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Oxidation of Low Density Lipoprotein Particles Decreases Their Ability to Bind to Human Aortic Proteoglycans

DEPENDENCE ON OXIDATIVE MODIFICATION OF THE LYSINE RESIDUES*

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Oxidation of low density lipoprotein (LDL) leads to its rapid uptake by macrophages *in vitro*, but no detailed studies have addressed the effect of oxidation on the binding of LDL to proteoglycans. We therefore treated LDL with various substances: copper sulfate, 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH), soybean lipoxygenase, and mouse peritoneal macrophages, and determined the extent to which the oxidatively modified LDL bound to human aortic proteoglycans in an affinity column. Oxidation of LDL with copper, AAPH, or macrophages, all of which increased its electrophoretic mobility, was associated with reduced binding to proteoglycans, until strongly oxidized LDL was totally unable to bind to them. After treatment of LDL with soybean lipoxygenase, the change in electrophoretic mobility was small, and the amount of binding to proteoglycans was only slightly decreased. The increased electrophoretic mobility of oxidized LDL reflects modification of the lysine residues of apolipoprotein B-100 (apoB-100). To mimic the oxidative modification of lysines, we treated LDL with malondialdehyde. This treatment also totally prevented the binding of LDL to proteoglycans. In contrast, if the lysine residues of apoB-100 were methylated to shield them against oxidative modification, subsequent treatment of LDL with copper sulfate failed to reduce the degree of LDL binding to proteoglycans. Finally, the active lysine residues in the oxidized LDL particles, which are thought to be involved in this binding, were quantified with NMR spectroscopy. In oxidized LDL, the number of these residues was found to be decreased. The present results show that, after modification of the lysine residues of apoB-100 during oxidation, the binding of LDL to proteoglycans is decreased, and suggest that oxidation of LDL tends to lead to intracellular rather than extracellular accumulation of LDL during atherogenesis.

Human atherosclerosis is characterized by accumulation of low density lipoprotein (LDL)[1] in the arterial intima. In atherosclerostic lesions, LDL is modified and accumulates both intra- and extracellularly. The LDL modification that has been most studied is oxidation. The role of LDL oxidation in intracellular accumulation is well established. Thus, oxidized LDL is taken up by macrophage scavenger receptors, which transform the macrophages into foam cells (1).

In contrast, the role of oxidation of LDL in extracellular accumulation of LDL is not known. An important component of extracellular accumulation of LDL is binding of LDL to the extracellular matrix consisting mainly of proteoglycans (2, 3). Native LDL binds proteoglycans via ionic interactions between the positively charged lysine and arginine residues of the apolipoprotein component of LDL, the apoB-100, and the negatively charged sulfate and carboxyl groups of glycosaminoglycan chains of the proteoglycans (4–7). Upon oxidation of LDL, the unsaturated fatty acids of LDL lipids are decomposed, for instance, to malondialdehyde (MDA) and 4-hydroxynonenal. These compounds can then react with the lysine residues of apoB-100, thereby neutralizing them and so interfering with their ability to interact with other molecules. Indeed, Haberland *et al.* (8) found that MDA-modified LDL failed to bind to heparin.

In this study we oxidized LDL *in vitro* by several methods and analyzed the effect of oxidation on its degree of binding to proteoglycans isolated from human aortas. We also studied the effects of lysine modification on the degree of binding, by specifically modifying the lysine residues of apoB-100, and by preventing lysine oxidation by prior methylation of the lysine residues. Since the lysine residues of apoB-100 are of two types having different pK_a values, 8.9 and 10.5 (9), of which the former "active lysines" are thought to be located in the areas of apoB-100 that bind to proteoglycans, we also investigated the effect of oxidation on this population of lysine residues in LDL.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin, butylated hydroxytoluene (BHT), cholesteryl linoleate, ϵ-aminocaproic acid, and soybean lipoxygenase type V were from Sigma; copper(II) sulfate pentahydrate, EDTA, and CHOD-iodide kit (catalog no 14359) for peroxide measurement were from Merck; malondialdehyde bis(dimethyl acetal) was from Aldrich; and 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) was from Polysciences. Chondroitinase ABC and AC, chondroitin-6-sulfate, and the unsaturated chondro-disaccharide kit for high performance liquid chromatography (HPLC) were from Seikagaku Kogyo. [1,2-^{3}H]Cholesteryl linoleate was from Amersham. Phenylmethylsulfonyl fluoride was from Boehringer Mannheim, Celite 545 (acid-washed) was from Fluka, and the Schrynel nylon filter from Zürcher Beuteltuchfabrik AG. [^{13}C]Formaldehyde (99% isotope enrichment) as a 20% solution in water was from Isotec Inc., and [^{14}C]formaldehyde from DuPont. $NaCNBH_3$ from Sigma was purified by recrystallization from dichloromethane before use (10). Dulbecco's phosphate-buffered saline (PBS), RPMI 1640 culture medium with 25 mM HEPES, fetal calf serum, penicillin, and streptomycin were from Life Technologies, Inc. Female NMRI mice (20–30 g) were obtained from a licensed animal center (Poikkijoki). Superose 6 HR 10/30 columns, HiTrap *N*-hydroxysuccinimide activated columns,

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[1] The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; MDA, malondialdehyde; BHT, butylated hydroxytoluene; AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive substances.

HiTrap heparin columns, HiTrap Q columns, and PD-10 columns were from Pharmacia LKB Biotechnology; Bio-Gel A-0.5m gel filtration medium was from Bio-Rad; the 5-μm NH_2 (0.3 × 25 cm) and S5 ODS (0.3 × 25 cm) columns were from Spherisorb. Cholesteryl ester transfer protein was a kind gift from Drs. C. Ehnholm and M. Jauhiainen, National Public Health Institute, Helsinki, Finland.

Methods

Preparation and Labeling of LDL—Human LDL (d = 1.019–1.050) was isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mM EDTA (11). [^{3}H]Cholesteryl linoleate-LDL ([^{3}H]CL-LDL) was prepared by incubating LDL and cholesteryl ester transfer protein with solid dispersions of [^{3}H]cholesteryl linoleate on Celite for 18–24 h as described previously (12). The amount of LDL is expressed in terms of its protein concentration.

Oxidation of LDL—LDL was oxidized with copper by incubating LDL or [^{3}H]CL-LDL (1 mg/ml) in PBS supplemented with 5 μM copper sulfate at 37 °C for the time periods indicated (13). LDL was oxidized with AAPH by incubating [^{3}H]CL-LDL (1 mg/ml) in buffer A (150 mM NaCl, 1 mM EDTA, pH 7.4) supplemented with 25 mM AAPH at 37 °C for the time periods indicated (14). Oxidation was terminated by addition of BHT and EDTA (see below) and cooling the sample on ice. AAPH was removed from the sample over a PD-10 column equilibrated and eluted with ice-cold buffer A. LDL was oxidized by mouse peritoneal macrophages essentially as described elsewhere (15). Briefly, [^{3}H]CL-LDL (100 μg/ml) was incubated in 300 μl of RPMI 1640 cell culture medium supplemented with 300 nM copper sulfate in a 24-well plastic culture plate seeded with 1×10^6 macrophages in a humidified CO_2 incubator (5% CO_2 in air) at 37 °C for the time periods indicated. LDL was oxidized with soybean lipoxygenase by incubating [^{3}H]CL-LDL (2.5 mg/ml) in buffer A containing 1.25 mM linoleate-albumin and 60 units/μl soybean lipoxygenase at 37 °C for 18 h (16). LDL was reisolated by gel filtration chromatography on an A-0.5m column (1 × 50 cm). In all systems, oxidation was terminated by addition of BHT and EDTA to give final concentrations of 20 μM and 1 mM, respectively.

Treatment of LDL with Malondialdehyde—LDL was modified with MDA by incubating [^{3}H]CL-LDL (1 mg/ml in PBS) with increasing amounts of MDA on an ice bath for 3 h (17). The final concentrations of MDA are given in the figure legends. After incubation, LDL was dialyzed extensively against buffer A.

Methylation of LDL—To 2 mg of [^{3}H]CL-LDL in 2 ml of buffer A (150 mM NaCl, 1 mM EDTA, pH 7.4), 0.2 M $NaCNBH_3$ was added to give a final concentration of 20 mM. After addition of 15×10^{-6} mol of formaldehyde to the sample, the mixture was incubated at 4 °C for 18 h (10).

Analysis of Modified LDL—β-Carotene was analyzed spectrophotometrically by measuring the absorbance of the β-carotene in LDL (1 mg/ml) at 482 nm (18). Reactive lysine residues (ϵ-amino groups of lysine residues) were determined with trinitrobenzenesulfonic acid (19), using valine as standard, and expressed as percentages of the value for native LDL. The quantity of conjugated dienes in LDL was assessed by measuring the absorbance of LDL (0.1 mg/ml) at 234 nm with a spectrophotometer fitted with a cuvette with a 1-cm light path (20). Lipid peroxides were measured as described by El-Saadani *et al.* (21) with a commercial CHOD-iodide kit. Thiobarbituric acid-reactive substances (TBARS) were measured essentially as described by Hessler *et al.* (22), and expressed as MDA equivalents, with 1,1,3,3-tetramethoxypropane as standard. The electrophoretic mobility of LDL was determined on cellulose acetate plates (Helena Laboratories). The extent of LDL aggregation was determined by analyzing oxidized [^{3}H]CL-LDL with gel filtration column chromatography on two Superose 6 HR 10/30 columns connected in series. The degree of aggregation was measured as the proportion of ^{3}H radioactivity eluting in the void volume of the column.

Preparation and Characterization of Aortic Proteoglycans—Proteoglycans from intima-media of human aortas obtained at autopsy within 24 h of accidental death were prepared essentially as described by Hurt-Camejo *et al.* (23). Briefly, proteoglycans were extracted from the intima-media at 4 °C for 24 h with 15 volumes of 6 M urea, 1 M NaCl in the presence of 10 mM EDTA, 10 mM ϵ-aminocaproic acid, 0.2 mM phenylmethyl sulfonyl fluoride, and 0.02% (w/v) NaN_3. After extraction, the mixture was centrifuged at 100,000 × g for 60 min. The supernatant was diluted with 6 M urea to give a final concentration of 0.25 M NaCl and loaded on a HiTrap Q column (5 ml) equilibrated with 6 M urea, 0.25 M NaCl, 10 mM $CaCl_2$, and 50 mM acetate, pH 6.2, and the protease inhibitors. The column was washed with the above buffer, and the proteoglycans were eluted with a linear gradient of 0.25 to 1.0 M NaCl in the buffer (120 ml) at a flow rate of 2 ml/min. The peaks at 280 nm were collected, dialyzed against water, and lyophilized. The disaccharide composition of the proteoglycans was analyzed by HPLC, using a 5-μm NH_2 column, after treatment of the proteoglycans with chondroitinase ABC and AC (24). The proteoglycan preparation used here contained 56% of chondroitin-6-sulfate, 25% of chondroitin-4-sulfate, and 19% of dermatan sulfate. The amounts of the proteoglycans are expressed in terms of their glycosaminoglycan content.

Chromatography of Oxidized LDL on a Proteoglycan, Chondroitin-6-Sulfate, or Heparin Affinity Column—Human arterial proteoglycans or chondroitin-6-sulfate were coupled to an *N*-hydroxysuccinimide-activated HiTrap column (1 ml or 5 ml, respectively) according to the manufacturer's instructions. For this purpose, 1.0 mg of proteoglycans or 10 mg of chondroitin-6-sulfate in 0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, were coupled to the column at 25 °C for 2 h. The column was blocked with 0.5 M ethanolamine, pH 8.3, containing 0.5 M NaCl. Under these conditions, 0.7 mg of the proteoglycans and 7.4 mg of the chondroitin-6-sulfate were found to be coupled to the column. The columns were equilibrated with buffer B (10 mM HEPES, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) before use.

LDL or [^{3}H]CL-LDL was oxidized as described above, and 20–30-μl samples of the incubation mixtures corresponding to 20–30 μg of LDL were analyzed on the proteoglycan or heparin affinity columns by elution with a linear gradient of NaCl (0 to 250 mM or 0 to 500 mM, respectively, in 10 min) in buffer B. Chromatography was performed at a flow rate of 1 ml/min. LDL was eluted from the chondroitin-6-sulfate column at a flow rate of 2 ml/min with buffer B containing 250 mM NaCl. Proteins were detected by UV absorbance at 280 nm or, in some experiments in which radiolabeled LDL was used, by collecting fractions and determining their radioactivity. The conductivity of the eluent was also monitored. The chromatographic apparatus was Smart system (Pharmacia).

Preparation of ^{13}C-Labeled LDL and ^{13}C-Labeled Oxidized LDL for NMR—LDL (30 mg) was incubated for 6 h with 5 μM $CuSO_4$ in 30 ml of PBS at 37 °C. Oxidation was terminated by addition of BHT and EDTA to give final concentrations of 20 μM and 1 mM, respectively. For NMR analysis, the amino groups of the free lysine residues of apoB-100 of native or oxidized LDL were ^{13}C-labeled by reductive methylation with [^{13}C]formaldehyde (9, 10). First, 0.2 M $NaCNBH_3$ was added to 30 mg of native or oxidized LDL in 30 ml of buffer A to give a final concentration of 20 mM. After addition of 2×10^{-4} mol of [^{13}C]formaldehyde to the samples, the mixtures were incubated for 18 h at 4 °C. The reactions were stopped by extensive dialysis against buffer A, and the labeled LDL solutions were concentrated in Amicon 100 concentrators with a 100-kDa cut-off membrane. The H^{13}CHO was doped with a trace of [^{14}C]formaldehyde to give a known specific radioactivity. The degree of reductive methylation was calculated by counting the amount of [^{14}C]formaldehyde incorporated into the lysine residues of apoB-100.

NMR Spectroscopy—Broad-band proton-decoupled ^{13}C NMR spectra were measured from LDL samples comprising 11.2–11.4 mg of protein/ml in a solution containing 150 mM NaCl, 1 mM EDTA, and 0.02% NaN_3, pH 7.4, and 10% of D_2O for the spectrometer field-lock. ^{13}C NMR spectra were obtained at 150.8 MHz with a Varian Unity 600 NMR spectrometer. All experiments were recorded at 37 ± 0.5 °C, and the spectral width was 250 ppm, corresponding to 16,000 points in 0.219 s. Pulse length was 8 μs. Proton decoupling was performed with a GARP sequence (25). Total running times varied from 4 to 6 h, depending on the sample. Relaxation delay was 1.0 s to gain a good signal-to-noise ratio. The repetition time of these measurements was on the order of the longitudinal relaxation time of the active lysines (26). Consequently, the number of active lysines in these measurements was underestimated compared with the normal lysines which relax faster. We therefore measured ^{13}C-labeled LDL using relaxation delays of 1.0 and 5.0 s and obtained a correction factor of 1.47 to subsequently multiply the integrated dimethyl-lysine resonances of the active lysines. Prior to Fourier transformations, free induction decays were filled to 32,000 and weighted by 2-Hz line broadening.

Isolation of Mouse Peritoneal Macrophages and Incorporation of Oleate into Cholesteryl Oleate by Macrophages—Macrophages from unstimulated NMRI mice were harvested into Dulbecco's PBS containing 1 mg/ml bovine serum albumin (27). The peritoneal cells were resuspended in RPMI 1640 medium containing 20% of heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells ($1–2 \times 10^6$) were seeded into plastic 24-well plates (Falcon) and incubated overnight in a humidified CO_2 (5% CO_2 in air) incubator. Before the experiments, the dishes were washed to remove nonadherent cells. Each monolayer received 300 μl of RPMI medium containing 10 mg/ml bovine serum albumin, 100 IU/ml penicillin, 100 μg/ml streptomycin, 200 μM oleate-albumin, and 30 μg of native or oxidized LDL. After incubation for 18 h at 37 °C, the amount of cholesteryl oleate in the cells

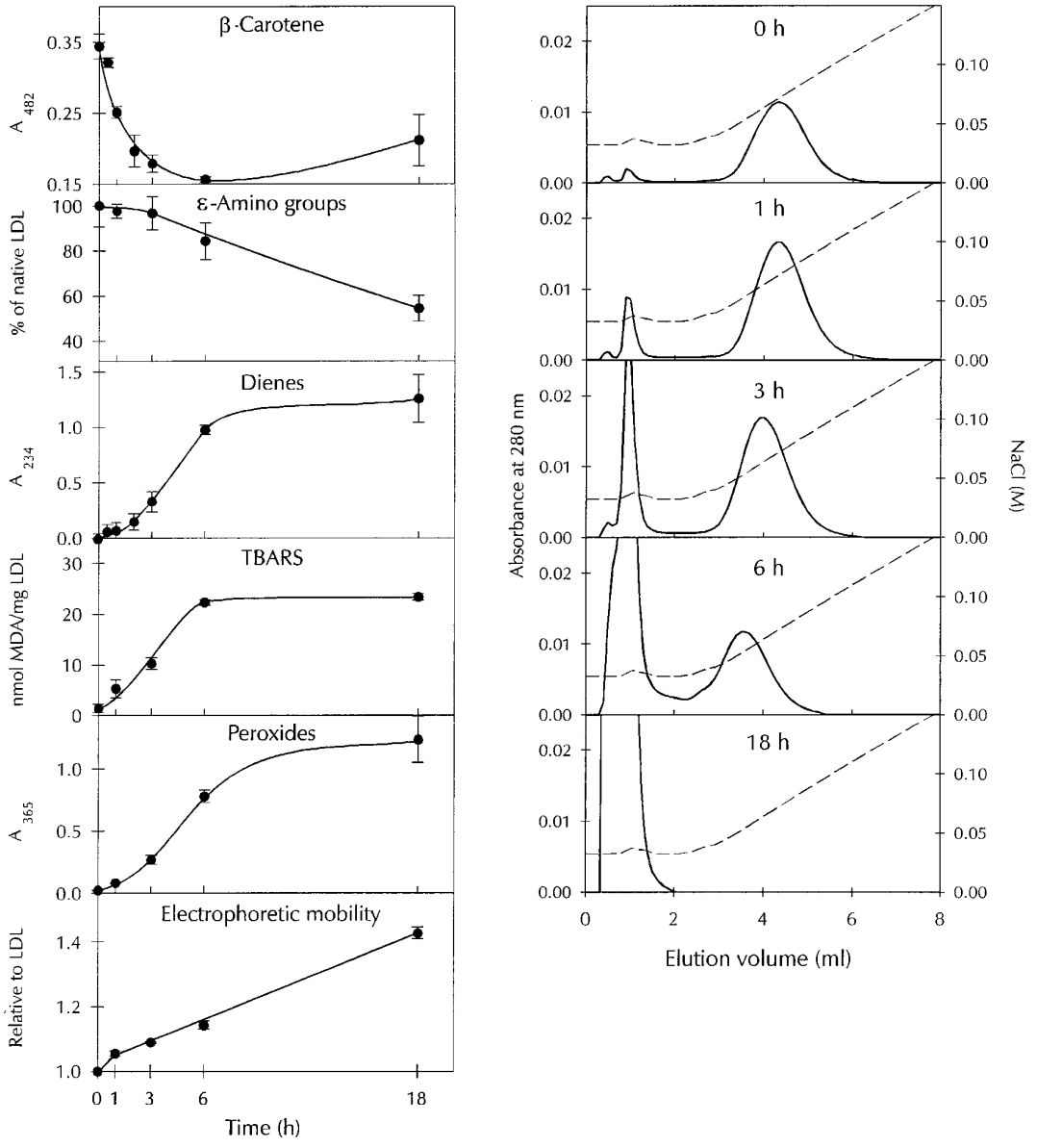

FIG. 1. **Affinity chromatography of LDL treated with copper sulfate on a human aortic proteoglycan column.** After incubation of LDL (1 mg/ml) in PBS containing 5 μM $CuSO_4$ at 37 °C for the indicated times, the amounts of β-carotene, ϵ-amino groups, conjugated dienes, TBARS, and peroxides, and the relative electrophoretic mobility were measured as described under "Experimental Procedures" (*left panels*). Samples (30 μl) of the incubation mixtures were analyzed by affinity chromatography on a 1-ml proteoglycan HiTrap column (*right panels*). Elution was carried out at 1.0 ml/min, using a linear NaCl gradient (0 to 250 mM in 10 min) in 10 mM HEPES, 2 mM $CaCl_2$, and 2 mM $MgCl_2$, pH 7.4. Elution was monitored by UV absorbance at 280 nm, and the NaCl gradient was controlled by measuring the conductivity of the eluent (*dotted lines*).

was determined with reverse phase HPLC, using an S5 ODS (0.3 × 25 cm) column as described previously (28).

Other Assays—Protein was determined by the method of Lowry *et al.* (29), with bovine serum albumin as standard. Glycosaminoglycans were determined by the method of Bartold and Page (30).

RESULTS

To study the effect of LDL oxidation on the amount of LDL bound to human aortic proteoglycans, LDL was oxidized by incubation with 5 μM $CuSO_4$ at 37 °C for various periods of time up to 18 h. At the indicated time points, the degree of LDL oxidation was analyzed by measuring changes in several parameters known to be associated with LDL oxidation (Fig. 1, *left panels*): loss of β-carotene and of ϵ-amino groups (lysine); formation of conjugated dienes, TBARS, and peroxides; and increase in the electrophoretic mobility of LDL. The amount of oxidized LDL bound to aortic proteoglycans was determined by applying aliquots of the incubation mixtures to a proteoglycan affinity column. The column was then washed for 1 min with buffer B, and the bound LDL was eluted with a 10-min linear NaCl gradient (0–250 mM NaCl) in buffer B. Elution was monitored with UV absorbance at 280 nm, and the gradient was checked by measuring the changes in conductivity (Fig. 1, *right panels*, *dotted lines*). Of the 20 μg of native LDL applied, 95% bound to the column and eluted as a single peak at 65 mM NaCl (Fig. 1, *right panels*, *top*). As shown by the elution profiles of the samples treated with $CuSO_4$ for various time periods, the higher the degree of oxidation (Fig. 1, *left panels*), the smaller was the amount of LDL bound to the proteoglycans, until, after oxidation for 18 h, the modified LDL particles failed to bind to the proteoglycans (Fig. 1, *right panels*, *bottom*).

Progressive copper-induced oxidation of LDL generates aggregation of the lipoprotein (31). To assess whether the observed loss in the ability of LDL to bind to proteoglycans is due to aggregation of LDL, we determined the degree of particle

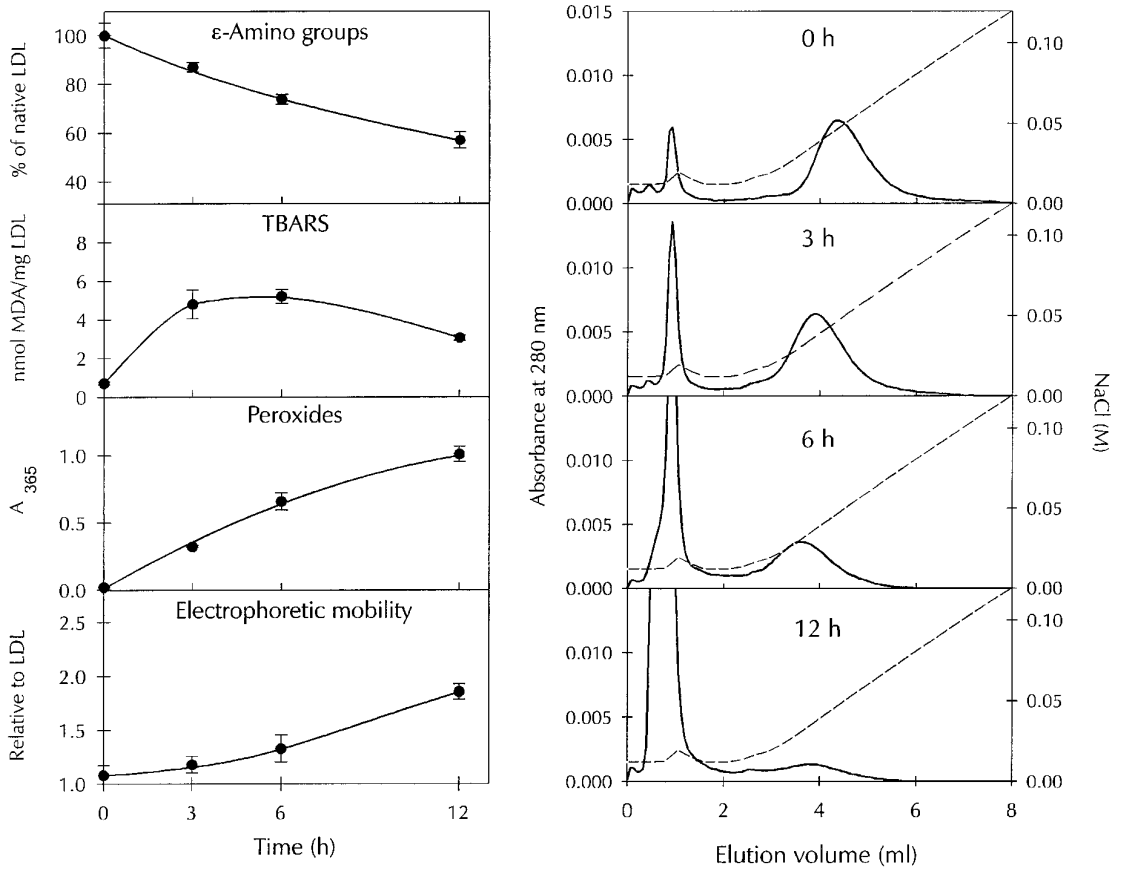

FIG. 2. **Affinity chromatography of LDL treated with AAPH on a human aortic proteoglycan column.** After incubation of [^{3}H]CL-LDL (1 mg/ml) in PBS containing 25 mM AAPH at 37 °C for the indicated times, the amounts of ε-amino groups, TBARS, and peroxides, and the relative electrophoretic mobility were measured as described under "Experimental Procedures" (*left panels*). Samples of the incubation mixtures were applied to PD-10 columns, and fractions containing [^{3}H]CL-LDL were pooled. Samples corresponding to 30 μg of LDL were analyzed by affinity chromatography on a 1-ml proteoglycan HiTrap column as described under "Experimental Procedures" (*right panels*).

aggregation in our oxidized LDL samples by gel filtration chromatography. When LDL was oxidized for 18 h, which fully abolishes binding of LDL to proteoglycans (Fig. 1), the amount of LDL aggregated (eluting in the void volume of the column) was 24%. Since only a fraction of the oxidized LDL particles had aggregated, aggregation cannot be responsible for the loss in the ability of LDL to bind to proteoglycans.

As oxidation progressed, the electrophoretic mobility of LDL increased (Fig. 1, *left panels*). Since only one band of LDL was formed, the particles must have been modified to roughly the same extent at any given time point. Yet, when analyzed with the proteoglycan affinity column, two populations of LDL emerged, one eluting without binding to the proteoglycans, and the other binding to the column and eluting only at the higher NaCl concentrations (Fig. 1, *right panels*). To examine whether these two populations of LDL had been differently oxidized, we performed an experiment in which LDL was oxidized by incubation with $CuSO_4$ for 6 h. The sample was then applied to a chondroitin-6-sulfate affinity column, the unbound LDL was collected in buffer B, and the bound LDL was eluted with buffer B containing 250 mM NaCl. Both fractions were concentrated in Amicon 10 concentrators with a 10-kDa cut-off membrane. Aliquots of the fractions were then rechromatographed on the same affinity column: most (90%) of the bound LDL did bind again, and most (85%) of the unbound LDL remained unbound. The same result was obtained when a proteoglycan affinity column was used. The bound and unbound oxidized LDL fractions were examined for their contents of peroxides and number of free ε-amino groups in lysines. No difference in peroxide content was found between the two fractions. In striking contrast, the amount of free lysine residues was 90% in the bound LDL and only 65% in the unbound LDL. When LDL was oxidized for 18 h, a similar difference in the number of lysine residues was observed between the bound and unbound LDL fractions, although the proportion of LDL that was bound was smaller than after oxidation for 6 h (above). In an additional experiment, LDL was oxidized for 18 h and chromatographed on a heparin HiTrap (1 ml) affinity column. Again, although there was no difference in peroxide content between the two oxidized LDL fractions, the bound LDL contained a higher proportion (75%) of free lysine residues than the unbound LDL (58%). As expected, the relative electrophoretic mobility of the unbound LDL was higher than that of the bound LDL. Taken together, the above results are compatible with the idea that the factor which had rendered LDL particles unable to bind to glycosaminoglycans or to proteoglycans was the reduction in the number of free lysine residues of apoB-100.

LDL was next oxidized with AAPH for various time periods. At the indicated time points, one sample of the incubation mixture was taken for analysis of the amounts of ε-amino groups, TBARS, peroxides, and relative electrophoretic mobility (Fig. 2, *left panels*), while another was applied to the proteoglycan affinity column (Fig. 2, *right panels*). As with copper-modified LDL, it was found that as oxidation advanced, the binding of LDL to the column decreased. After oxidation for 12 h, when 60% of the lysine residues were modified, the LDL failed to bind to the proteoglycans. Finally, AAPH-oxidized LDL, which contained 70% of free lysines, was applied to a chondroitin-6-sulfate affinity column, and the percentages of the free lysine residues in the bound and the unbound LDL

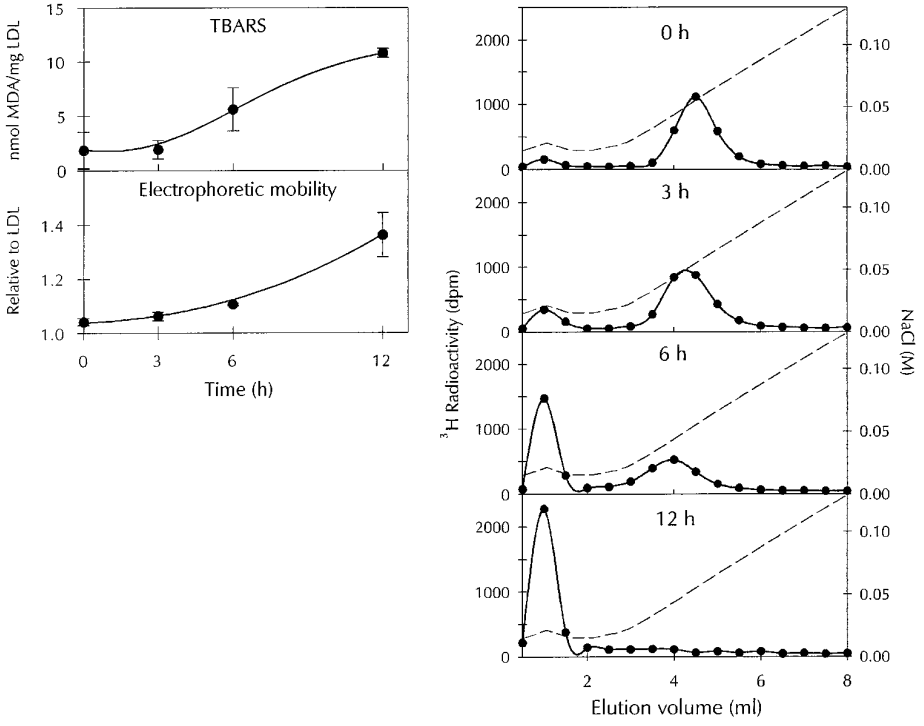

FIG. 3. **Affinity chromatography of LDL oxidized with mouse peritoneal macrophages on a human aortic proteoglycan column.** After incubation of [^{3}H]CL-LDL (100 μg/ml) with isolated mouse peritoneal macrophages in RPMI 1640 culture medium (300 μl) containing 300 nM $CuSO_4$ for the indicated times, the amounts of TBARS and the relative electrophoretic mobility were determined as described under "Experimental Procedures" (*left panels*). Samples (30 μl) of the incubation mixtures were analyzed by affinity chromatography on a 1-ml proteoglycan HiTrap column as described under "Experimental Procedures" (*right panels*). Elution was monitored by collecting 500-μl fractions and determining their radioactivities.

TABLE I
Oxidation of LDL with soybean lipoxygenase

After incubation for 18 h of [^{3}H]CL-LDL (2.5 mg/ml) in buffer A containing 1.25 mM linoleate-albumin and 60 units/μl soybean lipoxygenase at 37°C, the amounts of ε-amino groups and the relative electrophoretic mobility were measured as described under "Experimental Procedures." The ratio of ^{3}H radioactivity eluting from the proteoglycan column in peak II (see Fig. 1) to the total eluted ^{3}H radioactivity is used as a measure of the LDL bound to the proteoglycans.

LDL	ε-Amino groups	Peroxides	Electrophoretic mobility	LDL bound to proteoglycan column
	% of native LDL	*A_{365}/50 μg of LDL*	*relative to LDL*	*%*
Control LDL	100	0.05	1.1	92
Lipoxygenase-treated LDL	87	0.24	1.2	75

fractions were found to be 72 and 64%, respectively. Thus, in the AAPH-oxidized, as in the copper-oxidized LDL preparations, the number of free lysine residues was higher in the bound fraction than in the fraction that failed to bind to the glycosaminoglycans.

To obtain LDL oxidized in a more physiological way, LDL was incubated with mouse peritoneal macrophages for up to 12 h. At the indicated times, formation of TBARS and the relative electrophoretic mobility of the LDL particles were determined (Fig. 3, *left panels*), and the degree of binding to proteoglycans was determined on the proteoglycan column (Fig. 3, *right panels*). As the incubation time increased, LDL was progressively oxidized, the degree of oxidation being comparable to that obtained with $CuSO_4$ or AAPH. As in the previous experiments, the increasing extent of oxidation progressively decreased the amount of binding, so that after incubation with the macrophages for 12 h, when the production of TBARS had reached a value of about 10 nmol of MDA/mg of LDL protein, no LDL was bound to the proteoglycan column.

Oxidation of LDL by lipoxygenases has recently received attention as a possible mode of oxidation in the arterial wall (17). To investigate whether lipoxygenase-mediated oxidation of LDL might play a role in the interaction of LDL with arterial proteoglycans, LDL was next oxidized with soybean lipoxygenase in buffer A containing 1.25 mM linoleate-albumin. After oxidation for 18 h, the samples were chromatographed on an A-0.5m column (1 × 50 cm) to separate the linoleate-albumin from the LDL. Fractions containing LDL were pooled and concentrated, and their peroxide content, number of ϵ-amino groups, electrophoretic mobility, and degree of binding to proteoglycans were determined. As shown in Table I, soybean lipoxygenase oxidized LDL in the presence of linoleate-albumin. The peroxide content of the oxidized samples was similar to that observed with the other methods of oxidation. However, the changes in electrophoretic mobility were smaller and the differences in the degree of lysine modification less pronounced than with the other methods of oxidation, and, as expected, the amount of binding to proteoglycans was only slightly decreased.

Lysine residues of apoB-100 are known to be important in the binding of LDL to proteoglycans. Thus, modification of the positively charged lysine residues during oxidation is likely to reduce the degree of binding to the negatively charged proteoglycans. To test this hypothesis, we compared the binding abilities of two LDL preparations, in one the lysine residues had been modified with malondialdehyde to mimic their modification during LDL oxidation, while in the other the lysine residues had been methylated to prevent their modification during LDL oxidation. Treatment of LDL with increasing concentrations of malondialdehyde progressively increased TBARS in the LDL particles, reflecting the formation of MDA adducts, and this increase was accompanied by increased electrophoretic mobility of LDL (Fig. 4, *left panels*), and decreased ability to bind to proteoglycans (Fig. 4, *right panels*). Treatment of LDL with 10 mM MDA, which increased the TBARS to about

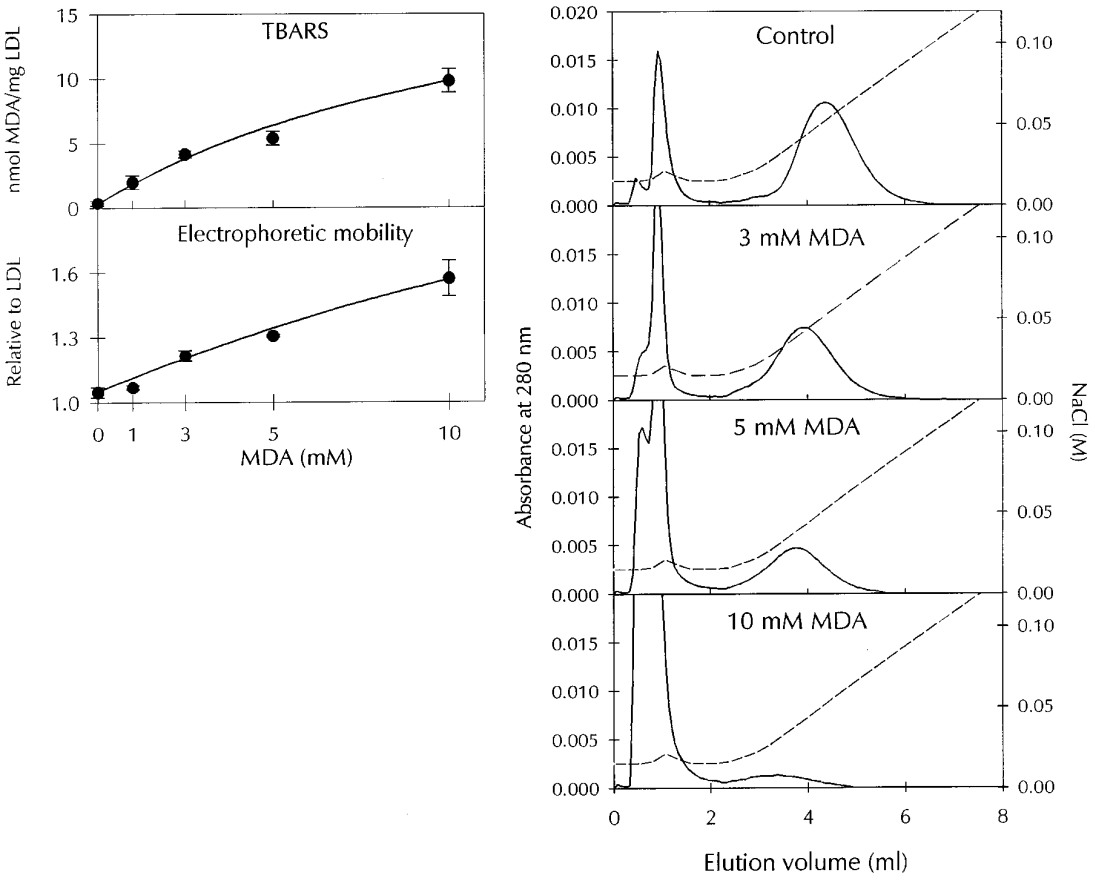

FIG. 4. **Affinity chromatography of malondialdehyde-LDL on a human aortic proteoglycan column.** LDL (1 mg/ml) was treated with 1–10 mM malondialdehyde as described under "Experimental Procedures." The TBARS and relative electrophoretic mobility of the samples were determined (*left panels*), and samples (30 μl) were analyzed by affinity chromatography on a 1-ml proteoglycan HiTrap column as described under "Experimental Procedures" (*right panels*).

TABLE II
Oxidation of methyl-LDL

[^{3}H]CL-LDL was reductively methylated and dialyzed against PBS. After incubation for 18 h of 500 μg of [^{3}H]CL-LDL or methylated [^{3}H]CL-LDL in PBS containing 5 μM $CuSO_4$ at 37°C, the amounts of ε-amino groups, TBARS, and relative electrophoretic mobility were determined as described under "Experimental Procedures." The ratio of ^{3}H radioactivity eluting from the proteoglycan column in peak II to the total eluted ^{3}H radioactivity is used as a measure of the LDL bound to the proteoglycans. Native LDL, methyl-LDL, and oxidized methyl-LDL each eluted as a single peak at 350, 350, and 250 mM NaCl, respectively.

LDL	ε-Amino groups	TBARS	Electrophoretic mobility	LDL bound to heparin column
	% of native LDL	*nmol MDA/mg LDL*	*relative to LDL*	*%*
LDL	100	2.5	1	98
Methyl-LDL	14	2.2	1.2	95
Oxidized LDL	68	25.2	1.9	13
Oxidized methyl-LDL	11	20.8	1.6	92

10 nmol of MDA/mg of LDL protein and the relative electrophoretic mobility of LDL to 1.5, virtually prevented any binding of LDL to the proteoglycan column. Methylation of native LDL, which led to modification of about 85% of the lysine residues in apoB-100, slightly increased the electrophoretic mobility of the sample. When this methylated LDL was oxidized with 5 μM $CuSO_4$ for 18 h, the TBARS of the sample increased to a degree similar to that observed in a control sample in which the lysine residues had not previously been methylated (Table II). The electrophoretic mobility also increased during oxidation, but to a lesser degree than in the control sample. The degree of binding was then analyzed by applying aliquots of the samples to a HiTrap heparin (1-ml) column. The column was washed with buffer B, and bound LDL was eluted with a 10-min linear NaCl gradient (0 to 500 mM NaCl) in buffer B, fractions of 500 μl were collected, and their radioactivities were determined. Of the LDL, 98%, and of the methylated LDL, 95% bound to the column, and were eluted with 350 mM NaCl. The oxidized LDL did not bind to the column, whereas 92% of the oxidized methyl-LDL did so, and was eluted with 250 mM NaCl, revealing that the factor mainly responsible for the observed decrease in binding during LDL oxidation was modification of the lysine residues of apoB-100.

To gain information about the effects of oxidation on the two populations of lysine residues, active and normal lysine residues, of apoB-100, we used NMR spectroscopy (9). For this purpose, LDL was first oxidized with 5 μM $CuSO_4$ at 37 °C for 6 h. Oxidation was terminated by addition of BHT and of EDTA to give final concentrations of 20 μM and 1 mM, respectively. Native and oxidized LDL were treated with [^{13}C]formaldehyde to add ^{13}C-labeled methyl groups to the lysine residues of apoB-100. The degree of labeling was monitored by co-incorpo-

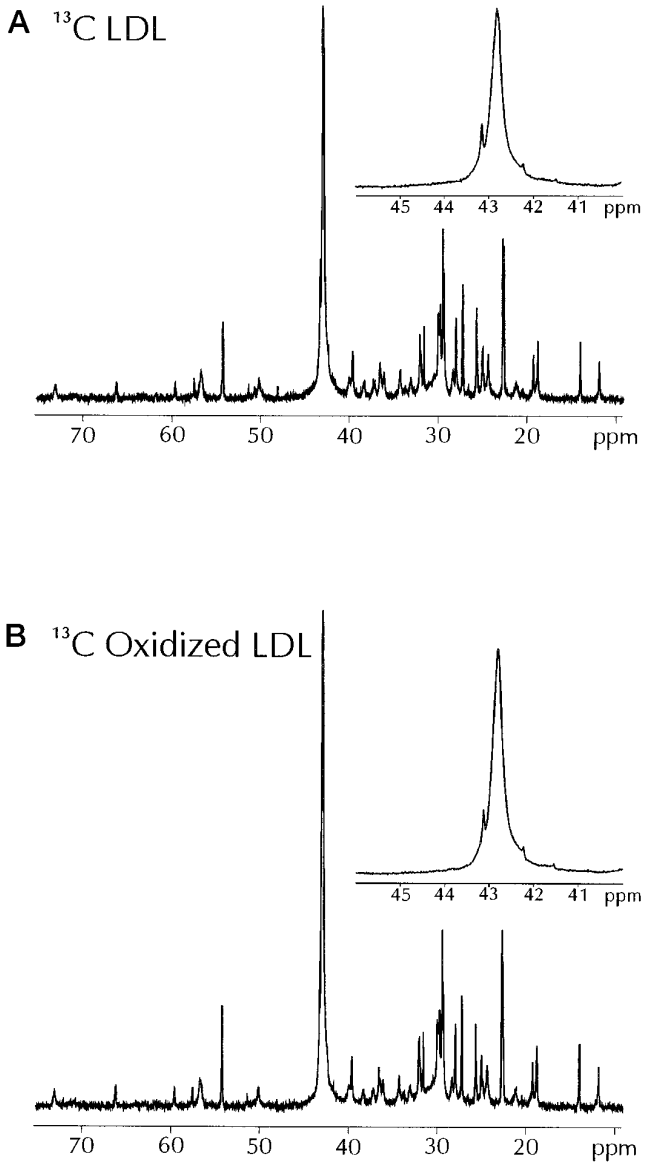

FIG. 5. **Proton-decoupled ^{13}C NMR spectra of native and oxidized LDL in which the lysine residues were converted to dimethyl lysines by reductive methylation.** The spectra (150.8 MHz) were recorded at 37 ± 0.5 °C as described in detail under "Experimental Procedures." The spectral widths were 250 ppm. Spectra of [^{13}C]LDL in which 66.7% of the lysine residues were methylated (*panel A*) and of ^{13}C-labeled oxidized LDL in which 69.0% of the lysine residues were methylated (*panel B*). The *insets* in each panel are expansions of the spectra showing dimethyl lysine resonances.

ration of [^{14}C]formaldehyde. The NMR spectrum of the ^{13}C-labeled LDL and ^{13}C-labeled oxidized LDL showed lipid resonances and, at 42.8 and 43.1 ppm, ^{13}C-labeled dimethyl lysine resonances (Fig. 5, *A* and *B*, respectively) which is in accord with the results of Lund-Katz *et al*. (9). At pH 7.4, active lysine residues are known to have an average chemical shift of 43.1 ppm, whereas normal lysine residues have a chemical shift of 42.8 ppm (9). The NMR spectrum of oxidized LDL showed similar resonances (Fig. 5*B*). The *insets* in each panel of Fig. 5 show the ^{13}C-labeled dimethyl lysine resonances in more detail. Integration of the two resonances gives the numbers of active and normal lysine residues in the samples (Table III). In the oxidized sample, the number of normal lysine residues was about the same as in LDL, but the number of active lysine residues was reduced.

Finally, we compared the effects of progressive LDL oxidation on the degree of binding to proteoglycans and on the rate of uptake by mouse peritoneal macrophages. For this purpose, LDL was oxidized with 5 μM $CuSO_4$, and, at the indicated time points (Fig. 6), divided into three aliquots: one was used for determination of the degree of oxidation, one for studying the degree of binding to the proteoglycan affinity column, and one for studying the rate of cellular uptake in macrophage monolayers. As oxidation progressed, the ability of LDL to bind to the proteoglycans rapidly decreased, in accordance with the above results. Incubation of the various oxidized samples of LDL with macrophages for 18 h and measurement of the cholesteryl oleate content of the cells revealed that the higher the degree of oxidation, the more LDL was taken up during the 18-h incubation. Taken together, it is evident that as the degree of LDL oxidation increased, the ability to bind to proteoglycans was rapidly lost, while the rate of uptake by macrophages steadily increased.

TABLE III
Effect of oxidation on the number of active and normal lysine residues in LDL particles

The percentages of active and normal lysine residues in LDL and oxidized LDL were derived by integration of the ^{13}C-labeled dimethyl lysine resonances shown in Fig. 5. The numbers of active and normal lysine residues were calculated from the percentages and rounded to the nearest whole number. In LDL, 238 (66.7%) and, in oxidized LDL, 246 (69.0%) of the 357 lysine residues of apoB-100 were methylated and observable in NMR spectroscopy. The integrals are accurate to ±15%.

LDL	Active lysine residues	Normal lysine residues
LDL	17 (7.2%)	221 (92.8%)
Oxidized LDL	10 (4.0%)	237 (96.0%)

DISCUSSION

This study shows that, after oxidation, the ability of LDL to bind to human aortic proteoglycans is decreased. We used several established methods to oxidize LDL: copper sulfate, AAPH, mouse peritoneal macrophages, and soybean lipoxygenase. In each case, the net negative charge of the LDL particles increased as the degree of binding of the particles to proteoglycans decreased. The increase in the net negative charge on oxidized LDL was accompanied with loss of free lysine residues. These results are compatible with the finding that during oxidation, the decomposition products of LDL lipids react with the lysine residues of apoB-100, thereby neutralizing them (32–35).

Affinity chromatography of oxidized LDL yielded two fractions of LDL, one eluting without binding to the proteoglycans and the other binding to the column and eluting at higher salt concentrations. The degree of lipid peroxidation in these fractions was similar, as judged by their lipid peroxide contents. However, the bound LDL contained more free lysines than did the unbound LDL in both the copper-oxidized and the AAPH-oxidized LDL preparations. It is not known whether this difference results from a difference in the rate at which the lysine residues in LDL particles are modified during oxidation or whether originally there were LDL particles having different numbers of free lysines. Interestingly, Hurt-Camejo *et al.* (23) have separated LDL particles into fractions with different abilities to bind to proteoglycans; the smaller LDL particles bound to proteoglycans more tightly than the larger ones. The difference in the binding to proteoglycans was suggested to be due to a difference in the conformation of the apoB-100 moiety on particles of different sizes. This hypothesis is supported by the evidence of Aviram *et al.* (36), who showed that changing the triglyceride content and size of LDL particles changes the conformation of apoB-100.

The higher the degree of oxidation of LDL, the fewer the number of lysine residues of apoB-100 that remained unmodi-

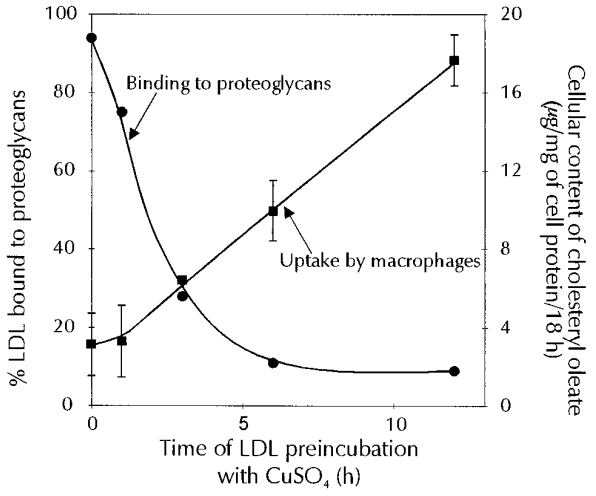

FIG. 6. **Affinity chromatography on a human aortic proteoglycan column and uptake by mouse peritoneal macrophages of LDL oxidized with $CuSO_4$.** [^{3}H]CL-LDL (1 mg/ml) was incubated in PBS containing 5 μM $CuSO_4$ at 37 °C for the indicated times. Samples (30 μl) of the incubation mixtures were applied to a proteoglycan affinity column as described under "Experimental Procedures." Fractions of 500 μl were collected, and their radioactivities were determined. The ratio of ^{3}H radioactivity eluting from the proteoglycan column in peak II (see Fig. 1) to the total eluted ^{3}H radioactivity is used as a measure of LDL bound to the proteoglycans. Other samples of the incubation mixtures corresponding to 30 μg of LDL were added to monolayers of mouse peritoneal macrophages containing 300 μl of culture medium and 200 μM oleate-albumin. After incubation for 24 h at 37 °C, the cellular content of cholesteryl oleate in each monolayer was determined by HPLC as described under "Experimental Procedures."

fied, and the smaller the fraction that bound to the proteoglycans. Irrespective of differences in the degree or type of oxidation or the type of sulfated polysaccharides used for binding, the number of free lysines was higher in the bound than in the unbound fraction. Since the binding of LDL to negatively charged sulfate and carboxyl groups of proteoglycans can be blocked by removing the positive charge from lysine residues of apoB-100 (37), the above experiments suggested that the decreased binding of oxidized LDL to proteoglycans depended on some modification of the lysine residues. To test this idea further, we specifically modified the lysines of apoB-100 with malondialdehyde. As expected, this modification completely prevented the binding of LDL to proteoglycans, a result in accord with those of Haberland *et al.* (8). We also incubated copper sulfate with LDL in which the lysines had been methylated. Prior methylation of lysine residues, which prevents their oxidative modification (32), also prevented the decrease in subsequent binding to proteoglycans. Most importantly, NMR spectroscopy revealed that oxidation had reduced the number of active lysine residues of apoB-100, *i.e.* those lysines that are thought to be involved in the binding of LDL to proteoglycans (9). In summary, the results show that modification of lysine residues by oxidation is a necessary and sufficient prerequisite for the decreased binding of LDL to proteoglycans.

As the degree of LDL oxidation increased and the degree of binding of LDL to proteoglycans decreased, the cellular uptake of modified LDL increased. The uptake of oxidized LDL is mediated by scavenger receptors, which recognize the oxidatively modified lysine residues of apoB-100 (35). The decrease in the number of free lysines of apoB-100 correlates with the uptake of oxidized LDL, and prior methylation of the lysine residues prevents the uptake of oxidized LDL via scavenger receptors (33). Thus, the same chemical modifications of the lysine residues that block the binding of LDL to proteoglycans (37) lead to recognition of the LDL particles by the scavenger receptors (1, 8, 38–40). This explains the observed parallel decrease in proteoglycan binding and increase in cellular uptake of oxidized LDL. Interestingly, Lougheed and Steinbrecher (31) have recently described a novel scavenger-receptor independent pathway for macrophage uptake of extensively oxidized LDL. This further supports the idea that oxidized LDL, regardless of the degree of oxidation, is destined to be taken up by cells rather than to be bound to proteoglycans.

Oxidative modification of LDL is thought to occur extracellularly in the arterial intima. Although immunohistochemical studies have shown that a fraction of the oxidized epitopes is located extracellularly (41), they cannot distinguish between oxidized LDL particles bound to extracellular proteoglycans and those remaining unbound in the fluid phase. The results of this study seem to favor the latter alternative, *i.e.* that the extracellularly located oxidized LDL particles are in the fluid phase. Some of these particles may have been released from proteoglycans, and then oxidized. Indeed, Camejo *et al.* (42) found that LDL released from proteoglycans is oxidized even more readily than is native LDL. Another possibility is that the LDL particles have been oxidized while bound to the proteoglycans. Arterial immobilization of LDL by proteoglycans has been suggested enable initial lipid hydroperoxide "seeding" and thereby facilitate progressive oxidation of the bound lipoprotein (43). Modification of the lysine residues of apoB-100 could then lead to release of the oxidized LDL from the proteoglycan. This suggestion is supported by the finding that LDL bound to heparin is released when treated with malondialdehyde (8). Taken together with the present results, it appears that oxidation of LDL, whether in the fluid phase or bound to proteoglycans, will modify the lysine residues of the apoB-100 moiety of LDL particles and prevent their interaction with proteoglycans. If this scheme is applicable to the arterial intima, oxidation of LDL will tend to lead to intracellular rather than extracellular accumulation of LDL.

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