copper (e.g. in the presence of excess citrate) the 2:1 copper–albumin complex is redox-active and that this complex is the major contributor to the initiation of lipid peroxidation in these systems and in our optimized assay. The possible relevance of the induction of peroxidation *in vivo* by the latter complex has yet to be studied.

Keywords: Lipoproteins; Liposomes; Peroxidation; Kinetics; Albumin; Copper–albumin complexes

Abbreviations: LDL, low-density lipoprotein; AAPH, 2′ azobis (2-amidinopropane) hydrochloride; HDL, high-density lipoprotein; PLPC, palmitoyllinoleoylphosphatidylcholine; OD, optical density; QLS, quasi-elastic light scattering; TMP, tocopherol-mediated peroxidation; WD, Wilson's disease; CSF, cerebro spinal fluid; RBCs, red blood cells

INTRODUCTION

In view of the putative involvement of oxidative modifications of low density lipoprotein (LDL) in atherogenesis,[1–4] a number of assays have been developed to monitor the susceptibility of LDL to peroxidation *ex vivo*.[5,6] The common procedures for isolation of LDL for these assays involve long ultracentrifugation and a subsequent dialysis to remove added preservatives like EDTA.[7,8] These procedures may affect the studied lipoprotein.[9] Moreover, lipoprotein peroxidation *in vivo* is likely to occur in the interstitial fluid surrounding the arterial wall.[10,11] Since the composition of the interstitial fluid is similar to that of plasma, particularly with respect to its content of water-soluble antioxidants,[10] relatively simple assays were developed to assess lipid peroxidation in a more natural milieu, i.e. in plasma or serum.[12–15]

Our previously developed method of evaluation of the susceptibility of lipids to peroxidation in unfractionated serum is based on assaying lipid peroxidation in a citrate containing medium.[13,14] Citrate was introduced because in its absence the dependence of peroxidation on copper concentration is "paradoxical" in that increasing the concentration of copper beyond a certain level results in prolongation of the lag preceding peroxidation.[13] Our interpretation of this finding was that the water-soluble reductants present in serum and the chloride ions present in the diluted serum act synergistically

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to stabilize Cu^{+} and by that prolong the lag at high copper concentrations. For a copper concentration of 100 μM and citrate concentrations below 360 μM, LDL peroxidation was inhibited by citrate in a dose-dependent fashion, but increasing the citrate concentration to 720 μM had only a relatively slight effect on the kinetics of peroxidation.[13] Based on the fact that AAPH-induced peroxidation of lipoproteins was not affected by citrate and on the slight effect of increasing the citrate concentration from 360 to 720 μM, we proposed that the inducer of peroxidation in our optimized assay is a copper–citrate complex, whose concentration remains about 100 μM, independent of the citrate concentration.

This assumption is rather questionable because under the same conditions, increasing the citrate concentration from 360 to 720 μM inhibited the peroxidation of high density lipoprotein (HDL).[13] Furthermore, in our previous work we have not related to the possibility, observed for ascorbate peroxidation,[16–19] that in albumin-containing systems (e.g. serum) a copper–albumin complex may induce lipid peroxidation. Evidence for the formation of two copper–albumin complexes was given by Zgirski and Frieden.[20] Based on the published binding constants,[20] it can be concluded that both a 1:1 and a 2:1 copper–albumin complexes may exist in the range of concentrations studied in the present investigation. Both the stability[20–22] and the redox activity[16,18,19] of these complexes are different. As a consequence, the dependence of copper-induced peroxidation on the concentration of added albumin can be expected to be complex.

In the present study, we evaluated the above possibility by studying the effects of albumin on copper-induced peroxidation of PLPC liposomes, LDL, HDL and mixtures of HDL and LDL in the absence and presence of citrate. The major finding is that in the presence of citrate, albumin may promote lipid peroxidation in liposomes and lipoproteins and that in our optimized assay, peroxidation is probably induced by a copper–albumin complex.

MATERIALS AND METHODS

1-Palmitoyl-2-Linoleoyl-sn-Glycero-3-Phosphocholine (PLPC) was purchased from Avanti Polar-Lipids Incorporation (Alabaster, AL). $CuCl_2$, EDTA, NaCl, NaH_2PO_4 and Na_2HPO_4 were purchased from Merck (Darmstadt, Germany). Sodium citrate and human serum albumin were purchased from Sigma (St. Louis, MO).

Preparation of PLPC Liposomes for Kinetic Studies

PLPC liposomes were prepared as previously described.[23] Briefly, the chloroformic solution of PLPC was evaporated and lyophilized overnight to remove traces of chloroform. The resultant film was dispersed at room temperature in a saline solution (146 mM NaCl) containing 15 μM EDTA, to minimize the peroxidation during the preparative stages. The PLPC dispersion (3.75 mM) was mixed to homogeneity using a vortex-mixer. Liposomes were prepared by sonication (10–13 min) under nitrogen and ice cooling (Huang, 1969), using a Heat Systems Incorporation XL-2020 probe-sonicator.

The mean size of the liposomes was evaluated by quasi-elastic light scattering measurements (QLS) using ALV's high performance particle sizer, model ALV-NIBS/HPPS, equipped with a HeNe-laser at 632.8 nm. The mean diameter of the PLPC liposomes varied between 40 and 80 nm.

After sonication, the liposomes underwent further dilution in PBS (pH 7.4, 146 mM NaCl, and 3.3 mM sodium phosphate) to final concentrations of 250 μM PLPC and 1 μM EDTA. The concentration of linoleate in liposomal PLPC studied in the present study and preceding paper,[24] were higher than those commonly studied in the investigation of LDL peroxidation so as to obtain OD values in the preferable spectrophotometric range under conditions of slow peroxidation. Liposomes were stored at 4°C until being used, typically within one week of their preparation.

Isolation of LDL and HDL for Kinetic Studies

Isolation of lipoproteins was initiated immediately after drawing of blood into EDTA containing tubes and was carried out at 4°C by sequential flotation of the lipoproteins in KBr solutions containing 1 mM sodium EDTA, at 40,000 rpm, using a 60 Ti rotor and a L7-80 Beckman ultracentrifuge. LDL was fractionated by 18 h centrifugation at a density of 1.019–1.050 g/ml. HDL was fractionated by subsequent 40 h centrifugation at 1.063–1.210 g/ml. The cholesterol content of the isolated lipoproteins was determined using the commercially available kit of Olympus System Reagent Cholesterol (Olympus Diagnostica, Germany). Prior to oxidation studies, both lipoprotein fractions were dialyzed overnight at 4°C in the dark against 2000 volumes of PBS containing 146 mM NaCl. EDTA was included in the dialysis medium such that after dilution to the desired lipid concentration the medium contained 1 μM EDTA, to prevent oxidation by metal ions contaminating the buffer.[8] Prior to being diluted for oxidation studies, the LDL and HDL samples were stored at 4°C for up to 6 days.

Monitoring the Peroxidation Kinetics

Oxidation of PLPC liposomes and lipoproteins was monitored at 37°C by continuous recording of

the absorbance at 4 wavelengths, using a Kontron (Uvikon 933) double-beam spectrophotometer equipped with a 12 position automated sample changer. Measurements were carried out in quartz cuvettes containing PBS solution (final volume of 1.5 ml, optical pathway 1 cm).

In many experiments, the reaction mixtures contained 100 μM copper and 720 μM citrate, as in our optimized assay of peroxidation of serum lipids.[13] In other experiments we have varied the concentration of copper within the range of 2–100 μM and the concentration of citrate within the range of 130–1440 μM, and the citrate/copper ratio varied within the range of 7.2–40. Albumin-containing mixtures contained 1–24 μM albumin. Different concentrations of citrate and/or albumin were added from aqueous solutions to the diluted solution of either liposomes or lipoproteins. A freshly prepared solution of $CuCl_2$ was added to each of the reaction mixtures and subsequently mixed with a Pasteur pipette prior to being monitored for peroxidation. Peroxidation of lipoproteins was monitored for approximately 5 h with an interval of 3 min between measurements. Peroxidation of PLPC liposomes was monitored for 18 h, with 11 min intervals between measurements.

The initial optical density (OD) values were recorded immediately after the addition of $CuCl_2$. The presented time-dependencies of absorbance were corrected by subtracting the initial OD from the OD recorded at later time points.

The peroxidation kinetics was monitored at multiple wavelengths. Most of the data presented in this paper are of OD measurements obtained at 245 nm. Only at albumin concentrations higher than 18 μM, when the OD values at 245 nm were beyond the sensitivity of the spectrophotometer, the presented data are those obtained at 268 nm. In those experiments in which the data were obtained at both wavelengths, the kinetic profiles were very similar, as have been shown previously.[13] The lag preceding peroxidation was derived from the kinetic profiles of peroxidation as previously described.[13,25]

Given the critical dependence of oxidation on the concentration of pre-formed hydroperoxides in the liposomes (and, therefore, on the individual preparation and its freshness),[24] each kinetic profile was compared with control experiments conducted with identical liposomal dispersions within the same batch.

Each of the time-dependencies of absorbance given in the present study is a representative of at least three kinetic measurements. Data analysis was performed by the standard procedures provided by Microsoft EXCEL XP and MICROCAL ORIGIN 7.0 software.

RESULTS AND DISCUSSION

The Effect of Citrate on Copper-induced Peroxidation of PLPC Liposomes

In our previous study,[24] the rate of copper-induced peroxidation in the absence of citrate increased upon increasing the copper concentration up to a saturating level of about 5 μM copper. The close similarity between the kinetic profiles of PLPC peroxidation induced by 5 and 10 μM copper (Fig. 1) is consistent with these results.

Citrate decreased the rate of peroxidation of PLPC in a dose-dependent fashion (e.g. Fig. 1 for 30 μM copper). As an example, in the presence of 720 μM citrate, even a very high concentration of copper (e.g. 30 μM), induced a very slow peroxidation (Fig. 1), similar to the results reported previously for the fractionated lipoproteins LDL and HDL.[13]

In our previous study, we have proposed that the relatively slow peroxidation obtained in the presence of excess citrate is induced by a copper–citrate complex.[13] Theoretically, this assumption implies that the rate of peroxidation should depend only on the concentration of copper–citrate. Given the high binding constant of copper to citrate ($pK_D = 5.9$),[26]

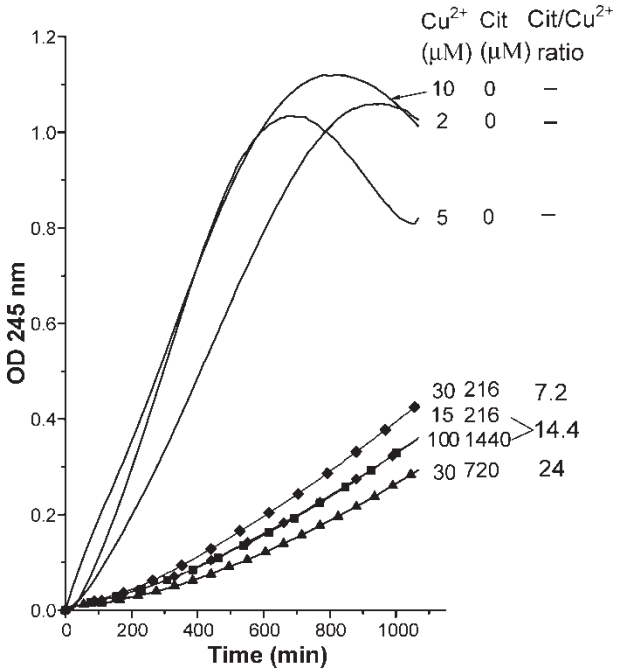

FIGURE 1 Time courses of the accumulation of peroxidation products during Cu^{2+}-induced peroxidation of liposomal PLPC (250 μM) in the presence or absence of citrate. The numbers alongside each graph indicate the concentrations of Cu^{2+} and citrate and the citrate/Cu^{2+} ratio for the specific kinetic profile. Formation of peroxidation products was monitored at 245 nm, at 37°C in the presence of 1 μM EDTA, so that the actual copper concentration is lower than its total concentration by 1 μM. Different symbols are used for the kinetics observed under different conditions in the presence of citrate (as given alongside each graph). The solid lines depict the kinetic profiles observed in the absence of citrate.

under conditions of a large excess of citrate, the concentration of copper–citrate is about equal to the total copper concentration. This means that for high citrate/copper ratios the rate of peroxidation by a copper–citrate complex should depend only on the total copper concentration, independent of the citrate/copper ratio. By contrast, peroxidation induced by residual free copper can be expected to depend on free copper concentration, which, in turn, can be shown to depend exclusively on the citrate/copper ratio. Specifically, in the latter case the rate of peroxidation should decrease upon increasing this ratio. Based on this analysis, the results described in Fig. 1 indicate that in the presence of excess citrate, peroxidation is induced by the residual free copper. Notably, in the absence of both EDTA and citrate, the lipid peroxidation of liposomal PLPC induced by 50 nM copper[24] resembled the slow peroxidation induced by 15–100 μM copper in the present study, in the presence of excess citrate (Fig. 1).

Specifically, comparison of the kinetic profiles observed for a constant copper concentration of 30 μM reveals that increasing the citrate concentration from 216 to 720 μM, i.e. increasing the citrate/copper ratio from 7.2 to 24, inhibited the peroxidation (Fig. 1), although the concentration of the copper–citrate complex is expected to be about 30 μM in both cases. In the context of the above considerations, this indicates that peroxidation is probably induced by free copper, whose concentration is very low in both cases, but considerably higher at 216 μM citrate than at 720 μM citrate. This conclusion is supported by two observations. First, in two mixtures in which the citrate/copper ratio was 14.4, the rate of peroxidation was slower than that observed at a citrate/copper ratio of 7.2, but faster than that observed when the latter ratio was equal to 24 (Fig. 1). Moreover, the two kinetic profiles observed at a citrate/copper ratio of 14.4 appeared identical (Fig. 1), in spite of the 6.7 fold difference in the total concentrations of both copper and citrate.

Hence, it can be concluded that in mixtures containing excess citrate, the rate of peroxidation is a function of the citrate/copper ratio rather than a function of the total concentration of copper. In view of our theoretical considerations, we can conclude that peroxidation in these systems is indeed induced by free copper.

The Effect of Albumin on Copper-induced Peroxidation in the Absence and Presence of Citrate

PLPC Liposomes

In the absence of citrate, albumin inhibits peroxidation. The magnitude of this effect depends on the copper/albumin ratio (Fig. 2). At copper concentrations lower than the concentration of albumin, e.g. in a mixture of 8 μM copper and 12 μM albumin (Fig. 2A), peroxidation was very slow. This observation accords with the paradigm that a 1:1 copper–albumin complex is a very weak inducer of peroxidation, if at all.[27] Addition of albumin (12 μM) to a system containing a higher concentration of copper (18 μM), also reduced the rate of peroxidation (Fig. 2B), although in the presence of albumin peroxidation at 18 μM copper (Fig. 2B) was much faster than at 8 μM copper (Fig. 2A). The acceleration of peroxidation upon increasing the copper concentration, in the presence of albumin (Fig. 2A,B), may of course be a result of an increase in the free copper concentration. Nonetheless, this acceleration may also be due to the effect of a relatively redox-active copper–albumin complex formed upon binding of a second copper ion to albumin. This possibility is consistent with the previous findings of Marx and Chevion,[19] Gryzunov *et al.*,[16] Lozinsky *et al.*,[18] and Lovstad,[17] that this complex can initiate oxidation of ascorbate in solutions. Our results (see below) are consistent

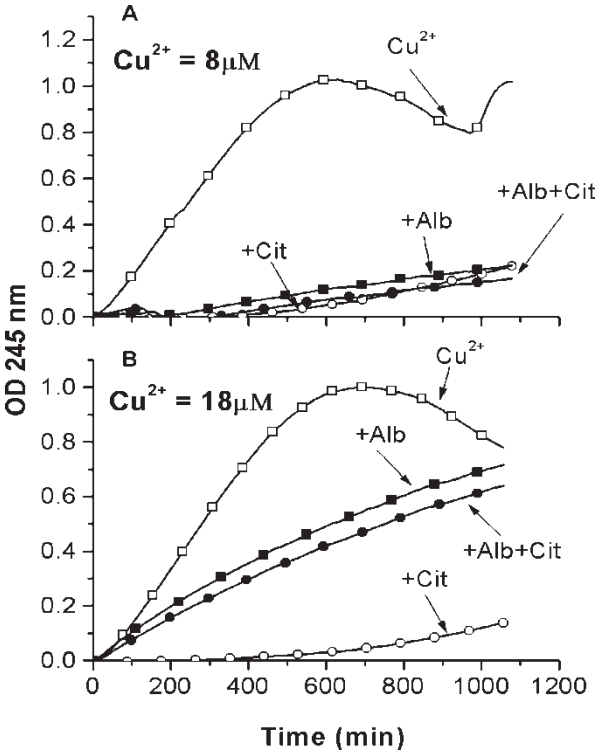

FIGURE 2 Time courses of the accumulation of peroxidation products during Cu^{2+}-induced peroxidation of liposomal PLPC (250 μM). Peroxidation was induced either by Cu^{2+} in the absence of citrate (squares) or by Cu^{2+} in the presence of citrate (720 μM, circles). The empty symbols represent mixtures containing no albumin and the filled symbols represent mixtures containing albumin (12 μM). The [Cu^{2+}]/[albumin] ratio was lower than 1 (0.67) in the experiments given in panel A, but higher than 1 (1.5) in the experiments given in panel B. Peroxidation was monitored at 245 nm, at 37°C in the presence of 1 μM EDTA.

with the hypothesis that the copper–albumin (2:1) complex is also capable of initiating peroxidation in lipidic systems.

The results obtained in the presence of 720 μM citrate support this conclusion (Fig. 2). Specifically, the addition of 12 μM albumin to 8 μM copper ($[Cu^{2+}] < [albumin]$) did not affect the peroxidation kinetics and the peroxidation remained very slow both in the absence and presence of citrate (Fig. 2A). By contrast, addition of 12 μM albumin to 18 μM copper ($[Cu^{2+}] > [albumin]$), inhibited peroxidation in the absence of citrate but promoted the peroxidation in the presence of citrate (Fig. 2B). Experiments conducted at different citrate/copper ratios within the range of 7.2–40 (results not shown) indicate that the latter effect is almost independent of the citrate to copper ratio. Binding of copper to added albumin in systems containing excessive citrate may only decrease the concentrations of both the free copper and the copper–citrate complex. Thus, the acceleration of peroxidation observed upon addition of albumin must be due to initiation of peroxidation by a relatively redox-active copper–albumin complex.

The assumption that peroxidation is induced by a 2:1 copper–albumin complex, whereas the 1:1 complex and the copper–citrate complex are relatively redox-inactive is supported by the dependencies of lipid peroxidation on the concentrations of copper and albumin in systems containing 720 μM citrate, as depicted in Fig. 3. In the latter figure, the square values of OD as observed after 18 h of continuous monitoring, are presented as functions of the concentrations of albumin (panel A), copper (panel B) and a 1.5:1 mixture of copper and albumin (panel C). Given the similar kinetic profiles observed in the presence of excess citrate (e.g. Fig. 1), the OD, as measured at a given time, is indicative of the rate of peroxidation. The rate of initiation of peroxidation can be expected to depend linearly on the concentration of the inducer of peroxidation.[28] In turn, the rate of peroxidation can be expected to depend on the square root of the rate of initiation[28] and is therefore likely to depend on the square root of the concentration of the redox-active complex.[28] Based on these considerations, we have chosen to describe the concentration dependencies of Fig. 3 in terms of OD square rather than OD. Accordingly, the results given in Fig. 3 can be interpreted as follows:

(i) For a constant copper concentration, increasing the concentration of albumin may result in acceleration of peroxidation due to the increase in the concentration of the redox-active 2:1 complex on the expense of the relatively redox-inactive copper–citrate complex. However, at further elevated albumin concentrations, increasingly higher fractions of copper are likely to form (redox-inactive) 1:1 copper–albumin complexes and, as a consequence, the rate of peroxidation can be expected to decrease. The observed "bell-shaped" dependence described in Fig. 3A accords with the above hypothesis, thus supporting it.

(ii) For a constant albumin concentration, when the concentration of copper is lower than that of albumin, peroxidation is expected to be very slow because the copper–albumin complexes will have a 1:1 stoichiometry. Further increase of the copper concentration will result in formation of increasing concentrations of the relatively redox-active 2:1 complex, hence in acceleration of peroxidation. At yet higher copper concentrations, after all the binding sites of albumin are saturated, we can only expect a minor acceleration because most of the added copper will be bound to citrate present in the solution, to form the relatively redox-inactive copper–citrate complex. This expected complex behavior was in fact observed experimentally (Fig. 3B), which lends further support to the above hypothesis.

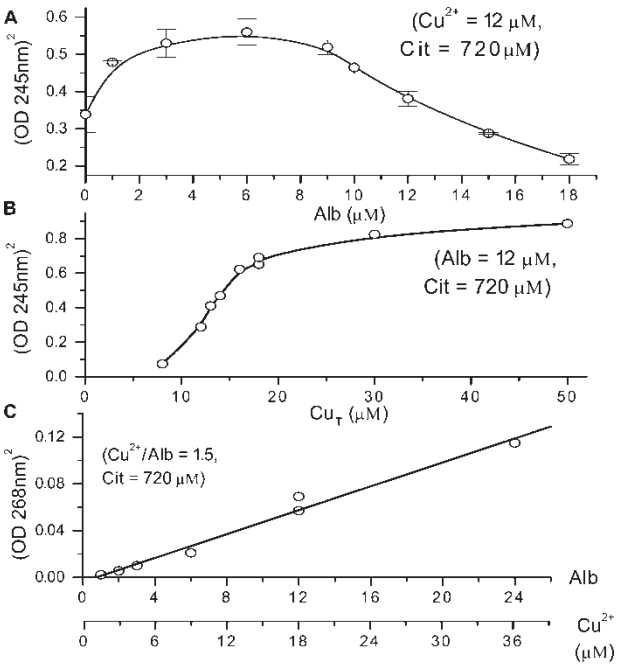

FIGURE 3 Dose-dependence of the kinetics of peroxidation of liposomal PLPC on the concentrations of albumin and copper in the presence of citrate (720 μM). Panel A depicts the dependence of peroxidation on albumin concentration (at a constant Cu^{2+} concentration of 12 μM). Panel B depicts the dependence on copper concentration (at a constant albumin concentration of 12 μM). Panel C depicts the effect of the concentrations of copper and albumin at a constant copper to albumin ratio of 1.5. All the kinetic experiments were conducted in the presence of 720 μM citrate and 1 μM EDTA, at 37°C. The square values of OD, as observed after 18 h of continuous monitoring, are described as functions of the concentration of albumin (panel A), copper (panel B) and a 1.5:1 copper:albumin mixture (panel C) (see text for details).

(iii) For a constant copper/albumin ratio, varying simultaneously the concentrations of both copper and albumin can be expected to result in a linear dependence of OD square on the concentration of the complex (i.e. of both copper and albumin). The observed linear dependence of OD square on the concentration of the complex (Fig. 3C) lends further support to the hypothesis that the 2:1 copper–albumin complex is indeed the inducer of peroxidation.

In conclusion, in the studied range of concentrations of copper, citrate and albumin, when the mixture contains no albumin, peroxidation of PLPC liposomes is induced by residual free copper, whereas in the presence of albumin, peroxidation is initiated by a 2:1 copper–albumin complex. Both the copper–citrate complex and the 1:1 copper–albumin complex contribute to the induction of peroxidation only slightly, if at all.

Fractionated Lipoproteins and their Mixtures

Similar to its effect on PLPC peroxidation, citrate (720 μM) prolongs the lag and decreases the maximal rate of copper-induced lipid peroxidation in LDL (Fig. 4A), HDL (Fig. 4B) and their mixtures (Fig. 4C). These results are similar to those obtained previously for the fractionated lipoproteins.[13] Notably, in the absence of albumin, the addition of citrate resulted in the appearance of a biphasic kinetic profile (Fig. 4), previously obtained in both lipoproteins and in their mixture and attributed to tocopherol-mediated peroxidation (TMP),[29] or to a protein-derived radical-mediated peroxidation.[30] Irrespective of the nature of the mediator, such behavior is typical for the conditions of low "oxidative stress", as induced under low copper:LDL ratio,[31] which in the present study might be either induced by copper–citrate or by residual, very low concentration of free copper. In view of our results in PLPC liposomes (see above), we think that free copper is the inducer of peroxidation rather than the copper–citrate complex.

The latter interpretation differs from that adopted in our previous work.[13] Therefore, we have reassessed the results of the previous study. Based on the data reported in that study on the rate of peroxidation of LDL and on its dependence on the concentration of citrate,[13] we evaluate that the dissociation constant of copper to LDL is approximately 0.5 μM. This value is quite close to the 1 μM estimate, based on LDL peroxidation in a citrate-free PBS buffered media.[28] It also accords with our present interpretation that free copper, rather than copper–citrate, is responsible for the lipoprotein peroxidation in the presence of excessive citrate.

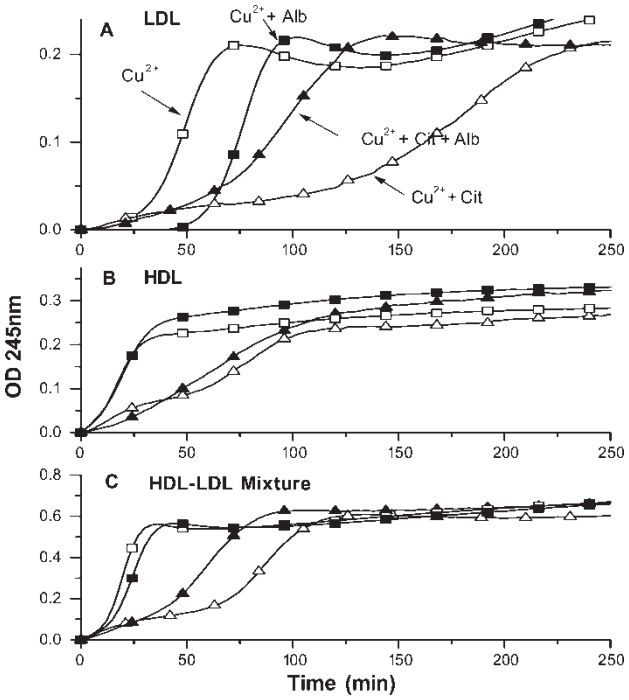

FIGURE 4 Time courses of the accumulation of peroxidation products during Cu^{2+}-induced lipoprotein peroxidation. Peroxidation was induced either by 10 μM Cu^{2+} in the absence of citrate (squares) or by 100 μM Cu^{2+} in the presence of 720 μM citrate (triangles). Panel A depicts the peroxidation of LDL (0.05 μM), panel B depicts the peroxidation of HDL (2.4 μM) and panel C depicts the peroxidation of a mixture of HDL (2.4 μM) and LDL (0.1 μM). The empty symbols represent mixtures containing no albumin. The filled symbols represent mixtures containing either albumin [1.5 μM; (squares)] or albumin and citrate [12 and 720 μM, respectively; (triangles)]. Peroxidation was monitored at 245 nm, at 37°C, in the presence of 1 μM EDTA.

The effect of albumin on copper-induced peroxidation of LDL resembles the effects described above for PLPC liposomes, both in the absence and presence of citrate. Specifically, in the absence of citrate, albumin prolongs the lag preceding peroxidation without affecting the maximal rate, whereas, in the presence of citrate, albumin shortens the lag and increases the maximal rate of peroxidation (Fig. 4A). Interestingly, in the absence of citrate, albumin affects only slightly the peroxidation of both HDL and mixtures of HDL and LDL (Fig. 4B,C). By contrast, in the presence of citrate, albumin affects the peroxidation of both HDL and mixtures of HDL and LDL, similar to the effects observed for LDL peroxidation under the same conditions, although in a less pronounced fashion (Fig. 4B,C).

In all the experiments presented in Fig. 4 the concentration of albumin was much lower than that of copper in order to evaluate the possible effects of albumin other than copper-binding. The effects of relatively high concentrations of albumin on copper-induced peroxidation of LDL, attributed mainly to copper-binding, were studied in our laboratory previously.[27] As shown in Fig. 4A, albumin in the absence of citrate prolongs the lag without affecting

the rate of peroxidation. Therefore, it may be concluded that its inhibitory effect is indeed not merely due to copper binding.[32] This conclusion is further supported by the fact that in the absence of citrate, the albumin concentration was 1.5 μM, and since the upper limit of copper binding to albumin is two ions per one albumin molecule,[20] it follows that only 3 μM (out of the 9 μM) can be bound to albumin, so that the concentration of free copper is at least 6 μM. This is a saturating concentration of copper for peroxidation of 0.05 μM LDL.[28] Hence, the anti-oxidative effect of albumin can not be merely due to copper binding, and must therefore involve other mechanism(s). One possibility is that albumin reduces the rate of inter-particle migration of hydroperoxides,[27] and by that inhibits peroxidation. For the given concentrations of copper and lipoproteins, the ratio of bound copper to oxidizable lipids is higher in HDL than in LDL,[33] which results in higher susceptibility of the HDL lipids to copper-induced peroxidation. As a possible consequence, peroxidation of HDL might be less dependent on inter-particle migration of hydroperoxides than peroxidation of LDL. This may explain the larger effect of albumin on LDL than on HDL peroxidation (Fig. 4, panels A and B, respectively).

In the presence of citrate, the addition of albumin to LDL, HDL or their mixtures shortens the lag preceding peroxidation, increases the maximal rate of peroxidation and abolishes the apparent TMP (Fig. 4). These effects are qualitatively similar to those observed for PLPC liposomes and are more pronounced for LDL than for either HDL or mixtures of HDL and LDL (Fig. 4).

In relating to the results obtained in media containing excess citrate, it is important to note that addition of albumin to lipoproteins, in copper-containing media results in complexation of part of the copper by albumin, thus reducing the concentrations of both residual free copper and copper–citrate. The prooxidative effect of albumin (Fig. 4) must therefore mean that a copper–albumin complex is the major initiator of lipoprotein peroxidation in the presence of citrate.

In conclusion, the present work demonstrates that similar to its role in the oxidation of ascorbate in aqueous solutions,[16,17,19] a 2:1 copper–albumin complex may also induce peroxidation of lipids in liposomes and lipoproteins. This inducer is less potent than free copper, at similar concentration but in systems containing low concentrations of free copper (e.g. in the presence of excess citrate), this complex may be the major contributor to the initiation of lipid peroxidation. Accordingly, this complex is likely to be the major initiator of peroxidation in our optimized assay of lipid peroxidation in unfractionated serum. This assay was based on the use of a high concentration of citrate in the medium to avoid its complication by the paradoxical dependence of peroxidation on the copper concentration, which occurs in the absence of citrate. Our modified interpretation regarding the inducer of peroxidation does not undermine our previous explanation of the paradoxical effect on the basis of the Cu^{2+}/Cu^{+} equilibrium, which, we think, remains valid.[13]

The possible involvement of a 2:1 copper–albumin complex in lipoprotein peroxidation *in vivo* is questionable because in biological media, in general, the concentration of copper ions is lower than the concentration of albumin. However, the latter complex might be relevant in pathological disorders involving metal ions overload. Specifically, Wilson's disease (WD) is characterized by defective biliary excretion of copper and impairment of its incorporation into ceruloplasmin. These defects result in accumulation of copper in the liver causing progressive liver damage,[35] which may result in leakage of free copper from the damaged hepatocytes. The free copper is "loosely" bound to albumin and amino acids in the circulation.[36] A possible result of such leakage is that serum lipoproteins of WD patients may be exposed to increased oxidative stress. However, the latter possibility is in apparent contradiction with the results of several studies. First, a rigorous examination of the concentrations of copper and albumin in the circulation of WD patients[36] reveals that the formation of a 2:1 copper–albumin complex is not very likely. Moreover, copper bound to albumin is taken up by red blood cells (RBCs), so that its concentration in the serum is lower than in the blood,[37] which reduces the possibility of induction of peroxidation in the serum by a copper–albumin complex. This is also consistent with the findings that both the serum TBARS level and the LDL susceptibility to peroxidation *ex vivo* in WD patients are similar to those observed in normal individuals.[36,38]

In spite of these considerations, a 2:1 copper–albumin complex might play a role in other body fluids of WD patients. This speculation is based on the available data on the copper concentration in the cerebro spinal fluid (CSF) of WD patients (1.1–1.5 μM)[39] and on our estimate of the concentration of albumin in the CSF of these patients, based on the following considerations: (i) the concentration of albumin in the serum of (the hypoalbuminemic) WD patients is approximately 25 g/l, i.e. about 350 μM,[40] and (ii) the concentration of albumin in the CSF of WD patients is merely 0.2–0.5% of its concentration in the serum,[41] namely, 0.7–1.8 μM (compared to normal values of 1.2–6 μM).[42] Hence, in extreme cases, the copper–albumin ratio may be higher than 2, so that a 2:1 copper–albumin complex may be formed in the CSF milieu of WD patients and initiate peroxidation.

We also considered the possibility that other polyvalent metal ions abundant in biological fluids (e.g. iron, zinc, magnesium and calcium) compete with copper for the first, tight, "redox-inactive" site of albumin and by that enhance copper binding to a second, "redox-active" site. This results in the formation of a "redox-active" copper–albumin complex, even when the total copper/albumin ratio is lower than one. The latter possibility is not likely in view of our experiments (not shown), in which neither Fe^{2+} nor Fe^{3+} affected the kinetics of copper-induced peroxidation in the presence of equimolar albumin. Yet, evaluation of the patho-physiological significance of peroxidation induced by a 2:1 copper–albumin complex in disorders associated with metal-ion overload requires further studies.

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