Modification of copper-catalyzed oxidation of low density lipoprotein by proteoglycans and glycosaminoglycans

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Abstract Chondroitin sulfate proteoglycans (CSPG) appear to contribute to retention of low density lipoproteins (LDL) in atherosclerotic lesions. In vitro, CSPG and glycosaminoglycans (GAG) modify LDL structure and increase its uptake by macrophages. This latter effect appears related to increased exposure of arginine- and lysine-rich segments of apoB-100. We explored whether alterations of LDL induced by human arterial CSPG and purified GAG alter the lipoprotein susceptibility to transition metals-catalyzed oxidation. Human LDL was complexed with human arterial CSPG and dissociated by raising the ionic strength. The nonaggregated, CSPG- and GAG-treated LDL was subjected to oxidation by micromolar amounts of Cu^{+}, Cu^{2+}, Fe^{2+}, and Fe^{3+}. This treatment increased LDL susceptibility to Cu^{2+} oxidation 3- to 5-times, as indicated by the degradation rate of phospholipids and cholesteryl esters and formation rates of dienes and thiobarbituric acid-reacting substances (TBARS). Also, human macrophages degraded the CSPG-treated, Cu^{2+}-oxidized LDL 3- to 6-times faster than native LDL similarly treated. No enhancement of oxidation was observed with Fe^{2+}, Fe^{3+}, and Cu^{+}. Quenching of the LDL intrinsic fluorescence by Cu^{2+} showed that heparin, CSPG, and chondroitin-6-SO_4 pretreatment increased the access of Cu^{2+} to hydrophobic chromophores, probably tryptophan, 6- to 7-, 3- to 4-, and 2- to 3-fold, respectively. Also, the affinity constant (K_a) of LDL for Cu^{2+} was increased from 0.12 μM to 0.20 μM by the treatment with CSPG and GAG. These results and evaluation of the fraction of surface-accessible LDL chromophores to acrylamide quenching suggest that the increased susceptibility to oxidation may be associated with an increase in the access of Cu^{2+} to hydrophobic regions in LDL caused by treatment with CSPG and GAG. This effect was not detected with Cu^{+}, Fe^{2+}, or Fe^{3+}. The phenomenon may contribute to acceleration of the oxidative modifications of LDL in cell culture models and in vivo. **—Camejo, G., E. Hurt-Camejo, B. Rosengren, O. Wiklund, F. López, and G. Bondjers.** Modification of copper-catalyzed oxidation of low density lipoprotein by proteoglycans and glycosaminoglycans. *J. Lipid Res.* 1991. **32:** 1983–1991.

Supplementary key words arterial proteoglycan • copper oxidation • macrophage uptake

Early atherosclerotic lesions are characterized by accumulation of apoB-containing lipoproteins focally in the extracellular matrix and by the presence of macrophages, possibly transformed into foam cells by nonregulated uptake of modified apoB lipoproteins (1–3). Immunohistochemical (4, 5) and biochemical evidence (6–11) supports the hypothesis that sulfated proteoglycans of the intimal extracellular matrix contribute to the deposition of apoB lipoproteins in atherosclerotic lesions of animals and man. In vitro experiments show that human LDL that has been complexed to chondroitin sulfate-rich proteoglycans (CSPG) and subsequently dissociated is taken up more avidly by human macrophages with the eventual formation of foam cells (12, 13). These effects are probably caused both by structural alterations of LDL induced by the formation of reversible complexes with the intimal proteoglycans and by selection of LDL particles with a high affinity for the CSPG (14). Such LDL alterations appear to involve a disruption of the lipid–protein organization, which was detected by low angle X-ray analysis (15), differential scanning calorimetry, proton nuclear magnetic resonance (16), and increased susceptibility to tryptic fragmentation (12, 17).

Once apoB-containing particles enter the intima, other types of structural modifications could take place besides those possibly induced by association with extracellular matrix components. An important contributor to the atherogenicity of LDL could be its oxidative modification in the extracellular environment (18, 19). This could cause rapid internalization of LDL by resident macrophages that accumulate at sites of lesion progression in response to chemo-attractants produced during lipid peroxidation.

Abbreviations: CSPG, chondroitin sulfate proteoglycan; GAG, glycosaminoglycan; LDL, low density lipoprotein (d 1.019–1.063 g/ml); HMDM, human monocyte-derived macrophage; TBARS, thiobarbituric acid-reacting substances; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Tris, tri(hydroxymethyl) aminomethane; BHT, butylated hydroxytoluene.

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Cytotoxic products of unsaturated fatty acid and cholesterol may also contribute to the formation of necrotic centers in lesions (20–22). Modification of lysine residues of LDL by reaction with malondialdehyde, one of the products of lipid oxidation, reduces its affinity for heparin (23). Also, any reduction in positive charges of LDL diminish its affinity for human arterial CSPG (24). It has been suggested that oxidation of LDL bound to CSPG in the intima could dissociate the complex, thus making the modified particle available to resident macrophages (25).

In the present study we explored the hypothesis of whether the structural modifications produced in LDL by the in vitro interaction with arterial proteoglycans and purified GAGs could modify its susceptibility to oxidation. We found that the reversible association with sulfated polysaccharides markedly increased the Cu^{2+}-catalyzed oxidation of LDL as evaluated by production of dienes, lipid modifications, formation of thiobarbituric acid-reacting substances, and increased uptake by human macrophages. Results obtained with spectrofluorimetric methods suggest that the enhanced rate of oxidation was related to increased access of Cu^{2+} to nonpolar regions of LDL.

MATERIALS AND METHODS

Preparation of human arterial proteoglycans

Chondroitin sulfate-rich proteoglycan (CSPG) was extracted from human aortic intima-media segments obtained within 24 h postmortem from victims of traffic accidents. The purification procedure and characterization of the CSPG aggregates used here has been described in detail (13, 14, 17). In brief, the intima-media segments were extracted for 24 h with 6 M urea, 1 M NaCl in 10 mM Tris-HCl buffer, pH 7.2, containing protease inhibitors. After centrifugation at 100,000 *g*, the CSPG aggregates in the supernatant were purified in two steps of ionic exchange chromatography in a DEAE Sephacell column (Pharmacia LKB Biotechnology, Uppsala, Sweden) using a 0.25–1.0 M NaCl gradient prepared in 10 mM Tris-HCl buffer, pH 7.2, containing 6 M urea. Further purification was obtained by gel filtration in a Sepharose 4B column (Pharmacia LKB Biotechnology) equilibrated with 10 mM Tris-HCl buffer, pH 7.2, that contained 6 M urea, the protease inhibitors, and 100 mM NaCl. The single hexuronate-containing peak collected contained 60–65% chondroitin-6-sulfate, 10–20% chondroitin-4-sulfate, and 10–20% dermatan sulfate as determined by high performance liquid chromatography of the unsaturated disaccharides produced by treatment with chondroitinase AC and ABC and the use of the appropriate standards (Seikagaku Co., Tokyo, Japan) (14). The proteoglycan moved as a homogeneous band in agarose electrophoresis with 50 mM Tris-glycine buffer, pH 8.2. The protein content was 20–25% of the proteoglycan dry weight, and the molecular weight of the aggregate, evaluated from the gel exclusion chromatography on Sepharose 4B, was greater than 4×10^6.

Low density lipoprotein preparation

Human plasma low density lipoprotein from normolipidemic male donors (d 1.019–1.063 g/ml) was prepared by differential centrifugation using KBr solutions containing 1 mg/ml ethylenediamine sodium tetracetate (EDTA) as previously described (12, 13). The lipoproteins were stored at 4°C in KBr in the presence of 0.1 mg/ml EDTA for not more than a week, and were equilibrated in the appropriate buffer or media before use with the aid of Sephadex PD-10 columns (Pharmacia LKB Biotechnology). With the aid of ^{14}C-labeled EDTA were established that this column removes more than 99% of the EDTA present in the KBr-containing solution. The procedures used for lipoprotein labeling with ^{125}I have been described in detail (13, 14). The lipoprotein to be used for cell culture experiments was labeled prior to treatment with proteoglycan and glycosaminoglycans.

Treatment of LDL with arterial proteoglycan, glycosaminoglycans, and agarose-heparin

The effect of CSPG and glycosaminoglycans (GAG) on LDL was studied under three experimental conditions: *1*) reversible complexes of LDL with CSPG and GAG formed at low ionic strength (20 mM NaCl), collected by centrifugation, washed, and solubilized with physiological ionic strength solutions (140 mM NaCl); *2*) LDL bound at low ionic strength (20 mM NaCl) to CSPG and GAG immobilized in agarose columns and eluted by raising the ionic strength (200 mM NaCl); and *3*) LDL that was mixed with CSPG and GAG at the physiological ionic strength (140 mM NaCl) and that formed only soluble complexes.

With the first treatment, LDL modified by arterial proteoglycan (LDL-CSPG), by chondroitin-6-sulfate (LDL-C6S), and heparin (LDL-Hep) were prepared by adding to solutions of lipoprotein (2 mg apoB protein/ml) sequential 10-μl aliquots of solutions containing 1 mg/ml of arterial CSPG, chondroitin-6-sulfate, or heparin. The lipoprotein, CSPG, and GAG had been previously equilibrated in 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer containing 20 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.2 (buffer A). Approximately 70–80% of the LDL could be precipitated by CSPG or GAG. The complex was collected by centrifugation at 10,000 *g* at 2°C for 10 min. The supernatant containing the nonprecipitated LDL was saved and the pellet was washed with buffer A and redissolved without mechanical stirring in buffer B (5 mM HEPES buffer containing 150 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.2) or in RPMI 1640 cell culture media. Under these conditions the CSPG- and GAG-treated LDL (LDL-CSPG, LDL-

C6S, and LDL-Hep) could be recovered as a nonaggregated solution with no signs of protein or lipid hydrolytic degradation and with a molecular size similar to that of native LDL (LDL-N) (12, 13). The solutions of modified LDL contained less than 10 μg of CSPG or GAG/mg LDL protein measured with a nanogram-sensitive Safranin O method (26).

LDL was also complexed to heparin and to C6S that were covalently bound to agarose (Pharmacia LKB Biotechnology) on 1 × 5 cm columns (agarose-Hep, agarose-C6S). A similar agarose column without the immobilized heparin or C6S was used as blank control. After washing and equilibration of the columns in buffer A, 1-ml aliquots of LDL (1 mg/ml), also in buffer A, were loaded on the agarose-Hep or agarose-C6S columns and the agarose blank columns. The LDL were allowed to remain on the columns for 2 h at 20°C and then eluted with a column volume of buffer B that was made 200 mM in NaCl. Under these conditions the eluted LDL from the experimental (LDL-Hep-agarose, LDL-C6S-agarose) and blank control columns (LDL-N-agarose) were collected in a solvent of similar composition and free of GAG. Heparin grade I was obtained from Sigma (St. Louis, MO) and chondroitin-6-sulfate from shark cartilage was from Seikagaku Co. In all the experiments, untreated LDL (LDL-N) designates lipoprotein that had not been in contact with CSPG or GAG.

Soluble complexes of LDL with CSPG, C-6-S, and heparin were prepared by mixing solutions of LDL at 0.1 mg/ml in buffer B with 0.1 mg/ml of the polysaccharides. In this excess of CSPG and GAG at physiological ionic composition (buffer B) no insoluble complex is formed.

Oxidation of LDL-N and LDL treated with CSPG and GAG

LDL-N, LDL-CSPG, LDL-C6S, LDL-Hep, LDL-Hep-agarose, LDL-C6S-agarose, and LDL-N-agarose were adjusted to 200 μg protein/ml with buffer B. The lipoproteins were incubated at 37°C under air with 5 or 10 μM, CuCl, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $FeCl_3 \cdot H_2O$. The incubation was carried out in acid-washed borosilicate glass tubes with Teflon-lined, loosely tightened screw caps for up to 48 h. At different time periods, 0.5-ml aliquots of the incubate were withdrawn and made 20 μM in butylated hydroxytoluene (BHT) with a 1 mM ethanolic solution to delay the oxidative process until they were analyzed. Control incubations at 37°C were done without the metals and with BHT added at zero time.

LDL degradation by human macrophages

Human monocyte-derived macrophages (HMDM) were prepared from "buffy coats" obtained from healthy donors (13, 27). LDL-N and LDL-CSPG labeled with ^{125}I to 50–60 dpm/ng protein (13, 14) were oxidized for 0, 1, 4, and 48 h as described above. HMDM that had been cultured for 7 days were incubated with lipoprotein-depleted RPMI 1640 medium for 24 h before adding the different labeled lipoproteins. LDL-N and LDL-CSPG oxidized for different periods and equilibrated in cell culture medium were added at two concentrations, 5 and 10 μg protein/ml medium, to cells cultured at 37°C in 35-mm diameter wells in a 95% air:5% CO_2 atmosphere. After 5 h of incubation with the macrophages, the extent of lipoprotein degradation was determined by measuring the content of chloroform-extractable, acid-soluble ^{125}I-monoiodotyrosine in the media (13). Parallel dishes without cells were included as control for unspecific degradation of the lipoproteins.

Fluorescence quenching experiments

Fluorescence measurements were made in an RF-5000 Shimatzu spectrofluorophotometer (Tokyo, Japan) using a thermostatted cell holder. Aliquots of the quenchers, CuCl, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $FeCl_3 \cdot 6H_2O$ and acrylamide, dissolved in buffer B, were added to solutions of LDL-N, LDL-CSPG, LDL-C6S, LDL-Hep, and LDL-agarose also prepared in buffer B containing 20 μM BHT to avoid oxidation during the measurements. The samples were incubated at 20 or 40°C for 30 min under air before measurements. To evaluate the effect of the quenchers on the intrinsic fluorescence of LDL, the emission spectrum of each sample was collected between 300 and 400 nm with excitation at 280 nm. The same samples were used for measurements of absorbance at 280 and 330 nm. Under these conditions no signs of oxidation were detected in the lipoprotein solutions after the measurements and the samples were not turbid.

Treatment of fluorescence data

In the quenching experiments, inner filter effects of the LDL and the quenchers were corrected with the formula:

$$Fc = (Fob)\ \text{antilog}\ \{[(A_{280} + A_{330})]/2\} \qquad \textit{Eq. 1)}$$

where Fc is the corrected integral of the emission spectra, Fob is the observed integral, and A_{280} and A_{330} are the absorbances at the excitation and emission maxima (28, 29). Stern-Volmer quenching constants were evaluated from the slope of the plot of Fo/Fc versus the quencher concentration according to equation 2:

$$Fo/Fc = 1 + K_{SV}[Q] \qquad \textit{Eq. 2)}$$

where Fo is the corrected integral of the emission spectra in the absence of quencher, Fc is the corrected integral of the emission spectra in the presence of quencher, K_{SV} is the Stern-Volmer constant, and [Q] is the quencher molar concentration (28, 29). The fraction of fluorescent chromophores (mainly tryptophan residues) accessible to the quencher (f_a) and the association constant for the

quencher (K_a) were calculated using the modified form of the Stern-Volmer equation for two populations of chromophores:

$$Fo/(Fo - Fc) = [(1)/(f_a K_a Q)] + [(1/f_a)] \qquad Eq.\ 3)$$

The parameters were obtained from the experimental observations using linear regression (28, 29).

Analytical procedures

Protein of the lipoproteins and cells was measured with a modification of the procedure of Lowry et al. (30) before oxidation. Estimation of thiobarbituric acid substances and formation of conjugated dienes were carried out essentially as described by Kim and Labella (31). The lipid composition of the native and oxidized lipoproteins was determined in chloroform-methanol 2:1 (v/v) extracts. The washed chloroform phase was evaporated under N_2 and redissolved in heptane for spotting in quartz chromatorods. The main lipid classes were measured after thin-layer chromatography by means of flame ionization in an Iatroscan TH-10 instrument (Iatron Lab. Inc., Tokyo, Japan) (14). With this procedure only nonoxidized lipids remaining in the samples are measured. The vitamin E content of lipids extracts of LDL was measured fluorimetrically (32). Radioactivity of ^{125}I-labeled samples was measured with an LKB 1282 Compugamma Counter (LKB, Bromma, Sweden). Agarose isoelectric focusing was performed in a Phast system as previously described (33). The statistical significance of the pertinent data was evaluated by Student's t test. In all analyses and preparations only analytical reagents were used.

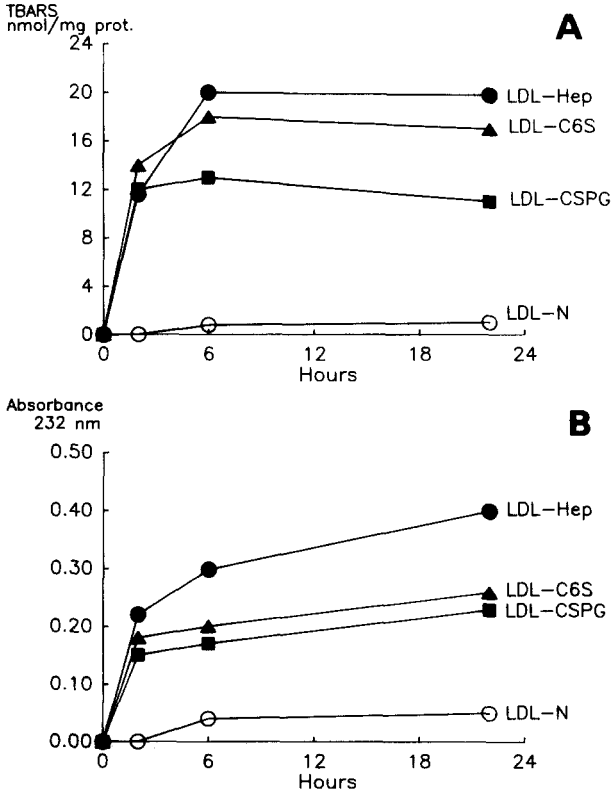

Fig. 1. Cu^{2+} oxidation of LDL that was complexed and dissociated from arterial chondroitin sulfate-rich proteoglycans (LDL-CSPG), chondroitin-6-sulfate (LDL-C6S), and heparin (LDL-Hep). Nontreated LDL (LDL-N) was used as control. The LDL were oxidized by 5 μM $CuSO_4$. Thiobarbituric acid-reacting substances (A) and formation of conjugated dienes (B) were measured at the indicated times.

TABLE 1. Alterations in lipid content induced by Cu^{2+} oxidation on native LDL (LDL-N) and LDL treated with arterial CSPG (LDL-CSPG)

Lipoprotein	Hours[a]	CE[b]	Chol[b]	PC[b]	SPC[b]	LPC[b]
LDL-N(BHT)	14	1.63	0.42	0.95	0.15	0.01
LDL-N(BHT)	24	1.66	0.40	0.87	0.17	0.03
LDL-N	14	1.48	0.30	0.64	0.12	0.09
LDL-N	24	1.21	0.30	0.60	0.12	0.18
LDL-CSPG(BHT)	14	1.71	0.35	0.81	0.24	0.23
LDL-CSPG(BHT)	24	1.60	0.30	0.38	0.15	0.35
LDL-CSPG	14	0.03	0.09	0.23	0.03	0.03
LDL-CSPG	24	0.04	0.09	0.26	0.05	0.03

[a]Hours of incubation with 5 μM $CuSO_4$.
[b]The lipids fractionated into cholesteryl esters (CE), free cholesterol (Chol), phosphatidylcholine (PC), and lysophosphatidyl choline (LPC). The values represent averages of duplicate measure in individual extracts and are expressed as mg of each lipid class/mg apoB protein.

RESULTS

Effect of CSPG and GAG on LDL oxidation rate

Low density lipoprotein that had been insolubilized with arterial CSPG, C6S, and heparin at low ionic strength (buffer A) and dissociated by raising the concentration of NaCl to physiological levels (buffer B), when exposed to Cu^{2+} was oxidized at a faster rate than native LDL. This was shown by the rate of appearance of TBA-reacting substances and conjugate diene formation (**Fig. 1**). Also the conversion of the lipids in LDL to products that were not extracted by chloroform-methanol 2:1 and that remained at the origin during thin-layer chromatography took place markedly faster in LDL that had been complexed and dissociated from arterial CSPG. This was prevented by 20 μM BHT added to the samples before adding Cu^{2+}. **Table 1** compares the rate of disappearance for the main lipid classes of LDL-N and LDL-CSPG. The results indicate that the cholesteryl esters were especially sensitive.

Agarose isoelectric focusing of LDL-N and LDL-CSPG (results not shown) indicate that after 4 h of oxidation by Cu^{2+} LDL-CSPG changed its isoelectric point from 5.20 to 4.80, whereas it took 48 h of oxidation to change the isoelectric point of LDL-N from 5.20 to 5.00. Therefore LDL-CSPG became a more negatively charged particle at physiological pH than LDL-N after a comparable oxidation period.

We found no differences between the vitamin E content of the LDL complexed with GAG and that of untreated LDL that could account for the dissimilarities in oxidation rate. In the LDL preparation used, the vitamin E content, expressed as α-tocopherol, was between 3.5 and 4.5 μg/mg apoB before and after exposure to CSPG and C6S. However, it may be possible that some of the differences could have been caused by changes in the concentration of other endogenous or exogenous antioxidants or chelators introduced during formation and solubilization of the complex. To distinguish this possibility from the effects associated with structural alterations induced by association with sulfated polysaccharides, the LDL was modified with heparin and C6S immobilized on agarose columns. With this design the only difference in the solutions of LDL eluted from the columns should be that introduced by the noncovalent association with the immobilized heparin and C6S. **Fig. 2** compares the extent and rate of Cu^{2+}-catalyzed oxidation for LDL that was bound to heparin-agarose and the one loaded on the control column as measured by formation of TBARS and dienes. Also, with this experimental design the modification caused by the sulfated polysaccharides accelerated the oxidative alterations when compared with those measured in LDL that was loaded on a similar column with only agarose. Experiments with LDL preparations from three donors gave similar results. Comparable results were obtained with LDL modified by C6S-agarose columns (results not shown). Incubation of LDL-N (200 μg/ml protein) with 5 and 10 μM CuCl, $FeCl_3$, and $FeSO_4$ for up to 24 h induced no measurable formation of TBARS or dienes above the values detected in controls containing 20 μM BHT. Pretreatment of LDL with C6S and heparin failed to increase the oxidation rate of LDL by CuCl, $FeSO_4 \cdot 7H_2O$, and $FeCl_3 \cdot 6H_2O$ above control values after 24 h incubation (0.2 nmol/mg apoB protein).

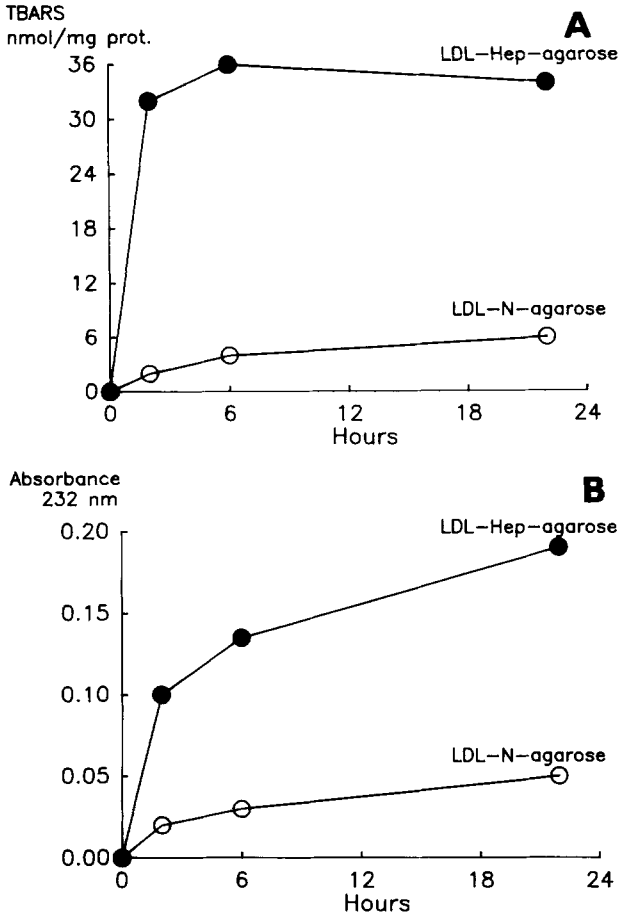

Fig. 2. Cu^{2+} oxidation of LDL that had been complexed and dissociated from a column of agarose-heparin. LDL at 1 mg/ml in buffer A (20 mM NaCl) was loaded on an agarose-heparin column (5 × 1 cm) and on a control column with only agarose. After 2 h in the columns, LDL from the agarose-heparin column (LDL-Hep-agarose) and the control column (LDL-N-agarose) were eluted with buffer B made 200 mM in NaCl. The lipoproteins were then subjected to Cu^{2+} oxidation and aliquots were used for measurements of thiobarbituric acid-reacting substances (A) and diene formation (B).

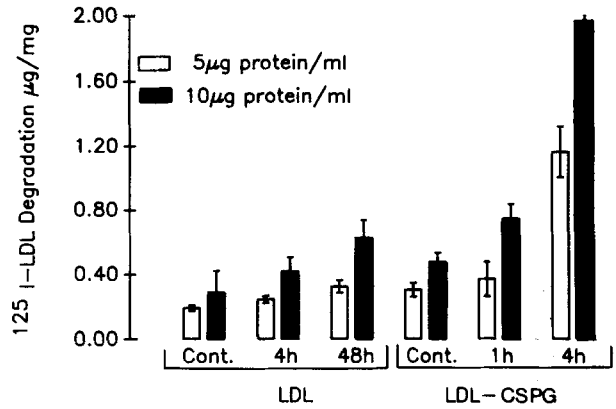

Fig. 3. Degradation of LDL by human macrophages. ^{125}I-labeled LDL that had been complexed with and dissociated from human arterial proteoglycans (LDL-CSPG) and native LDL (LDL-N) were oxidized with 5 μM $CuSO_4$ for the indicated periods. The oxidized lipoproteins were then added to cultures of monocyte-derived macrophages at 5 and 10 μg protein/ml of media. Degradation was estimated from the monoiodotyrosine in the media after 5 h incubation.

Degradation of LDL by human macrophages

Human monocyte-derived macrophages degraded arterial proteoglycan-modified LDL (LDL-CSPG) much more efficently than native LDL (LDL-N) exposed for comparable periods to Cu^{2+}-catalyzed oxidation. **Fig. 3** shows that LDL-CSPG oxidized for only 4 h was degraded 2- to 3-times more than LDL-N oxidized for 48 h. The increased degradation may reflect the higher extent of oxidative modifications and lower isoelectric point of LDL-CSPG when compared to LDL-N oxidized for similar periods. In these experiments we did not measure the rate of oxidation of the different lipoproteins by the cells and it is possible that the CSPG- and GAG-modified LDL may also have been more sensitive to cell-induced alterations.

Effect of copper and iron on the fluorescence of native LDL and LDL treated with sulfated polysaccharides

The quenching of tryptophan fluorescence by metals, non-metals, and acrylamide has been extensively used to measure affinity of the quenchers for proteins and to probe for the extent of exposure of the protein chromophores (28, 29). In our experiments, Cu^{2+} was a more efficient quencher of the LDL intrinsic tryptophan fluorescence after the lipoprotein had been treated with CSPG, heparin, or C6S and resolubilized than of the fluorescence of native LDL. **Fig. 4** shows the curves obtained for LDL-N, LDL-Hep, and LDL-CSPG after titration with Cu^{2+}. The fraction of chromophores (f_a) that were accessible to Cu^{2+} increased 3- to 4-fold for the LDL-CSPG, 6- to 7-fold for LDL-Hep, and 2- to 3-fold for LDL-C6S (the last one not shown in Fig. 4). K_a, the association constant between the chromophores and Cu^{2+}, also increased in the presence of the sulfated polysaccharides. **Table 2** presents a summary of the results.

Acrylamide is a very polar quencher of tryptophan chromophores in proteins (28, 29) and therefore only reaches those chromophores exposed to the solvent. **Fig. 5** presents the titration of LDL-N and LDL-CSPG with acrylamide. The curves had essentially the same intercept and slope, indicating that regions to which acrylamide had access (very polar) were not altered by the presence of arterial proteoglycan. This suggests that treatment with CSPG and GAG opens only nonpolar pockets of LDL to Cu^{2+}.

Quenching of protein chromophores could be caused either by collisional deactivation of the excited state by Cu^{2+} (collisional quenching) or by formation of complexes at the ground state level that do not fluoresce (static quenching). The first process is diffusion-controlled and therefore an increase in temperature should increase the slope of the titration curve described by the equation $Fo/Fc = 1 + K_{SV}[Cu^{2+}]$ (28). **Fig. 6** presents the curves obtained by titration of LDL-N and LDL-CSPG with Cu^{2+} at 20° and 40°C. The increase in the initial slope with temperature suggests that the effect of copper is caused by collisional quenching. The curves for LDL-N suggest that only a single class of chromophores was affected by the quencher, whereas those for LDL-CSPG indicate that there were at least two populations of chromophores interacting with Cu^{2+} since a downward nonlinear plot was obtained (28, 29).

To explore whether the alterations in the interactions with Cu^{2+} were caused only by formation and dissolution of LDL aggregates, the lipoprotein was bound and eluted from a heparin-agarose column or from a control agarose column as described above for the oxidation experiments. However, in this experimental condition the lipoproteins were always maintained in the presence of 20 μM BHT.

TABLE 2. Effect of arterial condroitin sulfate-rich proteoglycan (CSPG), heparin, and chondroitin-6-sulfate (C-6-S) on Cu^{2+}-induced quenching of LDL chromophores

Lipoprotein	Accessible Chromophore Fraction	K_a (μM^{-1})
LDL-Native	0.15 ± 0.05	0.12 ± 0.05
LDL-CSPG	0.60 ± 0.20	0.20 ± 0.04
LDL-Hep	0.80 ± 0.09	0.23 ± 0.04
LDL-C-6-S	0.35 ± 0.12	0.18 ± 0.06

The values represent averages ± standard deviation of four individual titrations using the same LDL preparation.

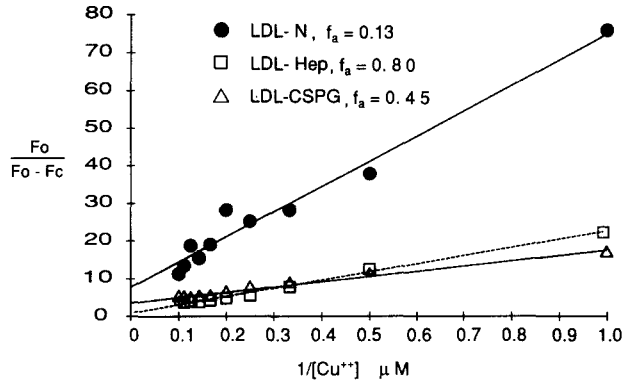

Fig. 4. Modified Stern-Volmer plots of the quenching by Cu^{2+} of the LDL intrinsic fluorescence. Native LDL (LDL-N) and LDL that was complexed with human arterial chondroitin sulfate proteoglycans (LDL-CSPG) and heparin (LDL-Hep) was dissolved in buffer B (HEPES 10 mM, 150 mM NaCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 20 μM BHT, pH 7.2) to 100 μg/apoB-100 protein/ml. All the solutions contained 20 μM BHT to avoid oxidation of LDL. Cu^{2+} was added to duplicate aliquots of these solutions to the indicated final Cu^{2+} concentrations. After 1 h at 20°C, the spectra were collected and processed as indicated in Methods; f_a gives an estimate of the fraction (0 to 1.0) of chromophores (mainly tryptophan residues) accessible to the quencher. The lines were drawn by linear regression.

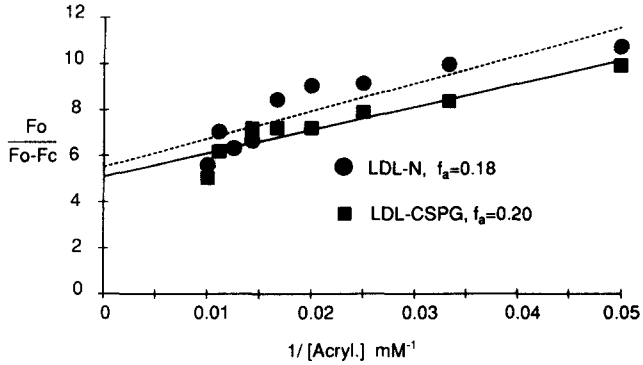

Fig. 5. Modifier Stern-Volmer plots of the quenching by acrylamide of the fluorescence of native LDL (LDL-N) and arterial proteoglycan-treated LDL (LDL-CSPG). Acrylamide was added at the indicated final concentrations to duplicate aliquots of the lipoprotein solutions. After 1 h at 20°C the spectra were collected and processed as indicated in Methods. Other conditions were as described in Fig. 4.

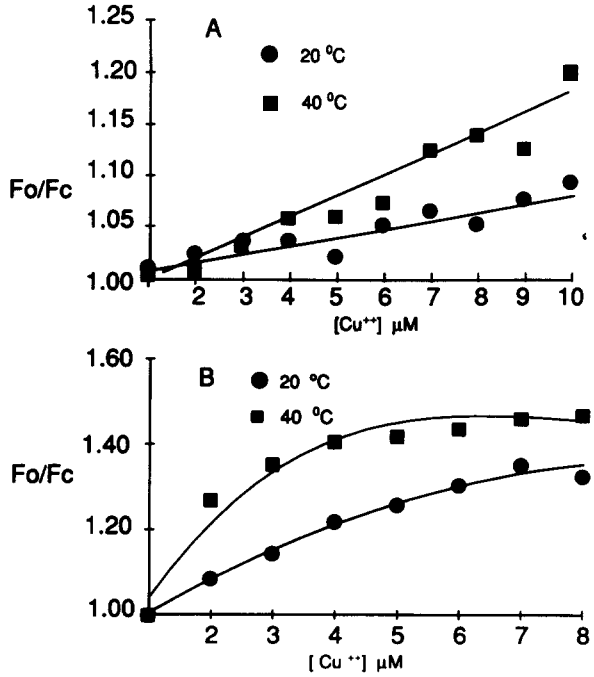

Fig. 6. Effect of temperature on the direct Stern-Volmer plots of quenching by Cu^{2+} of the fluorescence of native LDL (A) and LDL complexed to arterial CSPG (B). Native LDL (LDL-N) and to LDL that was complexed and dissociated from human arterial chondroitin sulfate proteoglycans (LDL-CSPG) were dissolved in buffer B to 100 μg/apoB-100 protein per ml. Cu^{2+} was added to the indicated concentrations to duplicate aliquots of this solution. After 1 h at 20° or 40°C, the spectra were collected and processed. Other conditions were as described in Fig. 4.

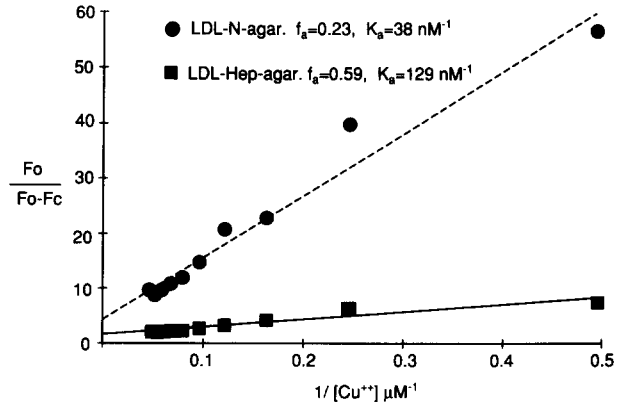

Fig. 7. Modified Stern-Volmer plots of the quenching of fluorescence by Cu^{2+} of LDL that was found and eluted from an heparin-agarose column (LDL-Hep-agar) and LDL that was loaded and eluted from a control column with only agarose (LDL-N-agar). Other conditions were as described in Fig. 4.

Fig. 7 shows that the LDL that was bound to the heparin-agarose column retained, after elution, the modifications responsible for the increased affinity for Cu^{2+}. Cu^{2+} also quenched fluorescence of LDL to a similar extent in the presence of either CSPG or C6S in buffer-B in solution (data not shown). This also indicates that the CSPG- and C6S-induced alterations responsible for the phenomenon were present in soluble associations. With the conditions used (see Methods) the fraction of accessible chromophores (f_a) increased to a level similar to that observed with the insolubilized-solubilized complex (not shown). No fluorimetric measurements were possible with soluble LDL-heparin complex because μM concentrations of Cu^{2+} produced immediate aggregation of the lipoprotein.

Cu^{2+} at micromolar concentrations is a better catalyst of oxidative modifications in LDL than Fe^{3+} and naturally Fe^{2+} and Cu^{+}. We found that only Cu^{2+} was capable of quenching the intrinsic fluorescence of tryptophan. Fe^{2+}, Fe^{3+}, and Cu^{+}, even at higher concentrations, altered neither the fluorescence of LDL-N nor that of LDL-CSPG and LDL-Hep. **Fig. 8** shows the curves with zero slope obtained with Fe^{2+} for LDL-N and LDL-CSPG. Similar curves were obtained with Fe^{3+} and Cu^{+} (not shown). These data also indicated that the 20 μM BHT used in the experiments was not capable of reducing Cu^{2+} to Cu^{+}.

DISCUSSION

Oxidation catalyzed by transition metals has been used extensively as a model to study the structural changes caused by reactive oxygen species and its consequences for lipoprotein–cell interactions (18, 19, 22, 23, 34). Recently, Thomas and Jackson (35) have provided evidence that Cu^{2+} oxidation of LDL requires the pre-existence of small amounts of lipid hydroperoxides (LOOH) in the lipoprotein. Although the exact mechanisms for LDL oxidative modifications in the arterial intima are not known, in vitro experiments indicate that release of transition metals by dying cells and the natural free radical generation by macrophages, smooth muscle cells, and endothelial cells may be implicated (19, 34). Focal accumulation of apoB-lipoproteins by specific retention in the extracellular compartment appears to precede lesion development (36). Intimal chondroitin sulfate-rich proteoglycans may increase the residence time of LDL in the intima allowing further modifications (25, 37). Our in vitro results suggest

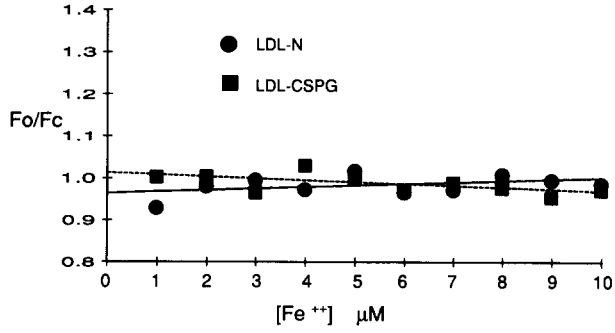

Fig. 8. Direct Stern-Volmer plot of the lack of effect of Fe^{2+} on the fluorescence of native LDL (LDL-N) and LDL treated with arterial CSPG (LDL-CSPG). The complexed LDL-CSPG was redissolved in buffer B. Other conditions were as described in Fig. 4.

that the sulfated glycosaminoglycans of arterial proteoglycans, besides forming complexes with LDL, may introduce structural modifications that increase the lipoprotein susceptibility to Cu^{2+}-catalyzed oxidation. Arterial proteoglycans in vitro alter the structure of both the lipid and protein moiety of LDL (12–17) and select LDL subclasses with higher susceptibility for these alterations (14).

Although we do not know which specific structural change in LDL may be responsible for the increased susceptibility to oxidation, the results from the fluorescence quenching experiments suggest that an elevation in the lipoprotein affinity for Cu^{2+} may be responsible. The previously observed loss of organization in the lipid core detected by low-angle X-ray diffraction and differential scanning calorimetry and increased susceptibility to proteolysis suggest a more open structure for proteoglycan- and glycosaminoglycan-treated LDL (12–17). Such structures appear to have more binding sites for Cu^{2+}. Our data indicate that Cu^{2+} is a poor tryptophan quencher for native LDL but a good one for CSPG- or GAG-treated LDL. On the other hand, the polar quencher acrylamide had very little access to the chromophores of native LDL and CSPG- or GAG-modified LDL. The newly CSPG- and GAG-created sites on LDL appear rather specific for the structure of Cu^{2+} and its associated hydration shell since Cu^{+}, Fe^{2+}, and Fe^{3+} did not alter the fluorescence of the GAG- or CSPG-modified LDL.

The affinity constants measured by tryptophan quenching give only relative estimates of the Cu^{2+} affinity for the sites containing the chromophore but not for the total LDL particle. However, the CSPG- and GAG-induced apparent increase in Cu^{2+} affinity and enhanced access to internal regions of the apoB-100 could account for the enhanced susceptibility to Cu^{2+}-catalyzed oxidation of the modified LDL. The net result could be an increased number of Cu^{2+} ions in hydrophobic regions of the LDL molecule whereas they could catalyze the formation of peroxyl ($LOO^{\cdot}$) and alkoxyl radicals ($LO^{\cdot}$) from the polyenoic fatty acids of phospholipids and cholesteryl esters (35). The observed increase of the Cu^{2+} quenching of modified and native LDL with increasing temperature points to diffusion limited interaction of Cu^{2+} with the particle. These barriers may be lowered by the disorganization induces by the GAG and by running the experiments just above the transition temperature of the LDL lipids (37°C) as those in Fig. 6 (17). Although we are not aware of detailed studies of metal binding to LDL (38), it has been found that in the presence of physiological concentrations of LDL no free Cu^{2+} is detectable in 1–80 μM Cu^{2+} solutions, which suggests a relative high affinity (39).

Previous in vitro findings indicate that once LDL has interacted with proteoglycans, its uptake by human or mouse peritoneal macrophages increases leading to foam cell formation (13, 14, 40). If, as shown here, proteoglycan-induced modifications also potentiate Cu^{2+} binding and subsequent lipoprotein oxidation, this process may be an additional contributor to lipoprotein modification and to transformation of macrophages into foam cells.

Partially supported by grants from the Swedish Medical Research Council (projs. 4531, 7142, 7115), the Swedish Association Against Heart and Chest Disease, AB Astra-Hässle, and Consejo Venezolano de Investigaciones Científicas (proj. SI. 1987). We are grateful to Dr. Daniel Steinberg for useful suggestions concerning the experimental design used and to Johanna Deinum for helpful discussions during the preparation of the manuscript. Part of the results in this manuscript were presented in the 63rd Annual Scientific Session of the American Heart Association, Dallas, TX 1990.

Manuscript received 18 June 1991 and in revised form 30 September 1991.

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