Free Rad. Res. Comms., Vol. 16, No. 4, pp. 239–246
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SCAVENGING OF LIPID PEROXIDATION PRODUCTS FROM OXIDIZING LDL BY ALBUMIN ALTERS THE PLASMA HALF-LIFE OF A FRACTION OF OXIDIZED LDL PARTICLES

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(Received September 25, 1991; in revised form February 3, 1992)

We analyse LDL oxidation *in vitro* in the presence of copper (II) ions and differentiate a lag phase and a rapid peroxidation phase. We demonstrate that a physiological concentration of albumin does not alter the kinetics of the dienes in the oxidizing LDL but reduces the fluorescence of the oxidizing LDL and alters the biological properties of oxidized LDL. We find in rats after intravenous administration of oxidized LDL, that it is rapidly cleared from the circulating blood. The presence of albumin during the peroxidation phase, however, reduces the fraction of oxidized LDL with rapid blood clearance. We propose that some lipid peroxidation products formed in oxidizing LDL are hydrophilic enough to diffuse into the aqueous buffer from where they react either with the ε-amino-groups of apolipoprotein B or albumin. Effective scavengers for these hydrophilic endproducts of the LDL oxidation pathways such as albumin might reduce modification of the LDL and might be useful to reduce its atherogenicity.

KEY WORDS: Low-density lipoprotein, lipid peroxidation, albumin, LDL oxidation.

ABBREVIATIONS: LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; PUFA, polyunsaturated fatty acid; TDS, tyramin-1-deoxy-sorbitol.

INTRODUCTION

The "modified LDL hypothesis" of atherogenesis suggests that the LDL is oxidized in a developing atherosclerotic lesion and that oxidized LDL is metabolized by monocytes/macrophages in the intima at increased rates causing their conversion to cholesteryl ester loaded "foam cells", the characteristic cell type of the early lesion.[1,2] Strong support for this hypothesis was obtained by *in vitro* studies with oxidized LDL and LDL-like material isolated from the arterial lesion and by antioxidant therapy in rabbits. The *in vitro* studies indicated that macrophages increase dramatically their cholesteryl ester content upon incubation with the lipoproteins.[3] In an animal model it was shown that antioxidant treatment with probucol reduces the LDL modification

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and LDL degradation in the lesions and that probucol attenuates the progression of atherosclerotic lesions in rabbits with familial hypercholesterolemia.[4]

In theory antioxidant therapy is not the only possibility to protect oxidizing LDL from modification. At least some intermediates of the LDL-PUFA oxidation pathway and aldehydic products are released from the oxidized LDL.[5] These products may react not only with apolipoprotein B of the LDL particle but also with reactive groups on other proteins or low molecular weight reactants. In this paper we ask therefore two questions:

(1) Do major plasma proteins like albumin influence the LDL modification process and do they react with LDL-derived lipid peroxidation products?

(2) If attenuation of LDL modification by albumin occurs, does it alter the biological fate of modified LDL?

We studied the influence of albumin on LDL modification *in vitro* in a simple assay. LDL is incubated with copper (II) ions as a prooxidant and A_{234} and Ex 340/EM 420 are measured to analyze the kinetics of the dienes and fluorescent oxidation products.[6,7] To study how their modification affects the biological fate of LDL particles we analyzed their plasma clearance in rats.

METHODS

Preparation and Characterization of Lipoprotein and Radioligands

Human LDL was prepared from the plasma of four male healthy blood donors (non-smokers) by sequential ultracentrifugation in NaBr solution and 0.01% EDTA.[8] The isolated LDL was stored in the presence of EDTA under nitrogen for up to 5 days before use. LDL was dialyzed before oxidation at 4°C for 24 h against 4 × 4150 mM Tris/HCl (pH 8.0)/150 mM NaCl (TN buffer) under nitrogen. The concentration of LDL was expressed in terms of protein content per ml solution and determined according to Lowry *et al.*[9] The integrity of the apolipoprotein B and the purity of the LDL were checked by SDS/polyacrylamide slab-gel electrophoresis.[10] When 2 μg protein were applied per lane apo B moved as a single band. No lp(a) was detected by silver staining with the BioRad silver staining kit.

Radioiodination of LDL was performed with $Na^{131}I$ tyramine-1-deoxysorbitol (TDS) and N-bromosuccinimide (NBS) as oxidant (1 μg NBS/1 mCi ^{131}I) and analyzed by thin layer chromatography as described.[11,12] The specific activity of ^{131}I-TDS-LDL was 3100 c.p.m./ng; more than 97% of the label was bound to the protein. The TDS label was used in these studies, because ^{131}I TDS is trapped in cells. It allows one to determine the clearance rate of ^{131}I I-TDS-LDL directly. Native LDL and ^{131}I-TDS-LDL (125 μg/ml or 0.25 μM) were oxidized in 2 ml 20 mM Tris/HCl (pH 8.0)/150 mM NaCl in a cuvette with 10 μM copper (II) as prooxidant at 42°C. We use Tris/HCl as buffer, because this buffer enables us to achieve a final concentration of all redox metals as high as 10 μM; the overall formation of dienes, fluorescent lipid peroxidation products, and thiobarbituric acid reactive substances during LDL oxidation is not different from the values obtained in phosphate buffer and F10 medium. We increased the incubation temperature from 37°C in our previous publication to 42°C.[7] This upshift of the temperature shortens the lag phase by 12 min but decreases the variability between LDL isolated from different donors. The formation of dienes at A_{234} and the appearance of fluorescence at Ex 340/Em 420 was recorded to measure the so-called

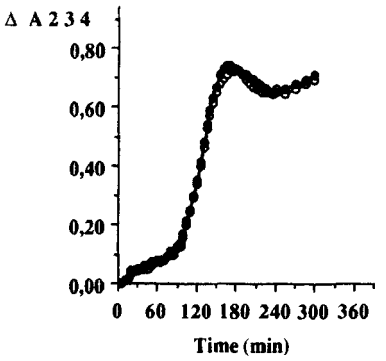

FIGURE 1 Kinetics of dienes (A_{234}) in oxidizing LDL in the absence and presence of albumin. 0.25 μM LDL with or with 3 μM rat serum albumin was incubated in Tris/HCl + 10 μM $CuCl_2$ at 42°C and room atmosphere: ○, A_{234} in absence of albumin; ●, A_{234} in presence of albumin.

"lag phase" (defined as the interval between the intercept of the tangent of the slope of the curve with the time-scale axis), and the maximal rate of A_{234} formation (calculated from the slope of the absorbance and fluorescence curve during the propagation reaction). To test for the albumin effect 3 μM albumin was added before the propagation phase. To stop the oxidative modification of LDL the samples were chilled on ice and EDTA as a chelator for the redox metal was added to a final concentration of 20 mM. The LDL was analyzed three times within 10 days. There was no evidence for a shortening of the lag phase over the 10 days of storage.

A TSK gel G 4000 SW preformed column 300 mm in length, with a diameter of 7.5 mm from LKB (S-16126 Bromma, Sweden cat. no. 2135-430) was used to separate albumin from oxidizing LDL. 200 μl samples containing 25 μg protein applied to the column were in 20 mM Tris/HCl, pH 8.3, 150 mN NaCl, 2 mM EDTA. Chromatography was performed at a flow rate of 0.8 ml/min with constant pressure.

In vivo *Studies*

Male-Sprague–Dawley rats (200–250 g) were anesthetized with ether and injected via a tail vein with ^{131}I-tyramin-1-deoxysorbitol (TDS)-LDL at a dose of 16 μCi (3100 c.p.m./ng). Blood samples were obtained through the tail vein. All data were plotted as a percentage of the total injected dose that remained in the plasma at each time interval.

RESULTS

LDL was oxidized in the presence or absence of albumin. The effect of albumin on the LDL oxidation kinetics was analyzed by following the dienes and the fluorochromic lipid-protein conjugates. 0.25 μM LDL (125 μg/ml) was oxidized at 42°C in the presence or absence of 3 μM albumin (200 μg/ml) in buffer containing 10 μM Cu^{2+}. These concentrations of albumin and LDL were chosen because they might occur under physiological conditions in body fluids.[13,14]

Figure 1 shows a representative A_{234} vs. time profile in the LDL oxidation assays. The lag phase, defined by the slow increase of dienes was 95 min and identical in the

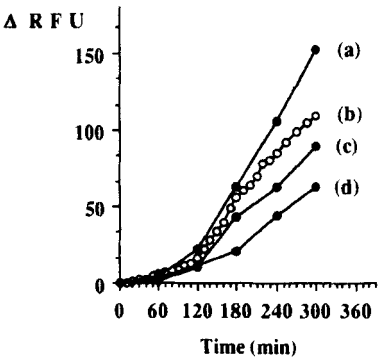

FIGURE 2 Kinetics of the formation of fluorochromic lipid protein conjugates on LDL and albumin. To separate oxidizing LDL and albumin, aliquots in the assay described under Figure 1 were subjected to gel permeation chromatography on a TSK gel G 4000 column equilibrated in 50 mM Tris/HCl pH 8.3, 2 mM EDTA, 20 mM NaCl at a flow rate of 0.8 ml/min. A_{234} nm and fluorescence with excitation at 340 nm and emission at 420 nm (Ex 340/Em 420) were monitored continuously: (a) unfractionated sample; (b) oxidizing LDL in absence of albumin; (c) oxidizing LDL in presence of albumin; (d) albumin separated from oxidizing LDL.

absence and presence of albumin. It is apparent that albumin does not influence the formation of dienes. Figure 2 shows the time course of the fluorescence (Ex 340/Em 420) in the same assays. Under both conditions increase of the fluorochromes was detected after 120 min. dEx 340/Em 420 was 140 units in the LDL (125 μg/ml) + albumin (200 μg/ml) mixture and 104 units in LDL (125 μg/ml) in absence of albumin. Compared to the control assay without albumin the presence of 200 μg albumin in the LDL oxidation assay increased the Δ Ex 340/EM 420 1.35-fold indicating formation of additional fluorochromic lipid-protein conjugates.

To examine the distribution of the fluorescent lipid-protein conjugates between albumin and LDL, the assay mixtures were subjected to h.p.l.c. gel chromatography on a TSK G 4000 column. Figure 2 shows the kinetics of the appearance of the fluorochromes on separated LDL and albumin as observed in 60 min intervals over 360 min. When LDL was oxidized in presence of albumin the production of the LDL fluorochromes was slower between 180 and 300 min resulting in a 18% reduction after 300 min. Concomitant with the reduction of the fluorochromes in the LDL there was an increase of the fluorochromes in the albumin. When albumin alone was incubated, no increase of the fluorescence was seen (data not shown). To obtain the maximal albumin effect on LDL fluorescence, albumin has to be present through the propagation phase. However, the albumin effect is not influenced when albumin was added at the beginning of the incubation or after 60 min at the end of the lag phase (data not shown).

To characterize the fluorescence of oxidized LDL and albumin isolated from the oxidation assay their emission spectra were compared. Both the oxidized LDL particles and the albumin formed characteristic fluorochromes with emission maxima between 410 and 430 nm when excitation was at 340 nm (Figure 3A, B). It is believed that these fluorochromes are Schiff bases.[5] We conclude that albumin was modified by LDL oxidation products and was developing fluorochromic lipid-protein conjugates similar to those of the oxidized LDL.

The effect of the protection of LDL by albumin in the oxidation assay on the

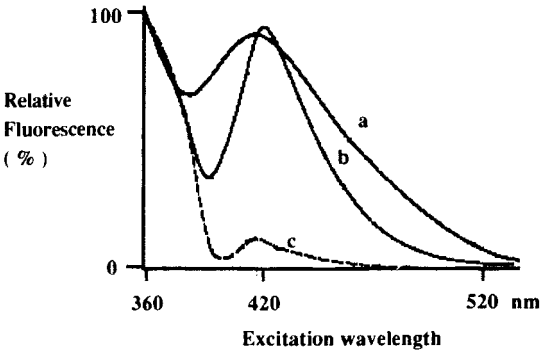

FIGURE 3 Emission fluorescence spectrum of oxidized LDL and albumin modified by oxidizing LDL. LDL and albumin were separated, after the oxidation, by h.p.l.c. as described in the legend of Figure 2. Emission spectra were measured at their corresponding excitation maximum at 340 nm. Fluorescence spectra: (a) oxidized LDL, (b) albumin modified by oxidizing LDL, (c) native albumin as a control.

biological behaviour of oxidized LDL was determined in rats. Because oxidation of LDL affects its clearance from the circulating blood into rats,[15] we conducted studies to compare the clearance rates of native LDL and oxidized LDLs in rats, with oxidized LDL obtained either in the absence or presence of albumin. We reasoned that the clearance rate of the modified LDLs can serve as an approximation of the magnitude of the LDL modification and therefore help to probe for LDL modification. In these experiments we used ^{131}I TDS-LDL and oxidized ^{131}I TDS-LDLs; albumin was added to oxidizing LDL 60 min after the beginning of the incubation at the end of the lag phase (albumin reduced the fluorescence in the oxidized ^{131}I TDS-LDLs but not the dienes to a similar extent as described in Figure 2). The disappearance of radioactivity from the blood after injection of the radioiodinated LDLs is given in Figure 4. Compared with native LDL ($t_{1/2}$ = 390 min) the oxidized LDLs were cleared extremely rapidly. More than 65% of the oxidized LDL obtained in the absence of albumin was cleared from the plasma of the rats within four min., but only 48% of the LDL oxidized in presence of albumin. This difference was significant and continued. After 30 min a mean of 30.6% vs. 19.8% of the injected radioactivity remained in the circulating blood of the rats. Since the administered amount of oxidized LDL was far too low to saturate the removal capacity of the liver, the rather high residual radioactivity in the blood after 30 min indicates that a fraction of the injected LDL is not behaving like "oxidized" LDL. This fraction remaining in the circulation after 30 min is apparently cleared at a much slower rate. It has a similar clearance rate as native LDL. Oxidized LDLs obtained in the presence of albumin must be treated *in vivo* as a heterogenous entity at least at the analyzed time point. It is obvious that differences in the fluorescence and turnover *in vivo* but not in the kinetics of the dienes of oxidizing LDL occur when LDL is oxidized in the presence of albumin.

DISCUSSION

In contrast to the formation of dienes the appearance of LDL fluorochromes of oxidized LDL could be reduced by albumin which was added at the end of the lag

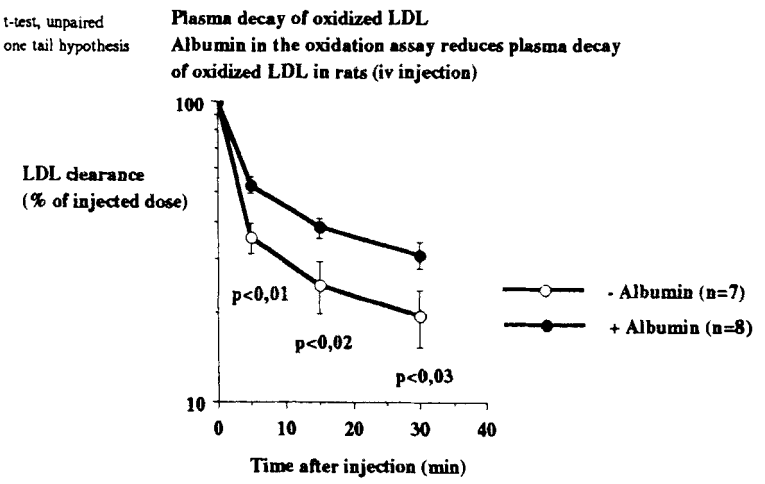

FIGURE 4 Percent of the total injected dose of oxidized human ^{131}I-TDS-LDL obtained in the absence (○) or presence of albumin (●) and that remaining in the plasma as a function of time after intravenous injection into rats. The mean ± SEM represents values obtained in the rats by isotope counting at each time point. Each rat received 16 μCi of oxidized LDL (125 μg/ml); 3200 cpm/ng) which was oxidized in the presence of 3 μM albumin or absence of albumin.

phase to an *in vitro* LDL oxidation system with Cu(II) ions as prooxidant. In a clearance study with oxidized LDL in rats we observed that the percentage of oxidized LDL with a rapid blood clearance after intravenous administration can be diminished by addition of albumin at the end of the lag phase. Thus we demonstrate a biological effect of albumin on LDL oxidation.

Albumin appeared to decrease the formation of fluorochromes but not the diene kinetics of oxidizing LDL. We propose that albumin is binding lipid peroxidation products, released from oxidizing LDL into the aqueous buffer, that would otherwise react with the LDL itself. This impression is based on studies showing that albumin treatment of oxidizing LDL incubated for 60 min with Cu(II) as prooxidant decreases the formation of fluorochromes on oxidized LDL. Since at the same time the fluorochromes on albumin increased in a LDL-dependent manner, it is apparent that some of the lipid peroxidation products leave the oxidizing LDL particle and react with albumin. It appears that the ε-aminogroups on LDL particles and reactive groups of albumin compete for aldehydes thereby reducing deleterious effects to LDL.

How is this possible? One possibility is phospholipid hydrolysis. It has been shown that phosphatidylcholine hydrolysis occurs in oxidizing LDL particles; phosphatidylcholine hydrolysis is believed to be essential for the continuing LDL oxidation process and a consequence of LDL oxidation.[16–18] On the other hand, albumin could have influenced the LDL modification by its antioxidant activity and by binding the Cu(II)ions.[19–21] This possibility is rather unlikely. Antioxidants and redox metal chelators in a LDL oxidation assay prolong the lag phase of the dienes.[6,7] Therefore at least a small effect on the dienes should have been observed, if these activities of albumin would be relevant. If in our assay albumin would have bound some copper, however, the formation of the reactive oxygen species and LDL dienes would have been substantially decreased.

Oxidized LDLs are rapidly cleared by hepatic scavenger receptors. It has been demonstrated that the Kupffer and hepatic sinusoidal endothelial cells are taking up oxidized LDL avidly.[15] The removal of oxidized LDL by the liver can not be competed by native LDL. The studies by van Berkel *et al.*[15] strongly support the concept that multiple hepatic scavenger receptors form a defence line against the accumulation of oxidized LDL in the circulating blood.[15] Our results demonstrate that oxidized LDL generated in the absence and in the presence of a physiological concentration of a plasma protein differ significantly in the rapid plasma clearance phase. After the rapid decay phase the plasma removal of remaining LDL radioactivity was similar to the rate of native ^{131}I TDS-LDL. A 12 h incubation of LDL in the oxidation assay increases the proportion of rapidly cleared LDL to >90% (Friedrich, unpublished observation). We assume that the fully oxidized LDL is modified and recognized by the scavenger receptors. The slowly cleared component could not be derived from cellular oxidized LDL breakdown in lysosomes, since the ^{131}I-TDS label is trapped in the cells. We do not know what the reason is for this heterogeneity. Possibly some "minimally-modified" LDL not recognized by the scavenger receptors is present in the albumin treated oxidized LDL samples. Repair of oxidized LDL and exchange of iodinated material to other blood components remains another possibility.

In summary, the presence of even a single plasma protein species in an LDL oxidation system influences the LDL modification process and the biological fate of the oxidized LDL. This excites us, since albumin as a scavenger of reactive lipid peroxidation products released from oxidizing LDL could produce protective effects and reduce the LDL catabolism by scavenger pathways. If it were possible to generalize this aspect, it can be hypothesized that drugs in an atherosclerotic plaque acting like albumin in the LDL oxidation assay might have beneficial effects.

Acknowledgements

We thank I. Schulz and R. Kühnlein for technical assistance and Boehringer Mannheim GmbH for the generous support.

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Accepted by Prof. Dr. H. Esterbauer