Decrease in pH Strongly Enhances Binding of Native, Proteolyzed, Lipolyzed, and Oxidized Low Density Lipoprotein Particles to Human Aortic Proteoglycans*

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Binding of low density lipoprotein (LDL) to proteoglycans and modification of LDL are key processes in atherogenesis. Recently, it has been demonstrated that during atherogenesis the extracellular pH of atherosclerotic lesions decreases. We have examined the effect of the decreased pH on the binding of LDL to human aortic proteoglycans. The binding of native, oxidized, proteolyzed (α-chymotrypsin-treated), or lipolyzed (sphingomyelinase- or phospholipase A2-treated) LDL particles to proteoglycans were measured in microtiter well assays at pH 5.5–7.5. We found that the lower the pH, the higher the amount of binding of LDL to proteoglycans. At the lowest pH tested (pH 5.5), the amounts of proteoglycan-bound native, proteolyzed, sphingomyelinase-, and phospholipase A2-treated LDL particles were 20-, 23-, 30-, and 37-fold higher, respectively, than at pH 7.5. Interestingly, although oxidized LDL failed to bind to proteoglycans at neutral pH, there was significant binding at acidic pH. Binding of native and modified LDL to proteoglycans at pH 5.5 was blocked by 1 mM NaCl, indicating that at neutral pH LDL binds to proteoglycans via ion interactions. Inhibition of this binding by acetylation and cyclohexanedione treatment of LDL showed that the positively charged amino acids of apolipoprotein B-100, lysine, and arginine, respectively, mediated the ionic interaction. Taken together, our results suggest that in areas of atherosclerotic arterial intima where the extracellular pH decreases, retention of LDL by proteoglycans is enhanced, leading to extracellular accumulation of LDL and progression of the disease.

Accumulation of cholesterol in the inner layer of the arterial wall, the intima, leads to the development of atherosclerotic lesions, the lesional cholesterol being mostly derived from circulating low density lipoprotein (LDL) particles (1). Once LDL particles have entered the subendothelial space they are entrapped by the extracellular matrix, particularly by intimal proteoglycans (2–4), which form an organized, negatively charged extracellular matrix, especially in atherosclerotic lesions, and LDL particles (11). Indeed, intimal LDL particles show signs of proteolysis, phospholipolysis, and oxidation, and intimal lipid droplets have features that suggest they are formed from modified LDL particles (11). Proteolysis, phospholipolysis, and oxidation induce changes in the surface monolayer of LDL particles. Sphingomyelinase (SMase) hydrolyzes sphingomyelin molecules to water soluble phosphocholine and ceramide, whereas phospholipase A2 (PLA2) hydrolyzes fatty acid esters of diacylglycerol phospholipids to form lysosphatidylcholine and fatty acids. Oxidation induces changes in both the protein and lipid components of LDL particles. Thus, lipid peroxides are formed that decompose further into aldehydes capable of reacting with apoB-100 and ultimately lead to cleavage of apoB-100 into peptides.

Based on their structural differences at different stages of atherogenesis, atherosclerotic plaques are classified as early, intermediate, advanced, and complicated lesions (12–14). Recently, other variables of the advancing lesions have also been described and related to the structural differences of the plaques. Plaques show heterogeneity both in temperature (15) and pH (16). Naghavi and co-workers (16) showed that in the lipid-rich areas of the plaques, the pH was more than one pH unit lower than in the calcified areas of the same specimens. This is expected because atherosclerosis is an inflammatory disease (17), and it is known that in areas in which inflammation takes place, the temperature rises and the pH decreases (18). Macrophages, which are abundant in atherosclerotic lesions, can substantially acidify their surroundings by extruding hydrogen ions (18). Moreover, in advanced atherosclerotic plaques there are hypoxic areas (19) in which cells may suffer from ischemia and therefore, to generate ATP, convert glucose into lactate, which once secreted will locally decrease the extracellular pH.

In the present study we have examined the effect of low pH on the binding of LDL particles to human aortic proteoglycans. In addition, we have proteolyzed, lipolyzed, and oxidized LDL and examined the binding of these modified particles to proteoglycans at different pH values.

**EXPERIMENTAL PROCEDURES**

**Isolation and Modifications of LDL**—Human LDL (d = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers (n = 8) by sequential ultracentrifugation in the presence of 3 mM EDTA (20, 21). The amounts of LDL are expressed in terms of their protein concentrations, which were determined by the method of Lowry et al. (22) with bovine serum albumin as a standard. Each experiment was performed with LDL from at least two donors.

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LDL (1 mg/ml) was incubated in Dulbecco’s phosphate-buffered saline (BioWhittaker, Verviers, Belgium) containing 2% (w/v) fatty acid-free bovine serum albumin (BSA, MP Chemicals) with 0.5 mg/ml of α-chymotrypsin (α-CT) (Sigma), 50 milliunits/ml of sphingomyelinase (Sigma), or 0.5 μg/ml of phospholipase A2 (Sigma) for 1 h at 37 °C. After the incubation, proteolysis was stopped by adding phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 1 mM, and lipolysis was stopped by adding EDTA to a final concentration of 10 mM. The degree of proteolysis was determined with SDS-PAGE, and the degrees of lipolysis were determined by thin layer chromatography, as described previously (23).

LDL (1 mg/ml) was oxidized by 5 μM copper sulfate in phosphate-buffered saline for 1, 3, 6, or 18 h at 37 °C. The degree of oxidation was determined by measuring thiobarbituric acid-reactive substances (24) and reactive lysine residues (ε-amino groups of lysine residues) (25). Lysine and arginine residues of LDL were modified by treatment with acetic anhydride (26) or 1,2-cyclohexanedione (20), respectively.

The degree of aggregation/fusion in the modified LDL samples was determined by gel filtration in Superose 6 10/300 GL column (Amersham Biosciences).

Preparation and Characterization of Aortic Proteoglycans—Proteoglycans from the intima-media of human aortas were obtained at autopsy within 24 h of accidental death and were prepared essentially by the method of Hurt-Camejo et al. (27), as described previously (28). Glycosaminoglycans were quantified by the method of Bartold and Page (29), and the amounts of proteoglycans are expressed in terms of their glycosaminoglycan content.

Binding of LDL to Human Aortic Proteoglycans—The wells of polystyrene 96-well plates (Thermo Labsystems, Vantaa, Finland) were coated with 100 μl of human aortic proteoglycans (50 μg/ml in phosphate-buffered saline) by incubation at 4 °C overnight. Wells were blocked with 3% BSA, 1% fat-free milk powder, and 0.05% Tween 20 in phosphate-buffered saline for 1 h at 37 °C. Wells without proteoglycans served as controls.

100 μl of LDL (0.1 mg/ml) in a buffer containing 1% BSA, 140 mM NaCl, 2 mM CaCl2, 2 mM MgCl2 and either 20 mM MES (pH 5.5 or 6.0), 20 mM PIPES (pH 6.5 or 7.0), or 20 mM HEPES (pH 7.5) was incubated in the wells for 1 h at 37 °C, unbound LDL was removed, the wells were washed with MES, PIPES, or HEPES buffered 50 mM saline, and the amounts of bound LDL were determined using the Amplex Red cholesterol kit (Molecular Probes, Eugene, Oregon). The amount of bound LDL is expressed as μg of LDL protein/well, which was calculated from the amounts of cholesterol (2.7 nmol of cholesterol/μg of LDL protein).

To determine the difference in the binding of unaggregated and slightly aggregated SMase-modified LDL particles to human aortic proteoglycans, LDL was first treated with SMase (50 milliunits/ml) at 15 °C for 1 h. Lipolysis was stopped with 10 mM EDTA, and half of the sample was incubated for a further 2 h at 37 °C, while the other half was kept at 15 °C. The binding of LDL to proteoglycans was determined by incubating the modified LDL particles in proteoglycan- or BSA-coated microtiter wells overnight at 4 °C.

RESULTS

The effect of pH on the binding of LDL to human aortic proteoglycans was examined in microtiter well assays at pH ranges 5.5–7.5. For this purpose, the wells were coated with the proteoglycans with BSA-coated wells serving as controls. LDL was incubated in the proteoglycan- or BSA-coated wells for 1 h. Lipolysis was stopped with 10 mM EDTA, and half of the sample was incubated for a further 2 h at 37 °C, while the other half was kept at 15 °C. The binding of LDL to proteoglycans was determined by incubating the modified LDL particles in proteoglycan- or BSA-coated microtiter wells overnight at 4 °C.

Next, LDL was modified proteolytically with α-CT or lipolytically with SMase or PLα2. α-CT degrades the apoB-100 of LDL particles, whereas SMase cleaves the sphingomyelin and PLα2 the phosphatidylcholine molecules of the particle surface. Polyacrylamide gel electro-
phoresis revealed that apoB-100 of the α-CT-treated LDL was completely degraded. Thin layer chromatographic analysis revealed that SMase treatment led to complete hydrolysis of the sphingomyelin molecules, and that PLA₂ treatment led to hydrolysis of over 90% of LDL phosphatidylcholine molecules (not shown). The extensively hydrolyzed LDL particles were next applied to proteoglycan- or BSA-coated microtiter wells, and the binding of the modified particles to them was determined at pH 5.5–7.5. As shown in Fig. 1, the same trend as with native LDL was also seen with modified LDL, the lower the pH, the higher the amount of binding. Moreover, when compared with the binding of native LDL, proteolytic and lipolytic modifications increased the amount of proteoglycan-bound LDL by ~1.5- and 3-fold, respectively (Fig. 1).

Aggregation and fusion of proteolyzed and lipolyzed LDL particles has been shown to enhance the binding of LDL to proteoglycans. In this study, short incubation times were used to minimize aggregation and fusion of the modified particles. Indeed, proteolyzed or PLA₂-treated LDL showed no evidence of aggregation/fusion when analyzed by gel filtration chromatography on a Superose 6 column. However, SMase-treated LDL was already slightly aggregated after the 1-h incubation used in this study. To determine whether the increase in the binding of SMase-treated LDL depended on aggregation of the particles, we next performed an experiment where particle aggregation was inhibited by performing the incubations at 15 °C. At this temperature, aggregation of SMase-modified LDL does not occur (30). After incubation for 1 h, lipolysis was stopped with EDTA, and half of the sample was further incubated for 2 h at 37 °C and the other half at 15 °C. After the incubations, the modified LDL particles were applied to proteoglycan- or BSA-coated microtiter wells and incubated at 4 °C at pH 5.5. The incubation temperature (either 37 or 15 °C) did not affect the binding of native LDL to human aortic proteoglycans (Fig. 2). In contrast, the amount of SMase-treated, proteoglycan-bound LDL depended on the incubation temperature. An increase in the amount of proteoglycan-bound LDL was observed only if SMase-treated LDL was incubated at a temperature that allowed particle aggregation/fusion (37 °C).

It is known that, at neutral pH, oxidation strongly decreases the binding of LDL to human aortic proteoglycans (28). To study the effect of pH on the binding of oxidized LDL particles, LDL was first treated with copper sulfate for various periods of time and the degree of oxidation determined by measuring the amount of thiobarbituric acid-reactive substances. As shown in Fig. 3A, the degree of oxidation increased progressively. The degree of lysine modification was also determined and it was found that after oxidation for 18 h, ~0.5 of the lysine residues had become modified (not shown). Binding of oxidized LDL particles to proteoglycans was then measured using the microtiter well assay. Oxidation progressively decreased the binding of LDL to proteoglycans at pH 5.5 (Fig. 3B). However, at pH 5.5, even the extensively oxidized LDL (oxidation for 18 h) was able to bind to proteoglycans. In fact, at pH 5.5, the amount of extensively oxidized LDL bound to proteoglycans was 3-fold higher than the amount of native LDL at pH 7.5.

The binding of LDL to proteoglycans is mediated by ionic interactions between the positively charged amino acids lysine and arginine of the apoB-100 of LDL particles and the negatively charged glycosaminoglycans of proteoglycans (31). To examine whether the binding of LDL to proteoglycans is mediated by ionic interactions also at low pH, we next performed a binding assay in the presence of 1 M NaCl. The effect of high salt concentration is seen in Fig. 4A. 1 M NaCl completely blocked the binding of LDL to proteoglycans, showing that the binding occurs via ionic interactions also at low pH.

Next, the role of lysine and arginine residues in the binding of LDL to proteoglycans was determined at low pH. Acetylation of LDL neutralizes the positive charge of lysine residues (26) and cyclohexanediol treatment neutralizes the positive charge of arginine residues (20), and each modification impairs the binding of LDL to proteoglycans at neutral pH. In this experiment LDL was first acetylated or treated with cyclohexanediol after which LDL was further treated with α-CT, SMase, or PLA₂. Acetylation or cyclohexanediol treatment did not have significant effect on the abilities of α-CT, SMase, or PLA₂ to hydrolyze the lipoproteins. As shown in Fig. 4B, acetylation completely blocked the binding of native and modified LDL at pH 5.5, although...
cyclohexanedione treatment blocked only the binding of unmodified LDL and only slightly decreased the binding of proteolylzed and SMase-modified LDL, whereas it did not affect the binding of PLA₂-modified LDL. One possibility for the difference is that during proteolysis and lipolysis, the conformation of apoB-100 becomes changed, so leading to exposure of unmodified arginine residues. To ensure that the result did not depend on this, we next performed an experiment, where LDL was first proteolyzed or lipolyzed and only then acetylated or treated with cyclohexanedione (Fig. 4C). In accordance with the previous experiment, acetylation completely blocked the binding of native, α-CT-, SMase- and PLA₂-modified LDL to proteoglycans. Cyclohexanedione treatment again fully blocked the binding of native LDL to proteoglycans but only partially blocked the binding of proteolyzed or lipolyzed LDL to proteoglycans. These findings suggest that although the arginine residues in apoB-100 are needed for binding of native LDL to proteoglycans, they are not critical for binding of proteolyzed or lipolyzed LDL to proteoglycans at acidic pH.

**DISCUSSION**

The high concentration of LDL particles in the arterial intima results from their selective retention in the intima (33–35), proteoglycans playing an important role in this process by binding LDL particles that have entered the intimal space (10). At neutral pH, however, native LDL is known to have a relatively low affinity for proteoglycans (4). We have observed that proteolytic and phospholipolytic modifications of LDL enhance the binding of LDL to proteoglycans, especially if the modified LDL particles are aggregated/fused (reviewed by Örni et al. (11)). Here, we show that a decrease in pH also enhances the binding of native LDL to human aortic proteoglycans and that the enhancement is even more pronounced for proteolyzed and PLA₂- and SMase-treated lipoproteins. In this experimental setup, LDL particles were first proteolyzed or lipolyzed at pH 7.4, after which the reactions were stopped by adding enzyme inhibitors. Thus, the degree of proteolytic and lipolytic modification was identical at the start of the binding experiments. Moreover, we aimed at conditions in which aggregation of the modified LDL particles was as minimal as possible. Under these conditions, SMase treatment induced slight aggregation of LDL and, in fact, the enhanced binding of SMase-treated LDL to proteoglycans was found to depend on particle aggregation. In contrast, proteolysis and PLA₂ treatment were found to enhance the binding of LDL to proteoglycans without particle aggregation.

Atherosclerotic plaques show heterogeneity in temperature (15, 36) and pH (16). Indeed, the hydrogen ion concentration in lipid-rich, vulnerable areas is over 10-fold higher than that in calcified areas of plaques (16). The decreased pH is likely the result of the metabolic activity of macrophages in the lesions, because macrophages can acidify their surroundings by extruding hydrogen ions (18). This notion is supported by the finding of De Vries et al. (37), who have shown that in the presence of oxidized LDL macrophages can acidify their environment to pH 5.5. In addition, under hypoxic conditions metabolically active macrophages secrete high amounts of lactate (38). Indeed, advanced atherosclerotic lesions show signs of hypoxia (19) with neovascularization of the plaques being a clear sign of local oxygen deprivation (39). Atherosclerotic arterial intima contains several proteolytic and lipolytic enzymes potentially capable of degrading LDL particles in the
extracellular milieu of the tissue either at neutral or at acidic pH. Thus, chymase, matrix metalloproteases, and cysteine proteases, all found in atherosclerotic lesions, can degrade apoB-100 in LDL particles in vitro (reviewed by Öörni et al. (11)). Of these proteases, cathepsin F is especially potent in degrading apoB-100 in LDL particles and has been shown to enhance the binding of LDL to proteoglycans in vitro (40). Of the extracellularly found intimal phospholipases both types La PL2 and V PL2 are able to hydrolyze LDL particles, type V being especially active toward the phosphocholines in LDL particles (41–43). Secretory SMase does not hydrolyze sphingomyelins of LDL particles at neutral pH unless the particles have been premodified e.g. with PLA2 (44). However, at acidic pH SMase readily hydrolyzes LDL. Interestingly, several studies have also indicated that oxidation of LDL is enhanced at acidic pH (reviewed by Leake (18)).

At neutral pH, oxidation has been shown to decrease the binding of LDL to proteoglycans (28), and even to induce release of bound LDL from glycosaminoglycans (45). The decreased binding of oxidized LDL results from the neutralization of the lysine residues of apoB-100 during oxidation. Interestingly, in the present work we found that oxidation decreased the binding of LDL to proteoglycans at acidic pH. Importantly, however, despite this decrease the binding of extensively oxidized LDL at pH 5.5 was still 3-fold higher than binding of native LDL to proteoglycans at neutral pH (see Fig. 3). In this extensively oxidized LDL, approximately half of the lysine residues had become modified during oxidation implying that the remaining half of the lysine residues mediated the binding to proteoglycans. Based on the above findings, we can speculate that provided unbound LDL becomes oxidized in an acidic extracellular compartment of the arterial intima, the oxidized particles may bind to proteoglycans and so contribute to the extracellular accumulation of cholesterol rather than being ingested by macrophages, as would occur if the pH is neutral (45).

In the present study, we showed that native, proteolyzed, and lipolyzed LDL binds to proteoglycans via ionic interactions at acidic pH. Neutralization of the lysine residues of apoB-100 by acetylation blocked the binding of native, proteolyzed, and lipolyzed LDL to proteoglycans, and neutralization of the arginine residues by cyclohexanedione modification blocked the binding of native LDL to proteoglycans, even at low pH. If the arginine residues were neutralized, however, the proteolyzed or lipolyzed LDL particles were able to bind to proteoglycans at pH values below 6.0, revealing that the arginine residues of apoB-100 had a minor role, if any, in the binding of proteolyzed or lipolyzed LDL to proteoglycans in this pH range. One possible explanation for this is that during proteolysis and lipolysis, conformational changes in apoB-100 reveal new binding sites where arginine residues are not critically positioned in terms of apoB-100-proteoglycan interaction. In fact, PLA2 treatment has been shown to alter the conformation of apoB-100 in LDL particles (46, 47). Similarly, treatment of LDL with SMase, especially when SMase-treated particles become aggregated, may lead to changes in the conformation of apoB-100. Finally, extensive proteolysis of apoB-100, which leads to the release of peptide fragments from LDL, is also likely to lead to spatial rearrangements of the remaining peptide fragments. Indeed, at neutral pH, an increase in the affinity of LDL for proteoglycans after treatment with PLA2 has been shown to depend on the exposure of a new binding site in apoB-100 (32). Interestingly, a decrease in pH appeared to have an additional effect on the conformation of apoB-100, because arginine residues were critical for the binding of proteolyzed and lipolyzed LDL to proteoglycans at pH 6.5–7.5, but not at pH 5.5–6.0.

The results of this study show that the affinity of LDL to proteoglycans increases as the pH decreases and that this affinity can be further increased if LDL particles are modified by proteolysis and phospholipolysis. Regarding the pathophysiology of the pH effect on LDL binding to intimal proteoglycans, the following points merit a comment. In advanced lesions, acidic environments develop in the deeper hypoxic areas whereas the pH of the subendothelial space is still close to neutral. Because LDL particles enter the intima by crossing the luminal endothelium and are then convected toward the deeper areas by the moving interstitial fluid, the particles are first exposed to neutral pH and only later to acidic pH. Accordingly, in the subendothelial neutral environment LDL particles are more likely to become modified by the neutral proteases and phospholipases, whereas in the deeper areas they are attacked by enzymes, which function optimally at acidic pH. Based on these findings, we suggest that a decrease in pH is a novel promoter in the processes that lead to enhanced retention of LDL in advanced atherosclerotic lesions.

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