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Lipoprotein Lipase Enhances the Binding of Native and Oxidized Low Density Lipoproteins to Versican and Biglycan Synthesized by Cultured Arterial Smooth Muscle Cells*

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Retention of low density lipoproteins (LDL) by vascular proteoglycans and their subsequent oxidation are important in atherogenesis. Lipoprotein lipase (LPL) can bind LDL and proteoglycans, although the effect of different proteoglycans to influence the ability of LPL to act as a bridge in the formation of LDL-proteoglycan complexes is unknown. Using an electrophoretic gel mobility shift assay, [^{35}S]SO_4-labeled versican and biglycan, two extracellular proteoglycans secreted by vascular cells, bound native LDL in a saturable fashion. The addition of bovine milk LPL dose-dependently increased the binding of native LDL to both versican and biglycan, approaching saturation at 30–40 μg/ml LPL for versican and 20 μg/ml LPL for biglycan. LDL was oxidized by several methods, including copper, 2,2-azo-bis(2-amidinopropane)-2HCl and hypochlorite. Extensively copper- and hypochlorite-oxidized LDL bound poorly to versican and biglycan. Proteoglycan binding to LDL was correlated inversely with the extent of LDL; however, the addition of LPL to oxidized LDL together with biglycan or versican allowed the oxidized LDL to bind the proteoglycans in an LPL dose-dependent manner. Addition of LPL had a greater relative effect on the binding of extensively oxidized LDL to proteoglycans compared with native LDL. LPL had a slightly greater effect on increasing the binding of native and oxidized LDL to biglycan than versican. Thus, LPL in the artery wall might increase the atherogenicity of oxidized LDL, since it enables its binding to vascular biglycan and versican.

A characteristic feature of atherosclerotic lesions is the accumulation of low density lipoproteins (LDL),[1] which undergo oxidative modification (1–3). Uptake of oxidized LDL via scavenger receptor(s) on macrophages can lead to the intracellular accumulation of lipid (1–3), but the mechanisms underlying extracellular accumulation of oxidized LDL, as well as native LDL, are not well understood. The binding and retention of lipoproteins by proteoglycans is believed to be critical in the initiation of atherosclerosis (4–6) and may be especially important for extracellular lipid accumulation.

Proteoglycans contain long carbohydrate side chains of glycosaminoglycans, which are covalently linked to a core protein by a glycosidic linkage. The interaction of proteoglycans with lipoproteins is thought to be primarily ionic in nature, such that negatively charged sulfate and carboxyl groups on glycosaminoglycan side chains interact with clusters of positively charged amino acid residues within apolipoproteins (apo) B100 and E on lipoproteins (7–9). The major proteoglycans present in atherosclerotic arteries are large chondroitin sulfate proteoglycans, such as versican, and small dermatan sulfate proteoglycans, such as biglycan (10–15), which are synthesized by vascular smooth muscle cells (16–19). In human atherosclerotic arteries, versican is found primarily in regions rich in smooth muscle cell, whereas biglycan is particularly abundant in the extracellular matrix immediately adjacent to areas of macrophage infiltration (11). Recently it has been suggested that biglycan plays an especially significant role in the trapping and retention of lipoproteins, since it was found to be the predominant proteoglycan that co-localizes with apolipoproteins B and E in human atherosclerotic lesions (11).

Lipoprotein lipase (LPL) has domains that can bind both lipoproteins and proteoglycans (20–23). It has been suggested that LPL may play a role in facilitating lipoprotein-proteoglycan interactions, independent of its enzymatic activity (24–26). LPL plays an important bridging role in the binding of triglyceride-rich lipoproteins to hepatic heparan sulfate proteoglycans, where it is involved in non-receptor-dependent lipoprotein removal by hepatocytes (26, 27). On the endothelium, triglyceride-rich lipoproteins bind to LPL that is anchored to cell surface heparan sulfate proteoglycans before the lipoproteins undergo hydrolysis (28). In addition to mediating the binding of lipoproteins to cell surface heparan sulfate proteoglycans, LPL also may act as a bridge between lipoproteins and extracellular vascular proteoglycans. Several studies have shown that the addition of LPL to endothelial cell extracellular matrices increases the retention of apoB- and E-containing lipoproteins (29, 30). Proteoglycans play a role in this interaction, as evidenced by the observation that the apolipoprotein binding is decreased following the enzymatic removal of heparan sulfate and chondroitin sulfate chains (31). Addition of LPL also has been shown to enhance the binding of arterial wall proteoglycans to native LDL immobilized to microtiter plates (22). Furthermore, LPL enhances the binding of oxidized LDL to extracellular matrix derived from endothelial cells (32), although the nature of the matrix molecules to which LPL and

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[1] The abbreviations used are: LDL, low density lipoproteins; apo, apolipoprotein; LPL, lipoprotein lipase; AAPH, 2,2-azo-bis(2-amidinopropane)-2HCl; HOCl, hypochlorite; TNBS, trinitrobenzenesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

the lipoproteins bind is not known. Oxidation of LDL inhibits its ability to interact with purified proteoglycans (33, 34), yet oxidized LDL appears to be present in the extracellular matrix of atherosclerotic lesions (35). Thus, LPL may play an important role in allowing oxidized LDL to bind to extracellular vascular proteoglycans.

Therefore, the present study was undertaken to determine whether LPL has specific affinities for the extracellular vascular proteoglycans, versican and biglycan, that are present in atherosclerotic lesions in regions associated with deposited LDL. Furthermore, studies were performed to determine whether different proteoglycans influence the ability of LPL to modulate the formation of native and/or oxidized LDL-proteoglycan complexes.

EXPERIMENTAL PROCEDURES

Chemicals and reagents were obtained from Sigma, unless otherwise stated.

Lipoproteins—LDL (d = 1.019–1.063 g/ml) was isolated from plasma from a pool of healthy human subjects by preparative ultracentrifugation in a Beckman Vti65 vertical rotor, as described previously (36).

LDL was oxidized by several different methods that resulted in different degrees of oxidative modification. Transition metal-dependent oxidation was achieved by incubating LDL (0.3 mg/ml) in phosphate-buffered saline in the presence of 5 μM copper sulfate for 4 or 18 h at 37 °C (37). Oxidative modification by hypochlorite (HOCl) was achieved by incubating 0.4 ml of LDL (1.5 mg/ml) with 5 μl of reagent grade HOCl (diluted 1:4, v/v with distilled water) for 20 min on ice (38). To prepare LDL with more moderate oxidative changes, LDL (0.5 mg/ml) was incubated with the thermally dependent free radical generator 2,2-azo-bis(2-amidinopropane)-2HCl (AAPH) (Polysciences, Warrington, PA) for 18 h at 37 °C (39). All oxidative reactions were terminated with the addition of butylated hydroxytoluene (25 μM final concentration). The LDL preparations were dialyzed for 18 h at 4 °C against HEPES sample buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) with 25 μM butylated hydroxytoluene, and stored at 4 °C in the dark, in preparation for the gel mobility shift assay, which evaluated lipoprotein-proteoglycan interactions. The extent of LDL oxidation was assessed by measurement of electrophoretic mobility on agarose gels in barbital buffer at pH 8.6 (36), the presence of conjugated dienes determined by measuring the absorbance at 234 nm (37), lipid peroxides (40), and trinitrobenzenesulfonic acid (TNBS) reactivity (41), which is a measure of free amino groups.

Protein concentrations were quantified by the Lowry method (42).

Lipoprotein Lipase (LPL)—LPL was isolated from fresh bovine milk using heparin-Sepharose chromatography, as described previously (43). Purity was confirmed by SDS-PAGE, followed by silver staining and Western blot analysis with the monoclonal antibody 5D2 (a kind gift from Dr. J. Brunzell, University of Washington, Seattle), which showed one distinct band with a molecular mass of approximately 55 kDa. For some experiments LPL was inactivated by incubating it at 50 °C for 4 h, as described previously (44).

Proteoglycan Isolation—To obtain radiolabeled proteoglycans, subconfluent human aortic smooth muscle cells (a generous gift of Elaine Raines, Department of Pathology, University of Washington, Seattle), grown in modified Eagle's medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum, were metabolically labeled with either 100 μCi/ml $Na_2[^{35}S]SO_4$ or 50 μCi/ml [^{35}S]methionine (ICN Biomedicals, Irvine, CA) for 24 h, as described previously (19, 45). The medium was combined with protease inhibitors (5 mM benzamidine (Eastman Kodak Co.), 100 mM 6-aminohexanoic acid, and 50 mM phenylmethylsulfonyl fluoride) and purified and concentrated by ion exchange chromatography on DEAE-Sephacel in 8 M urea buffer (8 M urea, 2 mM EDTA, 0.25 M NaCl, 50 mM Tris-HCl, and 0.5% Triton X-100 detergent, pH 7.4). Proteoglycans were eluted with 8 M urea buffer containing 3 M NaCl, concentrated on 50-kDa Centricon membranes (Amicon, Beverly, MA), and applied to a preparative Sepharose CL-2B molecular sieve column in 8 M urea buffer (46). Radiolabeled proteoglycans eluting at K_{av} <0.3 were pooled for versican, and those eluting at K_{av} 0.44 to 0.60 were pooled for biglycan. Aliquots containing approximately 30,000 dpm ^{35}S were precipitated in 80% ethanol and 1.3% potassium acetate and applied to gradient SDS-PAGE (4–12%). Prior to application to PAGE, some pellets were digested with 2.3 units per ml chondroitin ABC lyase in Tris-buffered solution (45 mM Tris, 0.09 mg/ml BSA, 2.7 mM sodium acetate, pH 8.0) in the presence of protease inhibitors at 37 °C for 3 h (47). Gels were dried, subjected to autoradiography, and quantitated using NIH Image software. Eluted material in each pool was applied to DEAE-Sephacel, washed extensively with 8 M urea buffer lacking detergent, and eluted with 4 M guanidine buffer (4 M guanidine, 10 mM EDTA, 50 mM sodium acetate, pH 7.4) (45). The eluted fractions were concentrated and exchanged into HEPES sample buffer on 50-kDa Centricon membranes, aliquoted, and stored at −80 °C for use in the gel shift assay.

The glycosaminoglycan content of the $[^{35}S]SO_4$-labeled versican and biglycan was quantitated using dimethylmethylene blue as described previously (48). Calculated molarities were based on the assumption that versican contained 15 chains of 70 kDa each and that biglycan contained 2 chains each of 59 kDa (16, 18, 49). Therefore, we estimated that 1 μM $[^{35}S]SO_4$-labeled versican was equal to approximately 1050 μg/ml glycosaminoglycan and that 1 μM $[^{35}S]SO_4$-labeled biglycan was approximately 118 μg/ml glycosaminoglycan.

Western Blot Analysis—Versican and biglycan preparations were digested with chondroitin ABC lyase or heparitinase I and II (Sigma) in Tris-buffered solution (45 mM Tris, 0.09 mg/ml BSA, 10 mM calcium acetate, pH 7.0) in the presence of protease inhibitors, applied to SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) using a Mini Trans-Blot Cell (Bio-Rad). The transferred proteins then were detected with a series of primary antibodies and appropriate secondary antibodies and enhanced chemiluminescence (Western Light Chemiluminescent Detection System with CSPD™ substrate; Tropix, Bedford, MA). Antibodies specific for biglycan (LF-51) and decorin (LF-136) (50) were a gift from Larry Fisher, Bone Research Branch, NIDR, National Institutes of Health. Antibodies to versican (51) were from Richard LeBaron, Division of Life Science, University of Texas, San Antonio, TX.

Gel Mobility Shift Assay—The interaction of lipoproteins with purified arterial proteoglycans was assessed using a modification of the gel mobility shift assay described by Camejo *et al.* (52, 53). In this assay, increasing concentrations of lipoproteins are mixed with a fixed amount (approximately 2000 dpm) of $[^{35}S]SO_4$-labeled versican or biglycan in HEPES sample buffer for 1 h at 37 °C, with or without the inclusion of LPL. The samples then were applied to wells in 0.7% (w/v) NuSieve-agarose (FMC Bioproducts, Rockland, ME), and electrophoresed in a HEPES running buffer that contains no NaCl (10 mM HEPES, 3 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.2) at 60 V for 3 h at 4 °C. $[^{35}S]SO_4$-labeled proteoglycans bound to lipoproteins remained at the origin, whereas free proteoglycans migrated into the gel. The gels were fixed in cetylpyridinium chloride (0.1% w/v, in 70% ethanol) for 1 h, air-dried, and exposed to Hyper-Film MP (Amersham Pharmacia Biotech) for autoradiography. The amount of complexed *versus* free proteoglycan in each lane was quantitated using a scanner (Hewlett-Packard ScanJet II cx) and ImageQuant software (Molecular Dynamics); apparent K_a values were calculated for each lipoprotein-proteoglycan interaction (SAAM Computer Software Package, Seattle, WA).

To visualize the migration of non-radioactive components (LDL and LPL) in the gel mobility shift assay, Western blotting was performed on some agarose gels immediately following the gel shift assay. Proteins in the agarose gel were electrophoretically transferred to nitrocellulose, and nonspecific sites were blocked with 1% (w/v) nonfat dry milk, 1% (w/v) fatty acid-free albumin in phosphate-buffered saline. LDL was detected using a monoclonal antibody (MB47; a kind gift from Dr. L. Curtiss, Scripps Research Institute, La Jolla, CA), and LPL was detected using the 5D2 antibody, followed by incubation with the appropriate IgG-peroxidase-linked secondary antibody. The peroxidase reaction was developed using a chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS

Radiolabeled proteoglycans that were synthesized and secreted into the culture medium were isolated by DEAE ion exchange chromatography in the presence of protease inhibitors (as described under "Experimental Procedures") and separated according to hydrodynamic size on Sepharose CL-2B columns under dissociative conditions. Washing the DEAE columns with buffer containing 0.25 M NaCl will remove any hyaluronan, the large, non-sulfated glycosaminoglycan produced by arterial smooth muscle cells (54). Purification of proteins by DEAE affinity chromatography will remove most proteins not modified by the addition of glycosaminoglycan chains (45).

DEAE-purified $[^{35}S]SO_4^-$ and [^{35}S]methionine-labeled prep-

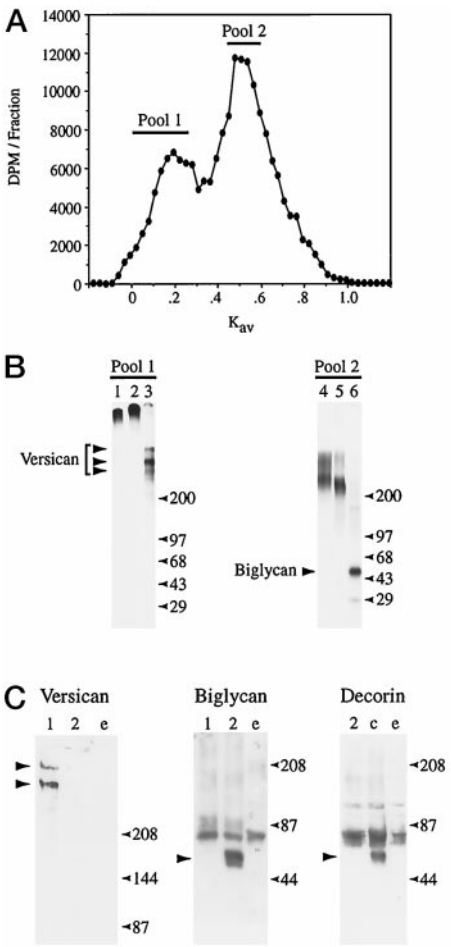

FIG. 1. **Proteoglycan isolation and characterization.** Arterial smooth muscle cells were metabolically labeled with [^{35}S]SO_4 or [^{35}S]methionine. Culture medium was collected, concentrated over DEAE-Sephacel, and subjected to Sepharose CL-2B chromatography (*A*). *Bars* indicate pooled fractions (K_{av} 0–0.3, *pool 1*; K_{av} 0.44–0.60, *pool 2*). Pools 1 and 2 from [^{35}S]SO_4- and [^{35}S]methionine-labeled media were applied to SDS-PAGE (*B*). *Lanes 1* and *4* are [^{35}S]SO_4; *lanes 2, 3, 5,* and *6* are [^{35}S]methionine. *Lanes 3* and *6* were digested with chondroitin ABC lyase prior to electrophoresis. *C,* Western blot analysis of pools 1 and 2. Versican, pools 1 and 2. Biglycan, pools 1 and 2. Decorin, pool 2 and positive control (*c*; isolated from CL-2B fractions at K_{av} 0.7). Prior to electrophoresis, the samples in *C* were digested with chondroitin ABC lyase and BSA. *Lane e,* enzyme and BSA only. *Arrowheads* indicate the position of versican, biglycan, and decorin core proteins.

arations were subjected to molecular sieve chromatography on Sepharose CL-2B. Fractions with K_{av} values of 0–0.30 and 0.44–0.60 were pooled (pools 1 and 2, respectively) (Fig. 1*A*). The pools were subjected to SDS-PAGE (4–12%) before and after chondroitin ABC lyase treatment (Fig. 1*B*). Pool 1 remained at the top of the 3.5% stacking gel and was sensitive to chondroitin ABC lyase digestion, generating 3 bands of [^{35}S]methionine-labeled material ranging from 390 to 500 kDa. The most predominant band electrophoresed at ~420 kDa. This pattern is typical of highly purified preparations of versican from arterial smooth muscle cells (16, 19, 55). Quantitation of the versican bands using NIH Image shows that they contain approximately 97% of the total detectable radioactivity in the [^{35}S]methionine-labeled pool 1. Western blot analysis of this pool demonstrated 2 bands at approximately 420–500 kDa with polyclonal anti-versican antibody (Fig. 1*C*). This peak was negative when tested by Western blot analysis for the presence of biglycan and decorin using antibodies generated against the core proteins of these proteoglycans (Fig. 1*C*).

Pool 2 (Fig. 1*A*) also was subjected to SDS-PAGE before and after digestion with chondroitin ABC lyase. Before digestion this pool ran as a broad band above the 200-kDa standard marker (Fig. 1*B*). Proteoglycans from primate arterial smooth muscle cells found in this location on SDS-PAGE have been identified as biglycan (17, 18). Decorin and biglycan core proteins electrophorese as doublets at ~50 kDa on SDS-PAGE (17, 18). Quantitation by NIH Image indicated that the 50-kDa doublet comprises about 84% of the total [^{35}S]methionine radioactivity detectable in pool 2 after chondroitin ABC lyase digestion (Fig. 1*B, lane 6*). Identification of the doublet was aided by Western blot analysis where it was stained by antibodies to biglycan but not by antibodies to versican or decorin. The decorin blot contained a positive control for that antibody (Fig. 1*C*), which was the CL-2B fraction that eluted at K_{av} 0.7. Digestion of [^{35}S]SO_4-labeled pools 1 and 2 resulted in the disappearance of any radioactive signal on SDS-PAGE (data not shown).

TABLE I
Binding affinities (K_a) of native and AAPH-modified LDL to [^{35}S]SO_4-labeled proteoglycans in the absence and presence of LPL

	Native LDL, K_a	AAPH-LDL, K_a
	M	
Versican	2.3×10^{-8}	1.0×10^{-6}
Biglycan	1.7×10^{-7}	3.0×10^{-6}
Versican + 10 μg/ml LPL	5.6×10^{-9}	1.1×10^{-7}
Biglycan + 10 μg/ml LPL	2.2×10^{-8}	3.3×10^{-7}

To determine if versican and biglycan exhibit different affinities for native LDL, a fixed amount (~2000 dpm per lane) of [^{35}S]SO_4-labeled versican or [^{35}S]SO_4-labeled biglycan was mixed with increasing concentrations of LDL and subjected to an electrophoretic gel mobility shift assay (52, 53), in which the interaction is performed under physiological conditions (*e.g.* salt and divalent cation concentrations, pH, temperature). This assay also allows determination of binding constants (*e.g.* K_a) for the interaction, and thus, estimated concentrations of each of the components, providing information helpful in defining the interaction in molecular terms. Results indicate that both versican and biglycan bound to native LDL with saturable kinetics and exhibited K_a values of 2.3×10^{-8} and 1.7×10^{-7} M, respectively (Table I). Furthermore, maximum binding to native LDL was achieved with a lower concentration (~8-fold lower) of [^{35}S]SO_4-labeled versican, 0.023 μM (Fig. 2*A*), compared with [^{35}S]SO_4-labeled biglycan, 0.187 μM (Fig. 2*B*), indicating that versican has a greater binding capacity than biglycan. By using K_a values generated with the SAAM statistical software, and assuming a molecular mass of 550 kDa for apoB on LDL, we can estimate the stoichiometry of the interaction. Therefore, these data indicate that at half-maximal binding, there are approximately 30–40 mol of native LDL per mol of versican and approximately 4–8 mol of native LDL per mol of biglycan. These estimates are consistent with the idea that several LDL molecules are able to interact with the long glycosaminoglycan side chains on proteoglycans (52) and that versican has a greater binding capacity than biglycan due to its greater number of chains (16, 17, 56).

To investigate how oxidative modification of LDL affects its interaction with proteoglycans, a number of different agents were used to produce LDL oxidized to varying degrees (Table II). Mild oxidation was achieved with a short term (4 h) incubation with copper sulfate or 18 h with AAPH. Extensively oxidized LDL was produced by a longer incubation (18 h) with copper sulfate or 20 min with HOCl. These latter LDL preparations had the greatest change in charge density (quantitated by relative electrophoretic mobility), free amino group content (quantitated by TNBS reactivity), and indices of lipid peroxi-

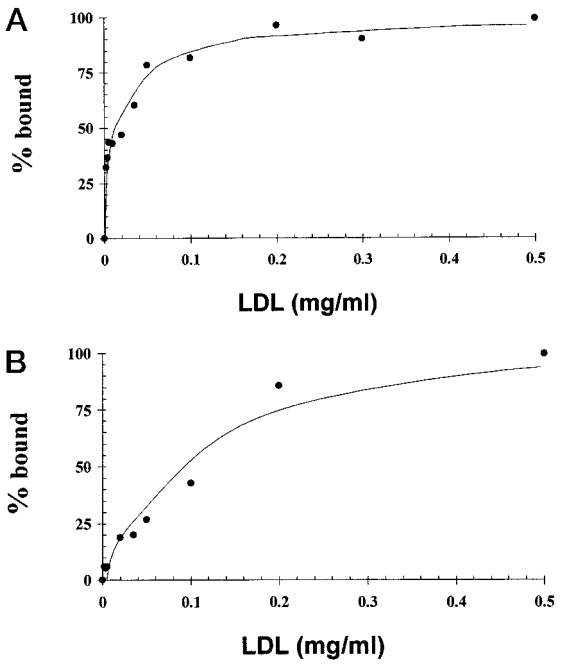

FIG. 2. **Binding of native LDL to vascular proteoglycans.** Binding curve from electrophoretic gel mobility shift assay to evaluate the interaction of native LDL with versican (*A*) or biglycan (*B*) is shown. Increasing concentrations (0–0.5 mg/ml) of native LDL were incubated with a fixed amount of [^{35}S]SO_4-labeled proteoglycan (0.023 μM [^{35}S]SO_4-labeled versican or 0.187 μM [^{35}S]SO_4-labeled biglycan) for 60 min at 37 °C prior to electrophoresis in agarose gels, as described under "Experimental Procedures." Dried gels were subjected to autoradiography, and the percent bound was calculated as the proportion of radioactivity remaining at the origin of the gel relative to the total radioactivity per lane. The data shown are from a single experiment, which is representative of 6 experiments, for both versican and biglycan.

TABLE II

Methods used to vary the extent of oxidative modification of LDL

The abbreviations used are: REM, relative electrophoretic mobility; LPO, lipid peroxides.

	REM (*vs.* native)	Conjugated dienes (A_{234})	LPO	TNBS reactivity
			nmol/mg protein	*% native LDL*
Native LDL	1.0	0.208	112	100
Method of oxidation				
4 h, $CuSO_4$	1.3	0.298	395	74
18 h, $CuSO_4$	3.6	0.491	1710	64
AAPH	2.2	0.303	832	96
HOCl	4.2	0.669	1289	60

dation (quantitated by conjugated dienes and lipid peroxides), as shown in Table II. The gel mobility shift assay with native LDL and [^{35}S]SO_4-labeled versican shows an increase in the amount of radioactivity retained at the origin of the gel with increasing LDL concentrations. This represents the proteoglycan that is bound, with a concomitant loss of free proteoglycan from the front of the gel (Fig. 3*A*). Interactions of native LDL with [^{35}S]SO_4-labeled biglycan demonstrated a similar pattern to that described with versican, except that the concentration of native LDL needed for half-maximal binding to versican occurred at a lower concentration (~0.025 mg/ml LDL protein; Fig. 3*A*) than for biglycan (~0.08 mg/ml LDL protein; Fig. 3*B*). This would be consistent with the higher affinity of native LDL for versican compared with biglycan, as shown in Table I. In marked contrast to native LDL, there was virtually no detectable binding of extensively oxidized LDL (18 h with copper sulfate) with either proteoglycan. This is evident from the

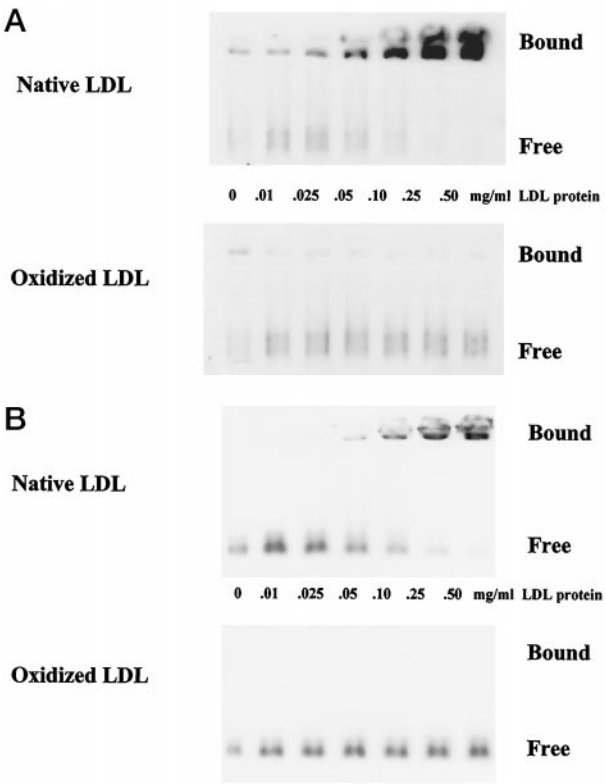

FIG. 3. **Binding of native but not extensively oxidized LDL to versican and biglycan.** Autoradiographs of gels from the electrophoretic gel mobility shift assay are shown. Increasing concentrations of native (*upper panels*) or extensively (18 h at 37 °C) copper-oxidized LDL (*lower panels*) were incubated with 0.023 μM [^{35}S]SO_4-labeled versican (*A*) or 0.187 μM [^{35}S]SO_4-labeled biglycan (*B*) for 60 min at 37 °C prior to electrophoresis in agarose, as described under "Experimental Procedures."

observation that even with the highest concentration of oxidized LDL (0.5 mg/ml) there is no [^{35}S]SO_4-labeled versican (Fig. 3*A*) or -biglycan (Fig. 3*B*) at the origin of the gel.

LPL facilitated the binding of lipoproteins to biglycan and versican in a dose-dependent manner (Fig. 4). Increasing concentrations of LPL, up to 40 μg/ml, resulted in increased binding of native and both mildly and extensively oxidized LDL to both proteoglycans, although it appears that it has a slightly greater effect on increasing the binding of oxidized LDL to biglycan compared with versican. Thus, for biglycan, the binding curves for the interaction began to plateau at 20 μg/ml LPL (Fig. 4*B*), whereas for versican, the curve did not plateau until closer to 40 μg/ml LPL (Fig. 4*A*). In experiments using a single concentration of LPL, 10 μg/ml was chosen for subsequent experiments since it lies on the steep (high affinity) part of the slope for the interaction. It is important to note that for both biglycan and versican, LPL was able to not only enhance the binding of native LDL to proteoglycans, but it also enabled oxidized LDL to increase substantially its ability to bind to proteoglycans.

Heat inactivation of LPL destroys its catalytic activity but not its structure (44). In the present study, heat-inactivated LPL (10 and 20 μg/ml) behaved in a manner similar to the native LPL (data not shown), indicating that the LPL's "bridging" role in facilitating lipoprotein-proteoglycan interactions is independent of its catalytic action.

By using a single concentration of LDL (0.1 mg/ml) from the different oxidized LDL preparations in the gel shift assay, it was demonstrated that the amount of modified lipoproteins that bound to versican and biglycan was reduced progressively as the extent of oxidation of LDL, assessed by relative electro-

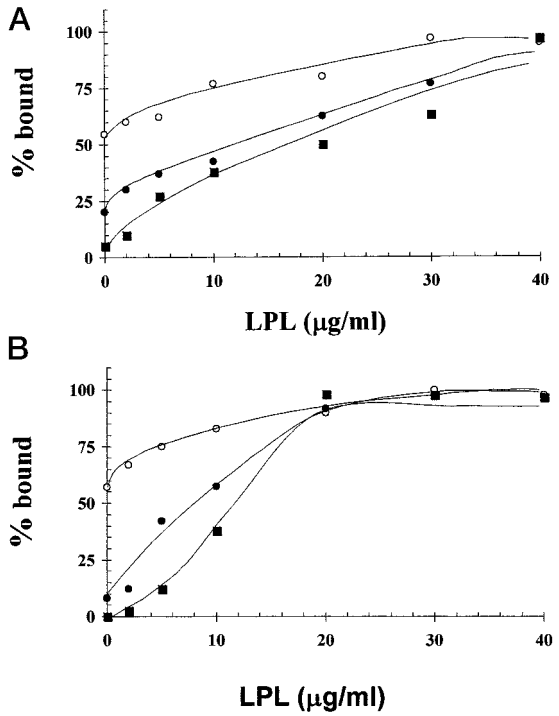

FIG. 4. **LPL-mediated increases in binding of native and oxidized LDL to proteoglycans: LPL dose-response curve.** Binding curves from electrophoretic gel mobility shift assay demonstrating the dose responsiveness of the ability of LPL to enhance the binding of native and oxidized LDL with proteoglycans are shown. Increasing concentrations (0–40 μg/ml) of bovine milk LPL were mixed with a single concentration of native (○), minimally oxidized (with AAPH, ●) or extensively oxidized (18 h with copper sulfate, ■) LDL (0.1 mg/ml) and 0.023 μM [^{35}S]SO_4-labeled versican (*A*) or 0.187 μM [^{35}S]SO_4-labeled biglycan (*B*). Samples were incubated for 60 min at 37 °C prior to electrophoresis in agarose gels, as described under "Experimental Procedures." Dried gels were subjected to autoradiography, and the percent bound was calculated as described in the legend to Fig. 2. The data shown are from a single experiment, which is representative of 3 experiments, for both versican and biglycan.

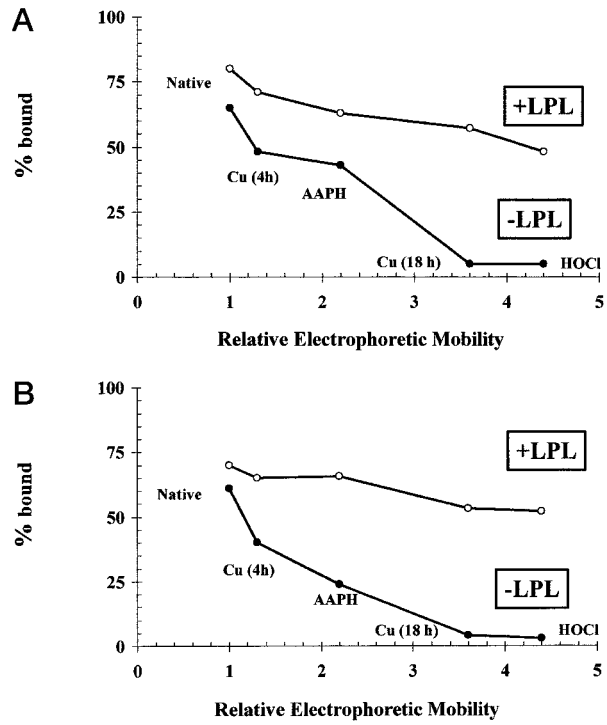

FIG. 5. **LPL increases the binding of native and oxidized LDL to vascular proteoglycans.** Results from gel mobility shift assay in which a single concentration of native and oxidized LDL (0.1 mg/ml) was mixed with 0.023 μM [^{35}S]SO_4-labeled versican (*A*) or 0.187 μM [^{35}S]SO_4-labeled biglycan (*B*), in the presence (○) or absence (●) of 10 μg/ml bovine milk LPL are shown. The extent of oxidation is expressed as relative electrophoretic mobility of the LDL preparation and is an estimate of the charge density. Samples were incubated for 60 min at 37 °C prior to electrophoresis in agarose, as described under "Experimental Procedures." Dried gels were subjected to autoradiography, and the percent bound was calculated as described in the legend to Fig. 2. The data shown are from a single experiment, which is representative of 3 experiments, for both versican and biglycan.

phoretic mobility, was increased (Fig. 5). When LPL (10 μg/ml) was added to the system, there was increased binding of LDL to versican (Fig. 5*A*) and biglycan (Fig. 5*B*) with all preparations of oxidized LDL. The pattern was similar for versican and biglycan, although it appears that LPL had a slightly greater effect on enhancing the binding of the more mildly oxidized LDL (4 h copper and AAPH-modified) to biglycan compared with versican. With the addition of LPL, the greatest relative enhancement in binding of the modified lipoproteins to either proteoglycan was observed with LDL that had been extensively oxidized by exposure to either copper sulfate or HOCl (Fig. 5, *A* and *B*).

In the presence of 10 μg/ml LPL the whole binding curves of the interaction of native and oxidized LDL with proteoglycans were shifted towards having a greater binding affinity, as demonstrated by the change in K_a values for these interactions (Table I). The change in the K_a with the addition of 10 μg/ml LPL was greater for the AAPH-modified LDL than for native LDL and even more so with biglycan compared with versican. This is consistent with our findings that demonstrate that the relative change in binding in the presence of LPL is greater for oxidized LDL than for native LDL and that biglycan appears to have a slightly greater effect than versican for the interaction with LPL and oxidized LDL. The interaction of AAPH-modified LDL and [^{35}S]SO_4-labeled biglycan, with and without LPL, is shown (Fig. 6). In the absence of LPL, binding of the [^{35}S]SO_4-labeled proteoglycans is observed only at the two highest concentrations of AAPH-modified LDL used (Fig. 6*A*). However, the addition of LPL (10 μg/ml) resulted in a greater amount of bound biglycan across all concentrations of LDL, including the lane in which there is only buffer (thus, 0 mg/ml LDL) (Fig. 6*B*). A similar finding was observed for versican (data not shown). The retention of proteoglycans at the origin in this gel shift assay, in which movement into the gel is dependent on charge rather than molecular size, indicates that LPL is able to bind versican and biglycan on its own, even in the absence of lipopro-

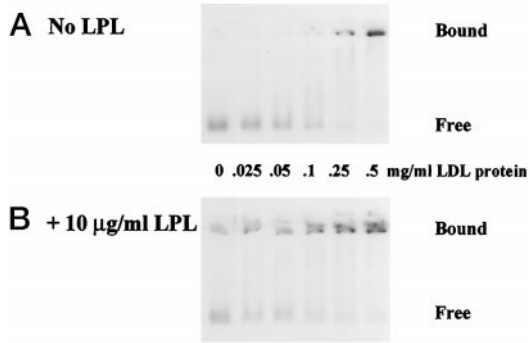

FIG. 6. **LPL increases the binding of minimally oxidized (AAPH-modified) LDL to biglycan.** Autoradiography of a gel from electrophoretic gel mobility shift assay is shown. Increasing concentrations of minimally oxidized (with AAPH) LDL were incubated with 0.187 μM [^{35}S]SO_4-labeled biglycan in the absence (*A*) or presence (*B*) of 10 μg/ml bovine milk LPL for 60 min at 37 °C prior to electrophoresis in agarose, as described under "Experimental Procedures."

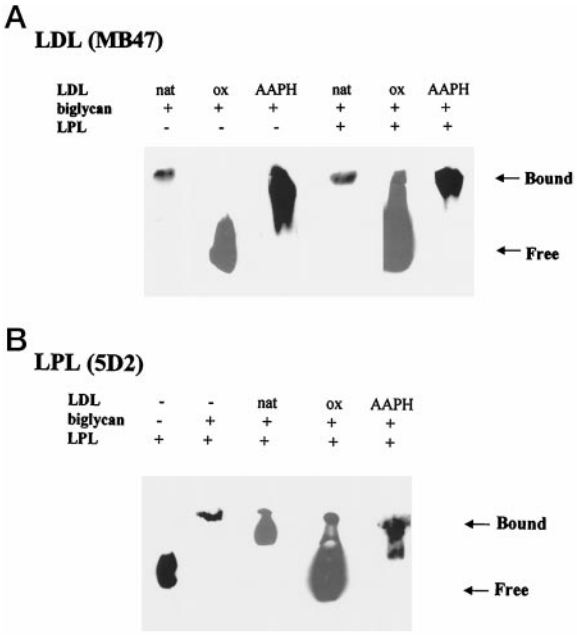

FIG. 7. **LPL facilitates the formation of a tertiary complex with LDL and biglycan.** Western blot analysis using chemiluminescence, following electrophoretic gel mobility shift assay is shown. Incubations were performed in the presence or absence of native (*nat*), extensively copper-oxidized (*ox*), or AAPH-modified LDL (0.1 mg/ml) with (+) or without (−) 0.187 μM [^{35}S]SO_4-labeled biglycan and/or 10 μg/ml bovine milk LPL for 60 min at 37 °C prior to electrophoresis in agarose, as described under "Experimental Procedures." Agarose gels were electrophoretically transferred to nitrocellulose and analyzed for LDL using the monoclonal antibody MB47 (*A*) or for LPL using the monoclonal antibody 5D2 (*B*), as described under "Experimental Procedures."

tein. This supports the idea that LPL has glycosaminoglycan binding domains, which can accommodate the extracellular vascular proteoglycans, versican and biglycan.

Western blot analyses of the gel shift experiments with native or oxidized LDL were performed to determine whether LPL co-localized with native or oxidized LDL and the bound proteoglycans. In the presence of proteoglycans, native LDL, visualized with the monoclonal LDL antibody MB47, remained at the origin (bound) (Fig. 7). However, in the presence of proteoglycans, extensively copper-oxidized LDL, which also is recognized by MB47, migrated part way into the gel (unbound). AAPH-modified LDL was detected at both the origin and part way into the gel (Fig. 7), which might be predicted since it is only mildly oxidized. LPL was visualized in the gel with the antibody 5D2. Incubations with LPL alone showed that it did not remain at the origin but also migrated part way into the gel (Fig. 7). However, in experiments containing LPL together with proteoglycan, LPL remained at the origin, bound to the proteoglycan. When all three components (LPL, LDL, and proteoglycan) were included in the incubation, both LPL and LDL were localized at the origin with the "bound" proteoglycans, indicating that a tertiary complex had been formed (Fig. 7). This was true for native LDL and different forms of oxidized LDL, although with the extensively oxidized LDL there was a broad band that extended part way into the gel from the origin, suggesting that not all of the extensively oxidized LDL was included in the complex.

Thus, these data indicate that in the absence of LPL, LDL loses its ability to bind to extracellular arterial proteoglycans as it becomes progressively more oxidized. The addition of LPL results in not only an increase in the ability of native LDL to bind to proteoglycans, but perhaps more significantly, it allows oxidized LDL to bind to arterial proteoglycans.

DISCUSSION

There is increasing evidence that extracellular matrix molecules, especially proteoglycans, play a critical role in trapping and retaining atherogenic lipoproteins in the artery wall. Once retained, LDL becomes susceptible to oxidative modification (57), which is believed to play an important role in atherogenesis (1–3). Oxidized LDL does not bind avidly to proteoglycans (33, 34) yet has been observed in the extracellular matrix in human coronary artery atherosclerotic lesions (35). Therefore, this oxidized LDL may have been trapped in its native state and subsequently modified or, on the other hand, could have been trapped following its oxidation. It is important to identify factors that modulate the interaction of native and oxidized lipoproteins with proteoglycans. One such factor may be LPL.

The lack of binding of extensively oxidized LDL to purified smooth muscle cell proteoglycans (33, 34) is most likely due to the loss in positive charges on the apoB100 molecule of oxidized LDL, since lipoprotein-proteoglycan interactions are primarily ionic in nature (7–9). The positive charges on apolipoproteins interact with negatively charged sulfate and carboxyl groups on the glycosaminoglycan chains of proteoglycans (7–9). We demonstrate here that the addition of bovine milk LPL to the system allows extensively oxidized (HOCl and 18-h copper-oxidized) LDL to bind to versican and biglycan and markedly enhances the binding of less extensively modified (AAPH- and 4-h copper-oxidized) LDL to these proteoglycans. Thus, LPL may be a mechanism that allows LDL to be retained by versican and biglycan, even if the LDL is oxidized. The fact that LPL may facilitate the trapping and retention of oxidized LDL has potential deleterious implications, since oxidatively modified LDL is known to contribute to the process of atherosclerosis (1–3). As LDL becomes progressively oxidized, it is no longer recognized by the LDL receptor. Intracellular accumulation of oxidized LDL leading to the formation of foam cells and plaque, which are hallmarks of atherosclerosis, occurs primarily via uptake by receptors on macrophages such as scavenger receptor type A, CD36, macrosialin, and other binding proteins (3).

LPL may have very different roles in the pathogenesis of atherosclerosis depending on its catalytic *versus* its structural (bridging) function. Recent investigations have found that individuals with naturally occurring LPL mutations that result in diminished activity have a higher incidence of ischemic heart disease, suggesting that high LPL activity may be protective against the development of atherosclerosis (58, 59). The mechanisms underlying this observation are not well understood but may relate in part to the increased circulating triglyceride and reduced high density lipoprotein concentrations observed in these individuals with LPL mutations (58, 60). From the data in the present paper, however, LPL would be predicted to be proatherogenic, acting as a bridging molecule between lipoproteins and proteoglycans and thus increasing the potential that native and oxidized LDL would be trapped and retained by proteoglycans of the extracellular matrix.

The anatomical location of LPL also would be predicted to be an important factor with regards to its pro- *versus* anti-atherogenic role. Active LPL on the endothelium in adipose tissue and skeletal muscle would be expected to be anti-atherogenic, as it functions primarily to reduce the circulating concentrations of atherogenic lipoproteins via lipolysis. This idea is supported by the observation that individuals with LPL mutations that confer low LPL activity are characterized by an atherogenic lipid profile (high triglyceride and low high density lipoprotein concentrations) and have an increased risk for ischemic heart disease, presumably related in part to this dyslipidemia (58, 60). Furthermore, LPL-mediated lipoprotein uptake by liver cells could reduce atherosclerosis via enhanced catabolism of

atherogenic particles. However, mice with heterozygous LPL deficiency (LPL+/−) had no more atherosclerosis compared with LPL+/+ mice, despite the presence of dyslipidemia (61).

Conversely, LPL in the vicinity of intra-arterial sites prone to atherosclerosis might exert proatherogenic effects by facilitating lipoprotein binding and retention. The plausibility of an atherogenic role for LPL is strengthened by observations that LPL is secreted by macrophages in atherosclerotic lesions (62, 63). Indeed, a recent study demonstrated that macrophage-specific overexpression of LPL in mice resulted in increased foam cell formation and atherosclerosis (64). Thus, LPL that is present in the arterial intima could function as a modifying molecule, thereby facilitating the initial retention and uptake of lipoproteins that is critical in the pathogenesis of atherosclerosis. The presence of LPL in the arterial intima also could enhance uptake of native or oxidized lipoproteins by macrophages. The ability of LPL to enhance the binding of native LDL to proteoglycans also would increase the retention of LDL in the artery wall, thereby rendering the lipoprotein susceptible to oxidative modification (3). It has been hypothesized that LDL bound to proteoglycans is more susceptible to oxidative modification than unbound LDL (57). The observations that versican and biglycan are secreted by vascular smooth muscle cells (16, 17) and several types of proteoglycans are synthesized by endothelial cells and macrophages (17, 65, 66) further strengthen the plausibility of this model existing *in vivo*.

We observed that the relative change in binding that occurs in the presence of LPL is greater for oxidized than for native LDL. This may be due to the fact that the "base-line" binding (*i.e.* in the absence of LPL) of native LDL to proteoglycans is so much greater than it is for oxidized LDL. There is a finite number of accessible proteoglycan/LPL-binding sites on LDL, and they could be closer to being saturated on native LDL in the absence of LPL and, therefore, not as responsive to the bridging effects of the lipase. The interaction of LPL and heparin, as well as proteoglycans, has been suggested to be ionic (20, 21), whereas the nature of the interaction between LPL and LDL is less clear. The fact that LPL is able to act as a bridge between proteoglycans and extensively oxidized LDL, which is depleted of enough positive charges to inhibit its binding to proteoglycans, suggests that there are charge-independent sites on LDL that are able to mediate its interaction with LPL.

The observation that LPL had a slightly greater effect on the interaction with LDL and biglycan compared with versican is supported by the findings of Margelin *et al.* (20) who demonstrated that dermatan sulfate glycosaminoglycans were more efficient than chondroitin sulfate glycosaminoglycans in dissociating LPL from immobilized proteoglycans. This may be due to differences in the ratios of iduronic to glucuronic acid units and to the distribution of sulfate groups along the carbohydrate backbone for these glycosaminoglycans. It also suggests that such differences may in part play a role in the observation that biglycan is co-localized with apolipoproteins in human atherosclerotic lesions to a much greater extent than versican (11).

It was important that we used an assay in which lipoproteins were interacted with proteoglycans under physiological conditions (*i.e.* 140 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$ buffer; pH 7.4; 37 °C incubation temperature), which is in contrast to many of the other assays used to evaluate this interaction. Other advantages of the system are that very small quantities of materials are needed and that from the binding curves that are generated, one can calculate affinity constants (K_a). As with all *in vitro* experiments, such as those presented here, it is important to know whether the components can occur at similar concentrations *in vivo*. However, it is difficult to measure subendothelial concentrations of LDL or LPL. Reichl *et al.* (67) reported that lymphatic LDL, which is a measure of LDL concentrations in the interstitial space such as the arterial intima, is approximately 10% of circulating levels. This would result in an estimate of lymphatic LDL of approximately 0.01–0.1 mg/ml. The affinity constants calculated from the gel shift assay for the interaction of native LDL with proteoglycans (Table I), therefore, are near this estimated physiological range. *In vivo* concentrations of LPL are more difficult to estimate. Circulating concentrations after heparin injection (approximately 1 μg/ml) (68) are much lower than the concentration we used in the experiments presented in this paper. However, it is feasible that high local concentrations of LPL exist within the artery wall, especially in the vicinity of macrophages. Furthermore, it has been demonstrated that circulating concentrations of inactive LPL are substantially higher than free (*i.e.* non-heparin-releasable) circulating concentrations of active LPL (approximately 100 ng/ml *versus* 4.2 ng/ml, respectively) (68). This inactive LPL is not influenced by heparin injection. Thus, there may be a significant pool of LPL that is available to act structurally as a bridging molecule between lipoproteins and proteoglycans and, therefore, be potentially atherogenic in the role outlined here. The concentrations of LDL (0.01 mg/ml) and LPL (10 μg/ml) that we have determined as approximate values needed for half-maximal binding are consistent with reports by others suggesting that 2–4 LPL dimers interact with one LDL particle (21). By using a molecular mass of 550 kDa for LDL and 60 kDa for LPL, we calculate that approximately 4 LPL dimers are present per molecule of LDL.

Given the above, retention of native and oxidized lipoproteins to proteoglycans on the arterial extracellular matrix is a potential mechanism by which LPL can be atherogenic. It can be envisioned that LPL acts structurally to form a bridge between arterial wall proteoglycans and atherogenic lipoproteins; native LDL would be more susceptible to oxidative modification, whereas LDL already oxidized would be able to be bound and to be retained after which it could be taken up by macrophages or have multiple other biological effects that could influence atherogenesis.

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