Role of Plasmalogens in the Enhanced Resistance of LDL to Copper-Induced Oxidation After LDL Apheresis

Daniela Hahnel, Joachim Thiery, Thorolf Brosche, Bernd Engelmann

Abstract—Extracorporeal reduction of plasma low density lipoproteins (LDLs) by LDL apheresis was shown to attenuate the proatherogenic influences of LDL, such as impairment of vasodilation and increased monocyte adhesion to the endothelium. In 16 patients with familial hypercholesterolemia, we analyzed whether LDL apheresis by the heparin precipitation procedure affected the oxidative resistance of LDL. Plasma LDL cholesterol concentrations were reduced by 65% after the apheresis. The lag time of copper-mediated LDL oxidation was increased from 103 to 117 minutes (P<0.0005). The LDL contents of α-tocopherol and β-carotene, as well as the ratio of monounsaturated to polyunsaturated fatty acids in LDL, were not altered. However, the LDL apheresis induced a 15% increase in the LDL contents of plasmalogen phospholipids (P<0.0005), a class of ether phospholipids that were recently shown to prevent lipid oxidation. The phosphatidylcholine (PC) to lysoPC ratio was elevated by 16% after the apheresis (P<0.0005). The percent increase in LDL plasmalogn phospholipids showed a close association with the increased lag time after apheresis (P<0.0005). The LDL plasmalogen contents of the blood samples from patients and from normolipidemic donors were also positively related to the lag time (P<0.005). In vitro loading of LDL with plasmalogen phospholipids resulted in a prolongation of the lag time and an increase in the PC/lysoPC ratio. In conclusion, the rapid rise in LDL contents of plasmalogen phospholipids most probably causes the increase in lag time after LDL apheresis. Plasmalogens appear to play an important role in the oxidation resistance of LDL in vivo. (Arterioscler Thromb Vasc Biol. 1999;19:2431-2438.)

Key Words: LDL apheresis ■ plasmalogen phospholipids ■ lag times ■ LDL oxidation ■ lysophosphatidylcholine

The hypothesis that oxidized LDLs play a relevant role in the development and maintenance of atherosclerosis is based on considerable experimental evidence. In vitro–oxidized LDL reproduces many features characteristic of atherosclerotic vascular damage, such as increased adhesion of monocytes to the endothelium, transformation of macrophages into foam cells, and impaired endothelium-dependent vasorelaxation. Oxidized LDL was shown to be present in the atherosclerotic plaque. Furthermore, the LDL isolated from the plasma of patients with atherosclerosis exhibited an enhanced susceptibility toward oxidation in most studies.

Extracorporeal LDL apheresis, the removal of the greater part of plasma LDL particles from the circulation, is currently used for the treatment of patients with severe hypercholesterolemia. The efficacy of this treatment in preventing fatal and nonfatal cardiovascular events has been demonstrated. Furthermore, LDL apheresis was shown to ameliorate the impaired endothelium-dependent vasorelaxation. The procedure also led to diminished expression of endothelial adhesion molecules. Thus, typical proatherogenic consequences of oxidized LDL are attenuated after therapeutic reduction of LDL. Although removal of the particles per se might partially account for these effects, changes in the oxidizability of the LDL induced by LDL apheresis could also be relevant.

In the present investigation, the oxidative resistance of copper-oxidized LDL was found to be enhanced after apheresis, in accordance with recent work. In view of these findings, we attempted to gain insight into the mechanism responsible for the increased oxidation resistance after the LDL apheresis. Several chemical factors are known to influence the susceptibility of LDL toward copper-mediated oxidation. These include α-tocopherol, β-carotene, ubiquinol-10, and plasmalogen phospholipids. The results indicated that the enhanced oxidation resistance after LDL apheresis was specifically related to an increase in LDL plasmalogens.

Methods

Subjects
Sixteen patients with familial hypercholesterolemia (FH; 10 men and 6 women; mean±SD age 49±6 years; range 42 to 61 years) who had already been treated biweekly by LDL apheresis for at least 14 months were included in the study. All patients had angiographically assessed coronary heart disease and were concomitantly treated with

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From the Physiologisches Institut der Universität München (D.H., B.E.) and the Institut für Klinische Chemie, Klinikum Grosshadern, Universität München (J.T.), Munich, and the Institut für Gerontologie, Universität Erlangen-Nürnberg (T.B.), Nürnberg, Germany.

Correspondence to Dr Bernd Engelmann, Physiologisches Institut der Universität München, Schillershr. 44, D-80336, Munich, Germany. E-mail Bernd.Engelmann@physiol.med.uni-muenchen.de

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hydroyxymethylglutaryl CoA-reductase inhibitors and aspirin. Six of the patients (5 men, 1 woman) were concomitantly supplemented with vitamin E (400 IU/d). The supplementation had been started at least 8 weeks before the study. In some experiments, the LDL particles from 10 normolipidemic donors (8 men and 2 women; mean age 49±10 years; range 39 to 64 years) were also analyzed. Fasting blood samples were drawn into EDTA-coated tubes before or after apheresis, or, in some experiments, 48 hours after apheresis. The LDLs were isolated by ultracentrifugation or heparin precipitation (as indicated), the particles being stored for <30 minutes at 4°C before the start of the analyses. Informed consent was obtained from all patients and volunteers. The studies were carried out according to the principles of the Declaration of Helsinki.

**Lipoprotein Analyses**

Plasma cholesterol and triglycerides were measured using enzymatic test kits from Boehringer Mannheim. Plasma LDL cholesterol was quantified by a direct precipitation procedure (Immuno). HDL cholesterol was determined enzymatically (Boehringer Mannheim) after initial precipitation of apo B-containing lipoproteins with phosphotungstic/MgCl₂.

**Procedure of the LDL Apheresis**

LDL apheresis was performed using the HELP procedure (Heparin-induced Extracorporeal LDL Precipitation; Plasmat-Secura, B. Braun). In brief, plasma obtained by filtration of whole blood (70 mL/min) through a plasma separator was continuously mixed with an acetate buffer containing heparin. At the resulting pH of 5.12, LDL, lipoprotein(a), and fibrinogen were precipitated. After removal of the precipitated heparin complex by filtration, excess heparin was adsorbed by an anion-exchange column, and the clear, plasma filtrate was subjected to bicarbonate dialysis to restore plasma volume as well as the physiological pH. The plasma thus obtained was returned together with blood cells to the patient. During a single HELP apheresis, 2.5 to 3 L of plasma was treated. The duration of the treatment was 2 to 3 hours.

**LDL Susceptibility to Oxidation (Lag Time) and Analysis of TBARS**

LDL was prepared within 10 hours after blood collection by sequential ultracentrifugation. Blood was drawn into tubes containing EDTA (1 mg/mL). Plasma was separated from the blood cells by a 10-minute centrifugation. The plasma with a density of 1.02 g/mL was centrifuged for 4 hours at 340 000g in polycarbonate tubes in a TL-100 ultracentrifuge with a TLA-100.1 rotor (Beckman Instruments). After recovery, the infranatant was adjusted to a density of 1.063 g/mL with NaCl and centrifuged for 4 hours at 340 000g. The supernatant solution containing the LDL was freed from the EDTA by passage through desalting columns (Econo Pac IODG, Bio-Rad Laboratories). The particles were resuspended in O₂-saturated PBS (pH 7.4) at a concentration of 0.26 mmol cholesterol per liter. CuCl₂ (final concentration 1.67 mM/mL) was added from a freshly prepared stock solution. The suspension was incubated at 30°C, and the absorbance at 234 nm was monitored at 5-minute intervals for 4 hours to follow the formation of conjugated dienes. The lag time was determined as the intercept of the baseline and the slope of the absorbance curve during the propagation phase.

Thiobarbituric acid-reactive substances (TBARS) were determined according to Wallin et al. with some modifications. In brief, to tubes containing 200 μL of 50% trichloracetic acid, 75 μL of 1.3% TBA (in 0.3% NaOH, wt/vol) was added. The tubes were heated for 60 minutes at 90°C and then cooled in ice water. After centrifugation, 200 μL of the supernatant was transferred to a 96-well plate, and the differences between the absorbances at 530 nm and those at 630 nm were determined. The values thus obtained were compared with those of a standard curve of malondialdehyde equivalents, generated by hydrolysis of 1,1,3,3-tetraethoxypropane.

**LDL Contents of α-Tocopherol and β-Carotene**

LDL was isolated from EDTA-plasma by precipitation with heparin-acetate buffer (0.3 mol/L sodium acetate, 100 μmol/L sodium heparinate, pH 4.85) and resuspended in a Tris buffer containing (in mmol/L) 154 NaCl, 3 Na₃, and 1 EDTA and 10 g/L albumin (pH 7.4). The α-tocopherol contents were measured fluorometrically by high-performance liquid chromatography in hexane extracts of the LDL suspensions as described previously. When the LDL particles were isolated by ultracentrifugation, the LDL contents of α-tocopherol agreed within 5.3±1.4% with those determined in particles prepared by precipitation with the heparin-acetate buffer. The contents of β-carotene were determined in the same extracts by separation using reversed-phase high-performance liquid chromatography and subsequent spectrophotometric detection at 450 nm (retention time 15.8 minutes) according to procedures described in Reference 29.

**LDL Contents of Plasmalogen and Ester Phospholipids**

LDL was recovered by precipitation with heparin-acetate buffer as described above. LDL lipids were extracted according to Bligh and Dyer by using chloroform containing 50 mg/L BHT. Phospholipids were separated by 2-dimensional thin-layer chromatography on DC 60 plates (Merck) by using the solvents proposed by Broekhuysen. In some of the samples, the percentages of the major LDL-associated phospholipids (phosphatidylcholine [PC], sphingomyelin, lysoPC, phosphatidylethanolamine [PE], and phosphatidylinositol) were subsequently determined by phosphate analysis. In other samples, the spots corresponding to PC and PE were scraped from the plate and the phospholipids eluted by addition of chloroform/methanol (1:4, vol/vol). The extraction was repeated twice. The phospholipids were applied to small thin-layer chromatography plates (10×10 cm), which were exposed for 3 minutes to HCl fumes (37% in water). After being dried, the plates were developed in chloroform/methanol/acetic acid/water (90:40:12:2, vol/vol/vol/vol). The phosphate contents of the spots corresponding to lysoPC and lysoPE, which reflected the amounts of plasmalenoylethanolamine, respectively, were measured. When 2 LDL samples obtained at the same time from the same donor were analyzed separately from the start of the analysis, the determinations of the plasmalenoylethanolamine agreed within 2.8±1.3%.

**Fast Protein Liquid Chromatography**

Gel filtration analysis of plasma was performed by means of a Superose 6 column (Pharmacia). Plasma (100 μL) was injected onto the column and eluted with a buffer containing (in mmol/L) 150 NaCl, 10 Na₂HPO₄, and 0.1 EDTA (pH 7.5) at a flow rate of 50 μL/min. Forty fractions of 50 μL were collected: fractions 15 through 19, VLDL and chylomicrons; fractions 20 through 26, IDL and LDL; and fractions 27 through 33, HDL. Cholesterol and triglyceride determinations were performed as described above.

**Determinations of Fatty Acids**

After precipitation of the LDL with the heparin-acetate buffer and extraction of LDL lipids (as per Reference 30 with chloroform containing 50 mg/L BHT), fatty acid methyl esters were generated by addition of BF₃-methanol. Subsequently, the fatty acid methyl esters were separated using a gas chromatograph (Hewlett-Packard HP-5880 A) equipped with a flame ionization detector.

**In Vitro Loading of LDL With Plasmalogen Phospholipids and α-Tocopherol**

Fresh venous blood from normolipidemic donors was drawn into tubes containing EDTA, and plasma was recovered by centrifugation. For the in vitro loading of LDL with brain plasmalenoylethanolamine (isolated as described previously in Reference 34), small, unilamellar vesicles were prepared. Aliquots (0.4, 0.7, and 1 μmol) of either brain plasmalenoylethanolamine or diacyl PE (16/0:18:2; PE) were dissolved together with 1.2, 2.1, and 3 μmol, respectively, of egg PC in 100 μL of ethanol (“low, medium, and high enrichment”; cf Table 3). 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 hours. The amount of plasmalenoylethanolamine and diacyl PE additionally present in the particles after this incubation period was comparable. Subsequently, LDL was prepared by ultracentrifugation. The amount of LDL-associated protein was determined by...
5000 IU of heparin was given intravenously to 3 normolipidemic donors as well as to 3 patients with FH (without vitamin E supplementation). The lag time of the isolated LDL oxidized with copper (1.67 nmol/mL) was not altered by the heparin administration (normolipidemic individuals: 99.8 ± 18.1 minutes before versus 100.7 ± 12.7 minutes 1 hour after heparin; patients with FH: 82.5 ± 12.3 minutes before versus 79.6 ± 10.2 minutes after heparin).

Elevation of LDL Plasmalogen Phospholipids After Apheresis

The oxidative resistance of LDL is governed by several factors, among which the LDL-associated antioxidants are of particular importance. 19 α-Tocopherol is considered to be 1 of the main LDL-based antioxidants. The α-tocopherol contents of the LDL from patients supplemented with vitamin E were 2.3-fold higher than those of the patients without vitamin E supplementation (Table 2). The LDL contents of α-tocopherol were not altered by the apheresis in the 2 groups of patients with FH (Table 2). Furthermore, the LDL contents of β-carotene as well as the ratio of monounsaturated to polyunsaturated fatty acids of the particles were also not significantly modified.
In vitro oxidation of LDL also induces changes in LDL phospholipids, in particular a reduction in LDL PC and a concomitant increase of lysoPC, its degradation product. The PC/lysoPC ratio of the particles was increased by 15% (without vitamin E supplementation) and 16% (with vitamin E supplementation) subsequent to the procedure (Table 2). After inclusion of all 16 patients into the analysis, the mean PC/lysoPC ratio was found to be elevated by 16% (from 20.9 ± 3.3 before to 24.2 ± 3.9 after apheresis, P < 0.0001).

Recent work indicates that plasmalogen phospholipids may play a relevant role in the oxidative resistance of LDL in vitro. The preapheresis values for the LDL-associated plasmalogens were 2.6-fold higher in patients with vitamin E supplementation compared with patients not supplemented with vitamin E (Table 2). After LDL apheresis, the plasmalogen contents were increased by 14% and 13% in patients without and with vitamin E supplementation, respectively (Table 2). When all patients were included in the analysis, the median of the LDL plasmalogen contents was elevated by 15% after the apheresis (from 20.9 before to 24.0 μmol/mmol of LDL cholesterol after apheresis, P < 0.0005).

Relations Between Lag Time and LDL Plasmalogen Contents
In view of these results, we analyzed whether the increase in LDL plasmalagens after apheresis was related to the prolongation of the lag time. As shown in Figure 2, there was a positive correlation between the percent elevation of the LDL plasmalogen contents induced by the apheresis and the degree of prolongation of the lag time. The positive associations were also noted when the 2 groups of patients without and with vitamin E supplementation were analyzed separately (without vitamin E: r = 0.66, P < 0.05; with vitamin E: r = 0.92, P < 0.005). The extent of increase in the lag time after apheresis was not related to the percent elevation of the PC/lysoPC ratio (r = 0.06, NS).

The LDL contents of α-tocopherol and of plasmalogen phospholipids as measured before apheresis in patients with FH and those of normolipidemic donors were compared with their lag time values. In the total group of 20 individuals thus evaluated, the LDL α-tocopherol contents exhibited a weak, positive association with the lag time values (Figure 3, upper panel). The plasmalogen contents showed a stronger relation to the lag time values in the same individuals (Figure 3, lower panel). The PC/lysoPC ratios as determined at the same time point in the same donors did not exhibit a significant relation to the duration of the lag time (r = 0.18). When the preapheresis values of all patients with FH and of the normolipidemic individuals were compared with the duration of the lag time, positive correlations were obtained (α-tocopherol versus lag time: r = 0.79, P < 0.0001; plasmalogen phospholipids versus lag time: r = 0.83, P < 0.0001; n = 26).
In Vitro Enrichment of LDL With Plasmalogen Phospholipids

To evaluate whether changes in the LDL contents of the plasmalogen phospholipids could be responsible for the variations in LDL oxidation resistance, LDL particles were enriched in vitro with 3 different quantities of plasmenylethanolamine and, as a control, with similar amounts of diacyl PE. After enrichment with the highest amount of plasmenylethanolamine, the oxidative degradation of PC was reduced by 35% (2 hours) and by 23% (4 hours) as a consequence of the plasmalogen enrichment (compared with the LDL loading with diacyl PE).

In LDLs with medium plasmalogen enrichment, the copper-mediated reduction of the PC/lysoPC ratio was prevented by 28% (2-hour incubation) and 32% (4-hour incubation) in relation to the respective control particles containing additional diacyl PE. After enrichment with the highest amount of plasmenylethanolamine, the oxidative degradation of PC was reduced by 43% and 39% after 2 and 4 hours of incubation of LDL with copper compared with the respective controls (Table 3).

Discussion

In the present investigation, we observed rapid changes in the oxidizability of the LDL particles after LDL apheresis in patients with FH. After the extracorporeal reduction of LDL, the resistance of LDL to copper-induced oxidation was enhanced, as indicated by a prolongation in the lag time of conjugated-diene formation. Concomitantly, the LDL contents of plasmalogen phospholipids were increased after apheresis, their elevation being most likely responsible for the strengthening of the oxidation resistance of the particles.

The reduced susceptibility of LDL toward copper oxidation observed in the present study after apheresis by the heparin precipitation method is in line with results of recent investigations performed with other procedures. Although relatively limited, the increase in lag time elicited by the apheresis might well be of physiopathologic relevance. Indeed, the previously observed differences in the lag time between healthy individuals and patients with coronary atherosclerosis and hyperlipidemia are mostly in a comparable percent range as those induced by the apheresis.

The oxidation resistance of LDL was determined in the present study by the use of a relatively high concentration of copper (1.67 nmol/mL). Previous work shows that at this and

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Figure 3. Relations between the lag time values and the LDL contents of α-tocopherol and plasmalogens. The LDL lag time values as well as the plasmalogen and α-tocopherol contents of 10 patients with FH (without vitamin E, measured before apheresis) and of 10 normolipidemic donors were determined concomitantly. Upper panel, Relations between the LDL contents of α-tocopherol and the duration of the lag time. Lower panel, Relations between the LDL contents of plasmalogen phospholipids and the lag time. ▲. Patients with FH (without vitamin E supplementation); ▲, normolipidemic donors.
higher concentrations of the metal ion, the length of the lag time is inversely related to the copper concentration. Although several findings indicate a relevant role for copper as a major oxidant under in vivo conditions (see, for example, References 41 through 43), the importance of the metal ion is still a matter of debate. Nevertheless, the increase in LDL plasmalogen contents after LDL apheresis is expected to also enhance the resistance of LDL to other oxidants. This can be deduced from earlier results indicating a comparable reduction in the lag time values measured. The PC/lysoPC ratios were determined as described in the legend to Figure 2. In some cases, the lipoproteins were oxidized with AAPH (2 μmol/mL) instead of copper under otherwise identical experimental conditions. Mean values are from a total of 4 or 5 determinations from 2 or 3 different lipoprotein preparations.

In the patients with FH analyzed in the present study, the percent increase in the LDL contents of total plasmalogen phospholipids (plasmenylethanolamine plus plasmenylcholine) exhibited a strong, positive relation to the prolongation of the lag time (Figure 2). When the preapheresis values for the LDL plasmalogen contents of the patients and those of normolipidemic donors were compared with the duration of the lag time, a close positive association between both parameters was again noted (Figure 3). The correlation between the α-tocopherol contents of LDL and the lag time was less pronounced in individuals not supplemented with vitamin E but became stronger after inclusion of the patients with vitamin E supplementation, in accordance with earlier data. Future studies are needed to evaluate whether the association between the plasmalogen contents and the oxidation resistance of LDL is also evident in larger populations of individuals. When LDL particles were loaded in vitro with plasmenylethanolamine (“low plasmalogen enrichment”; Table 3), comparable percent changes in the lag time of copper-oxidized LDL were obtained as by the increase in plasmalogen contents induced by the LDL apheresis. Thus, the elevated LDL plasmalogen contents are most likely responsible for the prolongation in the lag time values observed after LDL apheresis.

The reasons for the elevation in LDL plasmalogen contents after the apheresis are unknown at present. Plasmalogens were shown to be rapidly exchanged between lipid donors and erythrocytes. The plasmalogen pool of red blood cells,
which is >10 times higher than that of the LDL particles, is augmented after LDL apheresis.\textsuperscript{59} The enhanced delivery of erythrocyte-derived plasmalogens phospholipids to the LDL could contribute to the increase in LDL plasmalogen contents after apheresis. The steeper plasmalogens gradient between red blood cells and LDL induced by the rapid fall in LDL concentrations might favor such an enhancement of plasmalogens transfer.

LysoPC was previously reported to mediate the atherogenic effects of in vitro–oxidized LDL, including the impairment of endothelium-dependent vasodilation,\textsuperscript{50} the enhanced adhesion of monocytes to the endothelium,\textsuperscript{58} and the increased expression of endothelial growth factors.\textsuperscript{51} After LDL apheresis, the endothelium-dependent vasodilation was found to be ameliorated,\textsuperscript{12,52,53} and the adhesion of monocytes to the endothelium was observed to be diminished.\textsuperscript{13,14} On the basis of the results of the present study, these earlier findings might be partially explained by the diminished lysoPC contents of the particles (Table 2). The reasons for the increased PC/lysoPC ratios after apheresis are unknown. It is unlikely that the enhanced oxidative resistance of LDL after the apheresis might play a prominent causal role, because there was no correlation between this ratio and the duration of the lag time (see Results). Therefore, other factors may be relevant, such as alterations in the activities of enzymes known to remodel LDL-associated PC (e.g., lecithin:cholesterol acyltransferase).

In summary, the close associations between the LDL contents of plasmalogens phospholipids and the duration of the lag time observed in the present investigation indicate that plasmalogens are of considerable relevance for the resistance of LDL to copper oxidation. The diminished oxidizability of the LDL after LDL apheresis is suggested to contribute to the beneficial effects of the procedure on endothelial dysfunction and the prevention of coronary artery disease.

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