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Arterioscler Thromb Vasc Biol 2004;24;564-570; originally published online Jan 15, 2004;
DOI: 10.1161/01.ATV.0000117174.19078.85
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Objective—The aim of this study was to investigate the molecular mechanism for changes in proteoglycan binding and LDL receptor affinity on two compositional changes in LDL that have been associated with atherosclerosis: cholesterol enrichment of the core and modification by secretory group IIA phospholipase A2 ($sPLA_2$) of the surface.

Methods and Results—Transgenic mice expressing recombinant apolipoprotein (apo) B and $sPLA_2$ were generated. Recombinant LDL were isolated and tested for their proteoglycan and LDL receptor-binding activity. The results show site A (residues 3148-3158) in apoB100 becomes functional in $sPLA_2$-modified LDL and that site A acts cooperatively with site B (residues 3359-3369), the primary proteoglycan-binding site in native LDL, in the binding of $sPLA_2$-modified LDL to proteoglycans. Our results also show that cholesterol enrichment of LDL is associated with increased affinity for proteoglycans and for the LDL receptor. This mechanism is likely mediated by a conformational change of site B and is independent of site A in apoB100.

Conclusion—Site A in apoB100 becomes functional in $sPLA_2$-modified LDL and acts cooperatively with site B resulting in increased proteoglycan-binding activity. The increased binding for proteoglycans of cholesterol-enriched LDL is solely dependent on site B. **(*Arterioscler Thromb Vasc Biol*. 2004;24:564-570.)**

Key Words: lipoproteins ■ apolipoprotein B ■ proteoglycans ■ atherosclerosis ■ transgenic mice ■ phospholipase A2

High levels of plasma low-density lipoproteins (LDL) are a major risk factor for coronary disease and are the source of most of the cholesterol that accumulates in the arterial wall. Subendothelial retention of LDL is a key pathogenic process in atherosclerosis,[1,2] and several lines of evidence suggest that intramural retention of atherogenic lipoproteins involves the extracellular matrix, chiefly proteoglycans.[1] Subsequently, a series of biological responses to this retained material leads to an inflammatory response that promotes lesion formation and increased retention of atherogenic lipoproteins.[1]

Proteoglycans contain long carbohydrate side-chains of glycosaminoglycans, which are covalently attached to a core protein by a glycosidic linkage. The glycosaminoglycans consist of repeating disaccharide units, all bearing negatively charged groups, usually sulfate or carboxylic acid groups. In vitro, LDL binds with high affinity to many proteoglycans found in the artery wall. The interaction between LDL and proteoglycans involves basic amino acids in apolipoprotein (apo) B100, the protein moiety of LDL, that interact with the negatively charged glycosaminoglycans[3–5] or bridging molecules such as apoE or lipoprotein lipase.[1]

Isolation of large fragments of apoB100 from different regions characterized by concentrations of positive clusters indicated that up to 8 specific regions in delipidated apoB100 bind proteoglycans.[6–8] Two of these sites (site A at residues 3148 to 3158 and site B at residues 3359 to 3369) have been proposed to act cooperatively in the association with proteoglycans.[9] In the human sequence, a disulfide link between Cys-3167 and Cys-3297 has been postulated to bring the two sites into close proximity.[6] However, these cysteine residues are not conserved in other species.[7] We have earlier shown that LDL in which the basic amino acid residues of site B have been mutated to neutral amino acid residues (RK3359-3369SA LDL) fail to interact with proteoglycans.[8] This result indicates that site B is the primary proteoglycan-binding site in apoB100 when the protein is associated with native LDL.[8] However, the conformation of apoB100 on the surface of the LDL particle is likely dependent on the composition of the core lipids, the surface phospholipid content, and the diame-

Received July 3, 2003; revision accepted December 1, 2003.

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***Arterioscler Thromb Vasc Biol.* is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000117174.19078.85**

ter of the LDL particle. Thus, other binding sites may become functional in modified LDL.

An elevated plasma level of secretory group II phospholipase A2 ($sPLA_2$) is a strong independent risk factor for coronary heart disease.[10] Whether this is associated with the proatherogenic mechanisms of $sPLA_2$ activity in plasma or with its actions in the arterial wall remains to be elucidated. In blood plasma, $sPLA_2$ can modify the circulating lipoproteins, inducing the formation of small dense LDL particles, which are associated with increased risk of cardiovascular disease. Lipolysis of LDL by $sPLA_2$ alters the expression of apoB100 epitopes on the LDL particle,[11] and $sPLA_2$-modified LDL display increased interaction with glycosaminoglycans.[12] The plasma concentration of $sPLA_2$ is increased in systemic inflammations, such as rheumatoid arthritis (RA),[13] which is known to be associated with increased risk of coronary heart disease, and LDL from RA patients has increased binding affinities to glycosaminoglycans.[13]

Willner et al recently showed that the enrichment of cholesterol of LDL is another important risk factor for atherosclerosis; triglyceride-rich apoB-containing lipoproteins are not as atherogenic as those containing cholesteryl esters.[14] The authors proposed that the inflammatory response in atherosclerosis occurs primarily in response to toxicity from cholesterol accumulation in lesions.[14] However, it has also been shown that the triglyceride content of LDL are reciprocally related to the number of exposed free lysine amino groups of apoB100.[15] This could indicate that the increased atherogenicity of cholesteryl ester-rich LDL could, at least partly, be linked to an increased affinity for artery wall proteoglycans.

In this study, we tested the hypothesis that site A is important for proteoglycan-binding in $sPLA_2$-modified LDL. Here we show that site A acts cooperatively with site B to increase the affinity of $sPLA_2$-modified LDL for artery wall proteoglycans. We also show that cholesterol enrichment of the LDL particle is associated with increased affinity for proteoglycans. This mechanism is likely mediated by a conformational change of site B and is independent of site A.

Methods

Chemicals for SDS-PAGE were from Bio-Rad (Hercules, Calif). All other chemicals were from Sigma (Stockholm, Sweden). Horseradish peroxidase-conjugated polyclonal antibody against human apoB was from the Binding Site (Birmingham, UK); 1-Step Turbo TMB-ELISA was from Pierce (Rockford, Ill).

Generation of Transgenic Mice Expressing RK3148-3158SQ ApoB100-Containing LDL

The 95-kb apoB P1 plasmid p158[16] was prepared and modified by RecA-assisted restriction endonuclease cleavage.[17] See online Methods, available at http://atvb.ahajournals.org. The resulting P1 plasmid encodes RK3148-3158SQ apoB100 in which the basic residues arginine and lysine in site A are replaced with serine and glutamine, respectively. The plasmid also contains a CAA to CTA mutation in codon 2153 that effectively abolishes the formation of apoB48 in mouse livers.[17] The structure of human apoB100 containing the mutation is largely preserved when expressed in the mouse,[18] and the mutation does not affect proteoglycan binding or interaction with the LDL receptor.[7] P1 DNA was prepared and microinjected into fertilized mouse eggs (FVB/N) and back-crossed into C57BL/6J Bom. The two founders with the highest levels of plasma apoB were selected for breeding and further analysis.

Isolation of LDL

LDL (density=1.02 to 1.05 g/mL) were isolated from human apoB100 transgenic expressing RK3148-3158SQ LDL, RK3359-3369SA LDL[19] or recombinant control LDL[19] by sequential ultracentrifugation and dialyzed against 150 mmol/L NaCl and 0.01% EDTA, with pH 7.4.[19] Mouse apoE and apoB were removed by immunoaffinity chromatography with rabbit polyclonal antibodies.[19] Lion LDL was isolated identically[19] from lion plasma pooled from 8 lions of different ages and sexes.

Competition of Mouse apoE With Chemically Modified Human apoE

ApoB-containing lipoproteins (density=1.02 to 1.05 g/mL) isolated from transgenic LDL receptor-deficient ($Ldlr^{-/-}$ or $Ldlr^{+/+}$) mice were incubated with a 200-fold molar excess of cyclohexanedione-modified apoE from human very-LDL as described by Skålén et al.[2]

Characterization of Isolated Lipoproteins

The binding of LDL to biglycan or decorin was assessed with solid-phase assays as described in detail by Skålén et al.[2] Competitive LDL receptor-binding assays were performed as described,[19] and the amount of unlabeled lipoproteins needed to complete 50% with iodine (I) 125-labeled LDL after a 3-hour incubation at 4°C was determined from an exponential decay curve-fitting model.[19] Lipoprotein particle diameters were determined by dynamic light-scattering analysis with a Microtrac Series 250 Ultrafine particle analyzer fitted with a flexible conduit-sheathed probe tip (UPA-250; Microtrac, Clearwater, Fla).[21]

Lipolysis of Recombinant and Lion LDL With PLA_2

LDL (1 mg/mL) was incubated for 3 hours in 100 mmol/L HEPES, 5 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 140 mmol/L NaCl, and 10 μmol/L butylated hydroxytoluene, with pH 7.4, with or without 50 ng/mL PLA_2 (from bee venom) in the presence of 2% (weight/volume) bovine serum albumin.[22] Lipolysis was stopped by adding EDTA to achieve a final concentration of 10 mmol/L as described.[22] Samples of the reaction were separated by fast performance liquid chromatography gel filtration (ΔKTA Explorer; Amersham Pharmacia) with a Superose 6 HR 10/30 column. Aliquots of 200 μL were injected onto the column and separated with PBS buffer, with pH 7.4, at a flow rate of 0.2 mL/min. The fractions corresponding to non-aggregated LDL were subjected to binding studies.

Data Analysis

Curve-fit analysis was performed in GraphPad Prism version 3.00 for Windows using a nonlinear curve-fit assuming one-site binding (San Diego, Calif). Statistical analysis was performed using SigmaStat for Windows version 2.03 (SPSS, Chicago, Ill).

Results

Generation and Characterization of Recombinant RK3148–3158SQ LDL

To evaluate the importance of site A (residues 3148-3158) in the interaction between native apoB100-containing LDL and proteoglycans, we generated transgenic mice expressing human recombinant LDL in which site A in apoB100 was mutated by substituting serines and glutamines for arginines and lysines, respectively (RK3148-3158SQ). Recombinant control LDL, RK3148-3158SQ LDL, and RK3359-3369SA LDL[19] were isolated from mouse plasma by ultracentrifugation, and lipoproteins containing endogenous mouse apoE and apoB were removed by immunoaffinity chromatography;

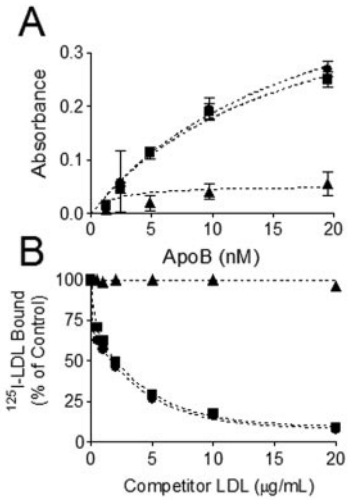

Figure 1. Solid-phase assays of the binding of LDL to biglycan (A) and competitive receptor binding assay of recombinant LDL (B). The abilities of recombinant control LDL (●), RK3148-3158SQ LDL (■), and RK3359-3369SA LDL (▲) to bind biglycan or compete with ^{125}I-labeled human plasma LDL (2 μg/mL) for binding to LDL receptors on normal human fibroblasts were determined. The recombinant lipoproteins were isolated from 20 mice, and endogenous apoE and apoB were removed by affinity chromatography. The results represent mean values (±SD) from 2 independent experiments, each performed with freshly isolated LDL (n=4 in each experiment) (A) or the mean of 2 independent experiments, each performed in duplicate (B).

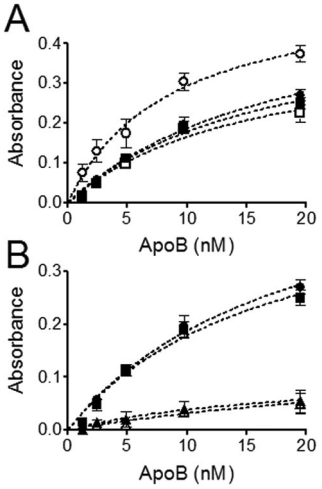

Figure 2. Binding of recombinant LDL isolated from sPLA$_2$ transgenic mice to biglycan. The abilities of recombinant control LDL (●) and RK3148-3158SQ LDL (■) and RK3359-3369SA LDL (▲) isolated from human apoB transgenic mice (●, ■, ▲) and from apoB×sPLA$_2$ transgenic mice (○, △, □) to bind biglycan were determined. Endogenous mouse apoB and apoE were removed from all lipoproteins by immunoaffinity chromatography. The results represent mean values (±SD) from two independent experiments, each performed with freshly isolated LDL from 15 mice of each group (n=4 in each experiment).

the removal of all endogenous apoB and apoE was confirmed by Western blot analysis (not shown). In RK3359-3369SA LDL, the arginine residues were converted to serines and the lysine residues were changed to alanines in site B of apoB100.[19] Previously, we showed that site B (residues 3359-3369) is the combined proteoglycan binding and LDL receptor binding site in apoB100 and that RK3359-3369SA LDL do not interact with proteoglycans or the LDL receptor.[7]

The isolated recombinant control LDL, RK3148-3158SQ LDL, and RK3359-3369SA LDL had almost identical lipid compositions (data not shown) and particle diameters (27.1±0.86, 26.7±0.20, and 26.8±0.85 nm, respectively; mean±SD).

To determine the ability of the recombinant LDL to interact with proteoglycans, we subjected recombinant control LDL, RK3148-3158SQ LDL, and RK3359-3369SA LDL to solid-phase assays with biglycan (Figure 1A) and decorin (not shown). In two independent experiments, recombinant control LDL and recombinant RK3148-3158SQ LDL bound biglycan with high affinity, whereas recombinant RK3359-369SA LDL displayed severely impaired binding (Figure 1A).

To investigate the importance of site A in the interaction with the LDL receptor, we isolated recombinant control LDL, RK3148-3158SQ LDL, and RK3359-3369SA LDL and tested them in an in vitro competitive receptor-binding assay (Figure 1B). Recombinant control LDL and RK3148-3158SQ LDL had almost identical receptor binding (ED_{50} 2.1 and 2.3 μg/mL, respectively), whereas recombinant RK3359-3369SA LDL displayed defective receptor binding (ED_{50} >20 μg/mL). Taken together, these results indicate that site A in native LDL is not involved in binding to proteoglycans or to the LDL receptor.

Solid-Phase Assays of Recombinant LDL Isolated From sPLA$_2$ Transgenic Mice

Next, we tested the hypothesis that site A can modulate the proteoglycan binding activity of modified LDL. Recombinant control LDL, RK3148-3158SQ LDL, and RK3359-3369SA LDL were isolated by ultracentrifugation from human apoB transgenic mice and from doubly transgenic mice expressing human apoB and sPLA$_2$.[23] After removal of endogenous mouse apoE-containing and apoB-containing lipoproteins by immunoaffinity chromatography, the isolated recombinant LDL were subjected to solid-phase assays with biglycan (Figure 2A) and decorin (not shown). In two independent experiments, recombinant control LDL from sPLA$_2$ transgenic mice displayed increased binding, whereas recombinant RK3148-3158SQ LDL from sPLA$_2$ transgenic mice and sPLA$_2$ nontransgenic littermate controls had almost identical binding (Figure 2A). These results indicate that site A is important for the increased interaction of sPLA$_2$-modified LDL with glycosaminoglycans. However, RK3359-3369SA LDL did not interact with biglycan, regardless of whether it was isolated from sPLA$_2$ transgenic mice or not (Figure 2B). These results indicate that site A in sPLA$_2$-modified LDL modulates the binding of the LDL particle to proteoglycans but cannot substitute for site B, which is essential for the interaction with proteoglycans.

Characterization of sPLA$_2$-Modified Recombinant LDL

sPLA$_2$ hydrolyzes glyceroacyl phospholipids to form nonesterified fatty acids and lysophospholipids. Therefore, we analyzed the phospholipid composition of recombinant LDL isolated from human apoB transgenic mice and from apoB transgenic mice with mice expressing sPLA$_2$ (apoB×sPLA$_2$

TABLE 1. Phospholipid Composition of Recombinant LDL Isolated From Human apoB Transgenic Mice and From Double-Transgenic apoB×sPLA$_2$ Mice

	Human Recombinant LDL	sPLA$_2$-Modified LDL
Lysophosphatidylcholine	7.43±1.4*	10.03±1.03*
Sphingomyelin	7.77±0.91	8.77±0.74
Phosphatidylcholine	79.8±3.41	77.17±0.21
Phosphatidylinositol	4.1±1.4	3.43±0.38
Phosphatidylethanolamine	0.87±1.5	0.57±0.98
Total phosphorus (nmol/μg protein)	1.83±0.024	1.68±0.023

Values are mean±SD of % phosphorous content (nmol) of three different experiments.

Comparisons between independent groups were performed with Mann–Whitney *U* test.

*Significant change; $P<0.05$.

transgenic mice). The sPLA$_2$-modifed LDL contained more lysophosphatidylcholine than nonmodified recombinant LDL (Table 1). We also analyzed the content of total cholesterol, free cholesterol, cholesteryl esters, and triglycerides. No differences were found in the content of these lipids in recombinant LDL isolated from apoB transgenic mice or from apoB×sPLA$_2$ transgenic mice (Table 2). Neither the phospholipid composition (Table 1) nor the content of the other lipids (Table 2) was affected by the type of apoB100 (control or the two mutations, ie, RK3148-3158SQ or RK3359-3369SA) in LDL isolated from apoB transgenic mice or from apoB×sPLA$_2$ transgenic mice (data not shown).

Dynamic light-scattering analysis of recombinant control LDL isolated from human apoB transgenic mice and from apoB×sPLA$_2$ transgenic mice showed that recombinant LDL from the singly human apoB transgenic mice was significantly larger (26.7±0.42 versus 25.8±0.38 nm;, mean ± SD; $P<0.05$). Taken together, these results show that in vivo modification of recombinant LDL in sPLA$_2$ transgenic mice induces the formation of smaller LDL enriched in lysophosphatidylcholine. Thus, it is possible that the conformational change of apoB in LDL modified with sPLA$_2$ in vivo is caused by the formation of smaller LDL and/or by changes in the phospholipids composition of the LDL particle.

Solid-Phase Assays of Recombinant LDL Isolated From sPLA$_2$ Transgenic Mice by Size-Exclusion Chromatography

To discriminate between these possibilities, recombinant control LDL and RK3148-3158SQ LDL were isolated from human apoB transgenic mice and from apoB×sPLA$_2$ transgenic mice by a combination of ultracentrifugation and size-exclusion chromatography and subjected to solid-phase assays with biglycan. The isolated LDL had almost identical size (26.4±0.23 and 26.3±0.15 nm). In two independent experiments, recombinant control LDL from sPLA$_2$ transgenic mice displayed increased binding, whereas recombinant RK3148-3158SQ LDL from sPLA$_2$ transgenic mice and sPLA$_2$ nontransgenic littermate controls had almost identical binding. The K_d for control LDL and RK3148-3158SQ LDL isolated from apoB transgenic mice were 16.3 and 17.1, respectively, and from apoB×sPLA$_2$ transgenic mice 10.3 and 16.6, respectively. The results also showed that the diameter of sPLA$_2$-modified and non-modified recombinant control LDL were inversely correlated to the binding affinity to biglycan; smaller LDL had higher binding affinity to biglycan than larger LDL (data not shown). Taken together, the results show that the conformational change of apoB in sPLA$_2$-modified LDL is caused by the formation of smaller LDL and by changes in the phospholipid composition of the LDL particle.

TABLE 2. Lipid Composition of Recombinant LDL Isolated From Human apoB Transgenic Mice and From Double-Transgenic apoB×sPLA$_2$ Mice

	Human Recombinant LDL	sPLA$_2$-Modified LDL
Total cholesterol	1.58±0.06	1.51±0.06
Free cholesterol	0.61±0.02	0.58±0.03
Triglycerides	1.32±0.11	1.24±0.07
Cholesteryl esters	0.97±0.04	0.93±0.03
Phospholipids	0.046	0.042

Results represent mean values (±SD) (n=5) in μg lipid/μg protein.

To investigate if in vitro digestion and in vivo modification of LDL with PLA$_2$ give similar results, recombinant control LDL, RK3359-3369SA LDL, and RK3148-3158SQ LDL were isolated and digested in vitro with PLA$_2$ for 3 hours. Samples of the reaction were separated by size-exclusion chromatography, and non-aggregated LDL was isolated and subjected to binding studies. This was performed because lipolysis of LDL with PLA$_2$ in vitro induces particle aggregation.[22] The results from these studies showed that in vitro modification of recombinant LDL with PLA$_2$ gave similar results as in vivo modification of LDL, ie, binding affinity increased on PLA$_2$ digestion and site A was required for the increased interaction of sPLA$_2$-modified LDL with glycosaminoglycans (data not shown). However, the intra-assay variations (ie, imprecision) and the inter-assay variations (ie, irreproducibility) were much more pronounced when analyzing the binding of LDL digested with PLA$_2$ in vitro compared with LDL modified in vivo.

Binding of Lion LDL to Biglycan and to the LDL Receptor

Our data indicate that site A becomes exposed and functional on sPLA$_2$ modification and that site A and site B act cooperatively in binding to proteoglycans. In the human sequence, site A and site B are separated by 202 amino acids, but a disulfide link between Cys-3167 and Cys-3297 has been postulated to bring the two binding sites into close proximity.[10] However, these cysteine residues are not conserved in other species;[6] but because of a naturally occurring deletion between site A and site B in several carnivores, including lions, the binding sites are separated by 76 amino acids only in these species.[24] Therefore, we investigated the proteoglycan-binding activity of lion LDL with and without PLA$_2$ digestion.

Lion LDL and human LDL were isolated and digested in vitro with PLA$_2$ for 3 hours and non-aggregated LDL was isolated by a combination of ultracentrifugation and size-exclusion chromatography. The PLA$_2$ modification induced

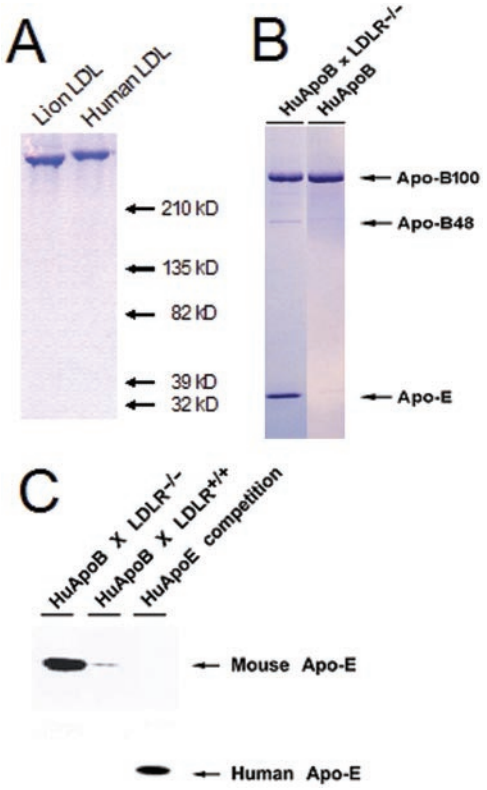

Figure 3. Coomassie staining and Western analysis of apoB-containing LDL. A, Lion and human plasma LDL (density=1.02 to 1.05 g/mL) were isolated by sequential ultracentrifugation. Five micrograms of LDL were separated by SDS-PAGE with 3% to 15% gels. Arrows indicate molecular mass markers. B, Recombinant LDL (density=1.02 to 1.05 g/mL) was isolated from *LDLR*$^{-/-}$ mice (HuApoB×LDLR$^{-/-}$) or *LDLR*$^{+/+}$ mice (HuApoB), and 2 μg were analyzed on SDS-PAGE with 3% to 15% gels. C, Endogenous apoB was removed by immunoaffinity chromatography, and 0.5 μg of recombinant control LDL (density=1.02 to 1.05 g/mL) from *LDLR*$^{-/-}$ mice (lane 1) or *LDLR*$^{+/+}$ mice (lane 2) was analyzed by Western blots with polyclonal antibodies against mouse apoE. Lane 3 shows recombinant control LDL (0.5 μg) from *LDLR*$^{-/-}$ mice analyzed by Western blots with polyclonal antibodies against mouse apoE (upper panel) and human apoE (lower panel) after incubation with CHD-modified human apoE (HuApoE competition). The CHD-modified samples were incubated with an equal volume of 1 mol/L hydroxylamine, 0.3 mol/L mannitol, at pH 7.0, at 37°C for 16 hours to reverse the modification of the arginine residues before sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the formation of smaller LDL enriched in lysophosphatidylcholine. The diameter of lion LDL and human LDL decreased from 27.2±0.33 to 26.1±0.25 nm and from 27.5±0.39 to 26.3±0.15 nm, respectively. The content of lysophosphatidylcholine in PLA_2-digested LDL increased 2.4-fold±0.3 and 2.8-fold±0.6, respectively.

As shown by SDS-PAGE, lion apoB migrated faster than human apoB100, and the LDL did not contain any apolipoprotein other than apoB (Figure 3A). In solid-phase assays, lion LDL bound with higher affinity than human LDL to biglycan; however, PLA_2 modification did not increase the binding of lion LDL to biglycan, as was seen in human LDL (Figure 4A). The K_d for lion and human LDL were 10.2 and 15.4, respectively, and for PLA_2-modified lion and human LDL 10.4 and 11.3, respectively. The binding capacity of lion

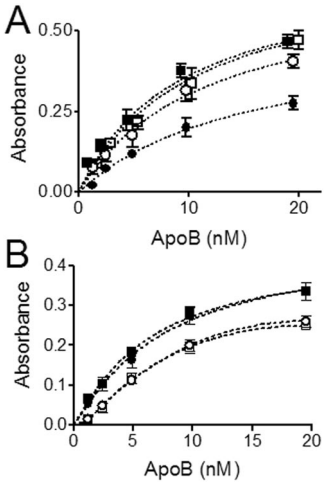

Figure 4. Solid-phase assays of lion LDL or recombinant LDL isolated from *Ldlr*$^{+/+}$ and *Ldlr*$^{-/-}$ mice to biglycan binding to biglycan. A, Lion (■) and human (●) plasma LDL (density=1.02 to 1.05 g/mL) were isolated by sequential ultracentrifugation and incubated with (○ and □) or without (● and ■) 50 ng/mL of PLA_2 (from bee venom). To prevent overlap in the figure, ■ and □ have been moved by −0.5 and +0.5 U on the X-axis, respectively. B, The abilities of recombinant control LDL (●) and RK3148-3158SQ LDL (■) isolated from *Ldlr*$^{+/+}$ (○ and □) and *Ldlr*$^{-/-}$ (● and ■) mice to biglycan were determined. Endogenous apoE was replaced with cyclohexanedione-modified human apoE, and endogenous mouse apoB was removed by immunoaffinity chromatography. The results represent mean values (±SD) from two independent experiments, each performed with freshly isolated LDL from 15 mice of each group (n=4 in each experiment).

and human LDL was similar (B_{max} 0.71 and 0.66, respectively), indicating that the deletion between sites A and B increases the affinity for proteoglycans but not the binding capacity. In competitive LDL receptor-binding studies with human ^{125}I-labeled LDL, lion and human LDL had almost identical receptor binding (ED_{50} 1.9 and 1.8 μg/mL, respectively). These results are consistent with the hypothesis that site A is functional on lion LDL with or without PLA_2 modification.

Solid-Phase Assays of Cholesterol-Enriched ApoB100 Recombinant LDL With Biglycan

To analyze if cholesterol enrichment of LDL is associated with increased affinity for proteoglycans, human apoB transgenic mice expressing recombinant control LDL, RK3148-3158SQ LDL, or RK3359-3369SA LDL were bred onto *Ldlr*$^{-/-}$ background. Recombinant LDL isolated from *Ldlr*$^{-/-}$ mice is enriched with cholesteryl esters. The total cholesterol to triglyceride (in mmol/L) ratios in recombinant LDL isolated from human apoB×*Ldlr*$^{+/+}$ transgenic mice and apoB×*Ldlr*$^{-/-}$ transgenic mice are 1.7 and 4.2, respectively.

Recombinant control LDL and RK3148-3158SQ LDL from *Ldlr*$^{+/+}$ and *Ldlr*$^{-/-}$ mice were isolated by ultracentrifugation, and endogenous mouse apoB and apoE were removed. LDL isolated from *Ldlr*$^{-/-}$ mice is greatly enriched in endogenous apoE (Figure 3B), and immunoaffinity techniques fail to remove all endogenous apoE from LDL isolated from *Ldlr*$^{-/-}$ mice.[1,2] Competition of mouse apoE with CHD-modified human apoE efficiently removes the endoge-

nous apoE from the recombinant LDL (Figure 3C), and the CHD-modified apoE fail to facilitate an indirect binding between the recombinant LDL and glycosaminoglycans.[1,2] The competition of mouse apoE with chemically modified human apoE did not affect the cholesterol or triglyceride content of the LDL particles. The recovery of these lipids were 94%±8% and 92%±11% after competition with CHD-modified apoE, respectively (mean±SD, n=5).

The purified recombinant control LDL and RK3148-3158SQ LDL from *Ldlr*$^{+/+}$ and *Ldlr*$^{-/-}$ mice were then subjected to size-exclusion chromatography to isolate LDL particles with almost identical size (diameters: 26.8±0.84 and 26.7±0.21 nm versus 26.9±1.87 and 26.8±0.42 nm, respectively; mean±SD). In two independent experiments, recombinant control LDL and RK3148-3158SQ LDL isolated from *Ldlr*$^{-/-}$ mice bound biglycan and decorin (not shown) with higher affinity than recombinant control LDL and RK3148-3158SQ LDL isolated from *Ldlr*$^{+/+}$ mice (Figure 4B). Within each genotype, recombinant control LDL and RK3148-3158SQ LDL displayed almost identical binding. The K_d for control LDL and RK3148-3158SQ LDL isolated from *Ldlr*$^{+/+}$ were 17.0 and 16.7, respectively, and from *Ldlr*$^{-/-}$ mice 9.1 and 8.2, respectively. The binding capacity of recombinant control LDL and RK3148-3158SQ LDL was almost identical, both when isolated from *Ldlr*$^{+/+}$ and from *Ldlr*$^{-/-}$ mice (B_{max} were 0.51, 0.47, 0.51, and 0.48, respectively). Control experiments showed that RK3359-3369SA LDL from both *Ldlr*$^{+/+}$ and *Ldlr*$^{-/-}$ mice had severely impaired binding (data not shown). Competitive receptor-binding studies with human ^{125}I-labeled LDL showed that cholesterol-enriched recombinant control LDL from *Ldlr*$^{-/-}$ mice bound with higher affinity to the LDL receptor than that from *Ldlr*$^{+/+}$ mice (ED_{50} 1.7 versus 2.5 μg/mL).

These findings indicate that cholesterol enrichment of the LDL particle is associated with increased affinity for proteoglycans and for the LDL receptor. This mechanism is likely mediated by a conformational change of site B and is independent of site A in apoB100.

Discussion

The aim of this study was to investigate the molecular mechanism for changes in proteoglycan binding and LDL receptor affinity on two compositional changes in LDL: modification by secretory group IIA phospholipase A2 ($sPLA_2$) of the surface and cholesterol-enrichment of the core.

Elevated plasma $sPLA_2$ activity has been shown to be an important risk factor for coronary heart disease.[8] Thus, clarification of the proatherogenic mechanisms by which $sPLA_2$ acts in the plasma and in the artery wall is important to our understanding of the pathogenesis of the disease. To investigate the molecular mechanism for how circulating $sPLA_2$ induces the increased binding affinities of LDL to glycosaminoglycans,[13] we crossed our human apoB transgenic mice with mice expressing $sPLA_2$.[23] The serum activity of $sPLA_2$ in these transgenic mice is elevated approximately 8-fold compared with nontransgenic littermates, but there is no evidence of systemic inflammation in the transgenic mice.[23] Here we show that in vivo modification of recombinant LDL in $sPLA_2$ transgenic mice induces the formation of slightly smaller LDL enriched with lysophosphatidylcholine. These results are in agreement with data of Grass et al[23] and with results from patients with rheumatoid arthritis who have increased plasma levels of $sPLA_2$.[13] Taken together, our results are consistent with the hypothesis that $sPLA_2$ modification of LDL in the circulation leads to exposure of site A, resulting in increased proteoglycan binding. Furthermore, the conformational change of apoB in $sPLA_2$-modified LDL is caused by the formation of smaller LDL and by changes in the phospholipid composition of the LDL particle.

However, it is conceivable that some of the observed effects could result from indirect secondary consequences. For example, $sPLA_2$ modification might cause LDL with low PG binding to be cleared, leaving behind a high-affinity subpopulation. It is also possible that $sPLA_2$ digestion in vivo allows a second enzyme to further alter the LDL, eg, sphingomyelinase, which has been reported to readily digest $sPLA_2$-treated LDL.[25] However, because a gentle PLA_2 digestion of recombinant LDL in vitro gives similar results, and because isolated LDL from apoB×$sPLA_2$ transgenic mice contain abundant sphingomyelin, these alternative explanations seems unlikely.

In some experiments, LDL was digested with PLA_2 in vitro. These incubations were performed for 3 hours only. The reason for this was to achieve similar modification of LDL as in patients with increased plasma levels of $sPLA_2$.[13] Prolonged digestion of LDL with PLA_2 in vitro results in hydrolysis of nearly all of the phosphatidylcholines, in pronounced aggregation, and in the presence of glycosaminoglycans fusion of LDL,[22] ie, the modifications of LDL that are more likely to occur within the extracellular matrix of the arterial wall.

The findings that lion LDL, with a 126-amino acid residue deletion between site A and site B, has increased affinity for artery wall proteoglycans and that PLA_2 modification of lion LDL did not increase the binding of lion LDL to biglycan are consistent with the hypothesis that site A on lion LDL is also exposed with or without $sPLA_2$ modification. However, studies with cross-linking or some other direct proof would be needed to draw this conclusion. It is also possible that other basic sequences or even other apolipoproteins might play a role in the unusual properties of lion LDL. However, the latter possibility seems unlikely because no apolipoproteins other than apoB was detected in isolated lion LDL (Figure 3A). The fact that two independent deletions have been found in apoB100 between site A and site B might indicate that these mutations provide an evolutionary advantage. In addition to the 126-amino acid deletion in several carnivores, including lions,[24] an 84-amino acid deletion has been found in elephants.[24] A tentative hypothesis is that an enhanced binding of LDL to matrix might promote wound healing, as has been proposed, although not proven, for Lp(a).[26]

Our results show that cholesterol-enriched LDL has increased affinity for glycosaminoglycans and the LDL receptor. This mechanism is likely mediated by a conformational change of site B and is, in contrast to $sPLA_2$-modified LDL, independent of site A. The finding that cholesterol-enriched LDL have increased affinity for artery wall proteoglycans offers an additional explanation for why the atherogenicity of apoB-containing lipoproteins correlates with their cholesteryl ester content.

The binding studies with LDL isolated from *Ldlr*$^{-/-}$ and *Ldlr*$^{+/+}$ mice were performed with LDL of almost identical

diameters, and the results are consistent with the triglyceride-dependent decrease in affinity for the LDL receptor reported in hypertriglyceridemic subjects.[27] However, our results are not consistent with the finding that in the absence of changes in the size of the particle, the relative content of neutral lipid in the core of LDL does not significantly affect apoB conformation or its affinity for the LDL receptor.[28] The reasons for these differences are unclear but could reflect differences in experimental design, because the secondary structure of apoB in LDL strongly depends on the physical state of the lipoprotein core.[29]

In this study, we tested the hypothesis that site A is important for proteoglycan binding of $sPLA_2$-modified LDL. The results confirm that site B is the principal proteoglycan-binding site in native apoB100-containing LDL and show that site A acts cooperatively with site B in the association with proteoglycans in $sPLA_2$-modified LDL. The results also show that cholesterol enrichment of the LDL particle is associated with increased affinity for proteoglycans and for the LDL receptor. This mechanism is likely mediated by a conformational change of site B and is independent of site A in apoB100. Taken together, the results show that LDL affinity for proteoglycans can be modified by size-dependent effects and size-independent compositional effects of either the surface or the core.

Acknowledgments

The authors thank K. Skålén, E. Björk, L. Lindgren, M. Heyden, B. Gabrielli, and K. Arnold for excellent technical assistance, S. Ordway and G. Howard for editorial assistance, and Dr Torsten Möller and Karin Magnusson at Kålmorden Zoo for lion plasma. This work was supported by the Swedish Medical Research Council, The Swedish Foundation for Strategic Research, and The Swedish Heart–Lung Foundation.

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SUPPLEMENTARY INFORMATION ONLINE

Generation of a P1 Plasmid Encoding RK3148-3158SQ apoB100—The 95-kb apoB P1 plasmid 6-GSBM (*i.e.* glycosaminoglycan-binding sites mutated)[1] was prepared and modified by RecA-assisted restriction endonuclease cleavage as described.[2-4] The P1 plasmid 6-GBSM encodes human apoB100 with mutations in the six carboxyl-terminal glycosaminoglycan-binding sequences in delipidated apoB100.[1] A 2.64-kb genomic DNA fragment containing the RK3148-3158SQ mutation was isolated from the P1 plasmid with oligomers *Eco*RI-38863 and *Eco*RI-41496 [3, 4] and ligated into a pBluescript vector containing a 3.1-kb genomic apoB fragment (*Eco*RI-35763 to *Eco*RI-38863) along with the chloramphenicol-resistance/*sacB* selectable markers.[5] The 5.7-kb apoB fragment together with the Cm/SacB sequence was isolated by RecA-assisted restriction endonuclease cleavage using the oligos 5′-gcatattttaaacacaagtgaaatatctggttaggatagaattctcccagttttcacaatgaaaacatc-3′ and 5′-gggaaaactcccacagcaagctaatgattatctgaattctcatgtttgacagcttatcatcg-3′ and ligated to a 5.7-kb-deleted "B100 Leu-Leu" P1 vector plasmid [3]. Plasmids containing the mutated apoB fragment and the chloramphenicol resistance/*sacB* genes were selected on chloramphenicol-containing plates.[5] A P1 plasmid was then isolated and characterized by restriction enzyme digestion. The chloramphenicol resistance/*sacB* genes were removed with selection on 5% sucrose, leaving the mutated 5.7-kb fragment within the apoB gene. The resulting P1 plasmid encodes RK3148-3158SQ apoB100 in which the basic residues arginine and lysine in Site A are replaced with glutamine and serine, respectively.

LDL lipid analysis—Total cholesterol and triglyceride concentrations were determined by enzymatic assays from Roche Diagnostic (Mannheim, Germany), whereas free cholesterol was estimated using a cholesterol oxidase kit from Wako Chemicals (Neuss, Germany). For phospholipid analysis, lipids from LDL were extracted with chloroform/methanol (2:1). For

each sample, four aliquots of extract were loaded on a multi channel (Baker Si-250) thin layer chromatography (TLC) plate. Phospholipids were separated in one dimension and their relative amount was quantitated by phosphorus assay in the presence of the silica gel.[6] Background phosphorus was assayed in an adjacent lane on the TLC plate and subtracted from each value. Protein concentrations were determined with the Lowry assay.[7]

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