Altered Susceptibility to In Vitro Oxidation of LDL in LDL Complexes and LDL Aggregates

Marcela Hermann and Bernhard Gmeiner

Low density lipoprotein (LDL) is known to form complexes with polysulfated compounds, like heparin, dextran sulfate (DS), and chondroitin sulfate. In particular, chondroitin 6-sulfate (C6S)-rich proteoglycans of the arterial intima can associate with LDL, resulting in accumulation of LDL in atherosclerotic lesions. Besides LDL complex formation, LDL self-aggregation has been recently suggested to play a role in atherogenesis. Oxidative modification of LDL has also been implicated as a factor in the generation of the atherosclerotic plaque. Assuming that LDL self-aggregation may alter the molecule's susceptibility to oxidative modification, we have studied the sensitivity of LDL in LDL aggregates as well as in insoluble and soluble LDL-C6S, LDL-heparin, and LDL-DS complexes to in vitro oxidation by copper ions. Complexing the LDL with C6S and heparin resulted in an increased susceptibility of LDL to in vitro oxidation, whereas the oxidation of LDL complexed with DS was unaffected. In great contrast to the oxidation of LDL in LDL complexes, the in vitro oxidation of LDL in LDL aggregates (self-aggregation by denaturation) was strongly reduced. The results suggest that complex or aggregate formation may alter the susceptibility of the lipoprotein to oxidative modification and finally its metabolic fate or biological activity. (Arteriosclerosis and Thrombosis 1992;12:1503-1506)

KEY WORDS • low density lipoprotein oxidation • low density lipoprotein–chondroitin sulfate complex • low density lipoprotein–heparin complex • low density lipoprotein–dextran sulfate complex • low density lipoprotein aggregates

I t has been suggested that the oxidative modification of low density lipoprotein (Ox-LDL) may play an important role in the initiation and progression of arteriosclerosis.1 Oxidation of LDL can occur in vivo, but many studies have been carried out with in vitro oxidized LDL (see additional studies cited in Reference 1). Ox-LDL is taken up rapidly by macrophages, resulting in the generation of foam cells. Foam cell formation is also facilitated by native LDL complexed with macromolecules, i.e., proteoglycans or fibronectin. Such complexes are avidly endocytosed by the macrophage and have been isolated from atherosclerotic lesions.2-6 Foam cell formation was also observed after incubation of macrophages with preparations of self-aggregated LDL,7 and therefore, LDL aggregates have been suggested to contribute to atherogenesis.7

Recently Camejo et al8 have shown that the complexing of LDL with arterial chondroitin sulfate (CS) proteoglycans (CSPGs) and glycosaminoglycans (GAGs) resulted in an alteration of the LDL structure, which was accompanied by a modification in its in vitro oxidation by copper ions.9 In their studies these authors have used resolubilized LDL particles from LDL-GAG and LDL-CSPG complexes.

Taking into consideration that the binding of proteoglycans to LDL can8,9 and the self-aggregation of the lipoprotein may alter the molecule's conformation, one can suppose that aggregate formation may lead to an alteration of the native LDL particle's susceptibility to oxidative modification. Using established in vitro model systems for 1) the formation of LDL aggregates,7 2) the interaction of LDL with C6S-rich proteoglycans,8,9 and 3) the in vitro oxidative modification of LDL by copper ions,10-12 in the present study we focused on the oxidation of LDL in LDL aggregates as well as insoluble LDL complexes, thus extending the study of Camejo et al9 to LDL particles that may have a completely different structure from that in resolubilized particles.

Methods

Preparation of LDL

LDL was isolated by preparative ultracentrifugation from fresh human plasma.13 Blood was obtained after overnight fasting from healthy, nonsmoking volunteers. The lipoprotein (d=1.019–1.063 g/ml) obtained was dialyzed extensively against 150 mM NaCl and 0.1 mM EDTA (pH 7.4), subsequently filtered through a 0.45-μm filter, and stored at 4°C. Before the experiments the LDL preparations were subjected to gel filtration on a Sephadex G-25 column (PD-10 column, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the desired buffer (20 mM NaCl and 5 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES] in the complex formation experiments and 150 mM NaCl and 5 mM HEPES in the aggregate formation experiments) to remove the EDTA.

LDL was radioiodinated by the iodomonochloride method according to McFarlane14 by using sodium [125I]iodide (specific activity, 296–555 GBq 125I/mg, Beh-
ringwerke, Marburg, FRG). After radioiodination the lipoprotein was dialyzed and filtered as described above. The final preparation had a specific activity of 300–400 cpm/ng protein.

Protein was measured by the Bio-Rad protein assay (Bio-Rad Chemical Division, Richmond, Calif.) by using bovine serum albumin as a standard. All experiments were done with the same LDL preparation and within 10 days after lipoprotein isolation.

**Preparation of LDL Complexes**

Insoluble and soluble LDL complexes with polysulfated compounds were prepared according to the model system of Olsson et al.\(^{15}\) LDL (290 μg protein) was incubated for 60 minutes at room temperature in 5 mM HEPES buffer with 5 mM Ca\(^{2+}\)/2 mM Mg\(^{2+}\) and 20 mM NaCl with C6S (from shark skin cartilage, Sigma Chemical Co., St. Louis, Mo.), heparin (177 USP units/mg, Sigma), or dextran sulfate (DS; molecular weight, 500,000; Serva). CS, heparin, and DS to LDL protein ratios (micrograms per microgram) were varied from 0.03 to 3.45 to obtain the insoluble or soluble complexes, respectively. Complex formation was calculated from parallel incubations containing radiolabeled LDL and the respective polysulfated compound. After 60 minutes at room temperature the tubes were centrifuged for 10 minutes at 10,000g, the pellets were washed with buffer, and the radioactivity in the precipitate was estimated.

**Preparation of LDL Aggregates**

LDL aggregates were prepared by vortexing LDL in buffer as published by Khoo et al.\(^7\) LDL preparations were vortexed in round-bottom tubes (12×100 mm) for as long as 60 seconds. The amount of LDL aggregates formed was estimated in a parallel series using radiolabeled LDL. After vortexing for the desired time the tubes were centrifuged for 10 minutes at 10,000g, the pellets were washed with buffer, and the radioactivity of the precipitated LDL was measured.

**LDL Oxidation**

LDL oxidation was performed by incubating the respective LDL preparation (total volume, 0.5 ml) with copper ions (10 μM) for 60 minutes at 37°C. Subsequently, 2.5 ml of 20% trichloroacetic acid (TCA) was added, and the extent of LDL oxidation in the precipitate was determined by quantification of thiobarbituric acid-reactive substances (TBARS) as given by Satho.\(^{16}\) Malondialdehyde (E. Merck, Darmstadt, FRG) was used as a standard.

**Results**

**Oxidation of LDL in Complexes**

A well-known feature of LDL is its ability to form complexes with polysulfated compounds, like heparin or DS.\(^{17,18}\) In addition, attention has been paid to C6S-rich proteoglycans and their interaction with LDL.\(^{15,19}\) These authors used an in vitro model system consisting of pure C6S or heparin/apolipoprotein B-100 complexes to study the interaction of LDL with arterial proteoglycans, a method that has the advantage of well-defined conditions. As shown in Figure 1A, the addition to LDL preparations of C6S, heparin, or DS in low concentrations resulted in the formation of insoluble complexes (compound to LDL ratio, 0.03). At higher ratios (up to 3.45), all compounds formed soluble complexes with the lipoprotein. These results are in good agreement with others.\(^{15,18,19}\) Next, insoluble and soluble LDL-C6S, LDL-heparin, and LDL-DS complexes were prepared and subjected to in vitro oxidation with copper ions (10 μM). Figure 1B shows that LDL complexed with C6S or heparin becomes more sensitive to in vitro oxidation. These results parallel the observations of Camejo et al.,\(^9\) who have studied the copper-catalyzed oxidation of LDL after resolubilization of the lipoprotein after com-
plexation with CSPGs or GAGs. The oxidation of LDL in the soluble complexes tended to mimic the control values (uncomplexed LDL), especially with C6S when the ratio of C6S to LDL was 3.45. In contrast, the oxidation of LDL in insoluble LDL-DS complexes was unaffected. Soluble LDL-DS complexes (DS to LDL ratio >0.5) could not be tested in this system (Figure 1B) because the LDL present in soluble DS complexes could not be precipitated by TCA, thereby making the estimation of TBARS associated with protein impossible with the assay system used.16 Omission of TCA and use of another acidification step (phosphoric acid) before the estimation of TBARS (as published in other methods) revealed that the sensitivity of LDL in soluble LDL-DS complexes to in vitro oxidation was not altered (results not shown). None of the compounds tested had any influence on the autooxidation of LDL, that is, TBARS formation in parallel incubations without copper ions.

Oxidation of LDL in Aggregates

Self-aggregating LDL may play a role in lipoprotein deposition in the arterial intima, and in vitro, such aggregates of LDL can be obtained by vortexing LDL.7 In view of the results already described with the complexes of LDL with sulfated polysaccharides, we decided to explore whether self-aggregated LDL was also altered in its susceptibility to oxidation. As depicted in Figure 2A, increasing the vortexing time of the LDL preparation resulted in an increased formation of LDL aggregates. After 60 seconds of vortexing, about 80% of the LDL was aggregated; these results are in good agreement with those of Khoo et al.7 As shown in Figure 2B, this increase in LDL self-aggregation was accompanied by a striking decrease in the oxidation of LDL by copper ions. After 60 seconds of vortexing, the amount of LDL oxidation was about 40% compared with control (100%). Vortexing for 60 seconds had no influence on the extent or rate of autooxidation of LDL.

Discussion

Both the complexing of LDL with C6S-rich proteoglycans and the oxidative modification of LDL appear to play an important role in atherogenesis.1,2 In addition to LDL complexes, the formation of LDL aggregates and their subsequent uptake by macrophages may also contribute to atherosclerosis.7 In this respect the oxidation of LDL in LDL aggregates (self-aggregated LDL) and LDL complexes was investigated by using well-established model systems. In contrast to LDL complexed with sulfated polysaccharides, self-aggregated LDL was not susceptible to in vitro oxidation. Resolubilized LDL from LDL-CSPG and LDL-GAG complexes has been shown to be more susceptible to in vitro oxidation than is native LDL.9 In our system insoluble complexes of LDL with heparin and C6S at low polysaccharide to LDL ratios (<0.5) showed increased sensitivity to oxidation. We do not know why the effect of complex formation becomes less pronounced at higher ratios. Our results indicate that although LDL is present as an insoluble form in complexes with sulfated polysaccharides as well as after self-aggregation, there is a marked difference in the oxidation rate of these two forms. The increased sensitivity of resolubilized LDL from LDL-CSPG and LDL-GAG complexes to oxidation by copper ions seems to be due to an increase in the access of Cu2+ to hydrophobic regions of LDL.9 Taking this suggestion into consideration, one could speculate that LDL aggregate formation may mask the copper-accessible regions of the lipoprotein, resulting in a decreased oxidation of the aggregated particle.

It is not known whether such a difference in LDL oxidation may also occur under in vivo conditions. A

![Figure 2. Line plots showing oxidation of low density lipoprotein (LDL) in LDL aggregates. Panel A: Formation of LDL aggregates by vortexing. Radio labeled LDL preparations were vortexed up to 60 seconds in round-bottom tubes. At the indicated time point the preparations were centrifuged for 10 minutes at 10,000g, and the amount of aggregated LDL was estimated by counting the radioactivity in the pellets. Panel B: Oxidation of LDL. Native LDL or aggregated LDL (LDL vortexed for the indicated time) preparations were incubated for 60 minutes at 37°C in the absence or presence of 10 μM copper ions. Subsequently the formation of thiobarbituric acid–reactive substances was estimated as described in the “Methods” section. ○, LDL aggregates; ●, oxidation of LDL in LDL aggregates. Each point represents the mean of duplicate experiments.](https://example.com/image.png)
phenomenon similar to the one described herein could contribute to enhanced oxidative modifications of LDL, once it is associated with the intimal extracellular matrix, therefore contributing to its potential atherogenicity.

Acknowledgment
We are grateful to Dr. G. Camejo, University of Gothenburg, Sweden, for helpful suggestions concerning the preparation of the manuscript.

References
Altered susceptibility to in vitro oxidation of LDL in LDL complexes and LDL aggregates.

M Hermann and B Gmeiner


doi: 10.1161/01.ATV.12.12.1503

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1992 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/12/12/1503

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/