Sphingomyelinase Induces Aggregation and Fusion, but Phospholipase A₂ Only Aggregation, of Low Density Lipoprotein (LDL) Particles

TWO DISTINCT MECHANISMS LEADING TO INCREASED BINDING STRENGTH OF LDL TO HUMAN AORTIC PROTEOGLYCANS

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During atherogenesis, low density lipoprotein (LDL) particles bind to extracellular matrix proteoglycans in the arterial wall, become modified, and appear as aggregated and fused particles. Sphingomyelinase (SMase) and phospholipase A₂ (PLA₂) have been found in the arterial wall, and, moreover, lesions LDL shows signs of hydrolysis of both sphingomyelin and phosphatidylcholine. We have now studied the effects of these two lipolytic modifications on the aggregation and fusion of LDL particles by hydrolyzing the particles with Bacillus cereus SMase or bee venom PLA₂. In addition, the binding strengths of the modified LDL to human aortic proteoglycans (PG) were analyzed on an affinity column. We found that SMase induced aggregation and fusion of LDL, but PLA₂ induced only aggregation of the particles. In addition, the SMase-induced aggregation and fusion of LDL was promoted by pretreatment of LDL with PLA₂. Determination of the binding strengths of the hydrolyzed LDL revealed that mere lipolysis of LDL without aggregation or fusion, either by SMase or PLA₂, did not affect the binding of the particles to PG. Aggregation and fusion of lipolyzed LDL particles, however, increased their strength of binding to PG. Active lysine residues in apolipoprotein B-100 (apoB-100) appear to be involved in the binding of LDL to PG, and, in fact, quantitative ¹³C NMR analysis revealed that, in the fused LDL particles, the number of active lysine residues per apoB-100 moiety was increased. Moreover, aggregation and fusion of LDL increased the number of apoB-100 copies and, consequently, the number of active lysine residues per aggregate or fused particle. Our present findings therefore (i) show that treatment of LDL with SMase and PLA₂ generates modified LDL particles, which then bind to human aortic PG with increased strength, and (ii) suggest that SMase- and PLA₂-induced aggregation and fusion of LDL are potential mechanisms leading to focal retention of extracellular lipid in the arterial wall.

In atherosclerosis, one important factor causing the retention of low density lipoprotein (LDL) particles within the arterial wall is their binding to intimal proteoglycans (1, 2). Indeed, complexes of apolipoprotein B-100 (apoB-100) containing lipoproteins and proteoglycans can be isolated from fatty streaks and fibrous plaques in the human aorta (3). Since a subendothelial proteoglycan-rich layer is present in all arteries, the atherosclerotic areas have been suggested to contain proteoglycans with a unique structure that favors entrapment of LDL (4, 5). Another possible cause of the focal accumulation of LDL-derived cholesterol is modification of the LDL in the atherosclerosis-prone intimal areas, with ensuing strengthened binding to proteoglycans. Of the potential modifications of LDL in the arterial intima, oxidation has been studied the most (6). However, oxidation of LDL inhibits rather than augments the interaction between LDL and proteoglycans (7). Another potential cause of LDL modification influencing the interaction between LDL and proteoglycans is proteolysis of the apoB-100 component of LDL. In fact, treatment of LDL with mast cell chymase (8) or a-chymotrypsin (9) increased the strength of LDL binding to proteoglycans. Treatment of LDL with these proteolytic enzymes induced fusion of the proteolysed LDL particles (10, 11), and the fused particles were found to bind more tightly than native LDL to proteoglycans (9).

A third possible type of modification of LDL in the arterial intima is lipolysis. Lesional LDL particles and extracellular lipid droplets are relatively enriched in sphingomyelin (SM) (12, 13), a finding suggesting hydrolysis of phosphatidylcholine (PC). In addition, aggregated lesional LDL contains ceramide, the lipolytic product of sphingomyelinase (SMase) (14). These findings, together with the fact that both secretory SMase (15) and secretory phospholipase A₂ (PLA₂) (16–18) have been found in the arterial intima, support the view that these lipolytic enzymes play a role in atherogenesis. After hydrolysis by either SMase or PLA₂, LDL is taken up by macrophages in vitro (19, 20). However, no studies have addressed the effects of these enzymes on the direct binding of LDL to proteoglycans.

In this study, we have examined the effects of hydrolysis of SM with Bacillus cereus SMase and hydrolysis of PC with bee venom PLA₂ on the morphology of LDL particles and on the interaction between LDL particles and human aortic proteoglycans. The distinct changes in the lipids of LDL induced by the two enzymes were found to lead to dissimilar aggregation/fusion behavior of the modified LDL particles. Despite these
differences, both modifications were found to increase the binding strength of LDL to human aortic proteoglycans.

**EXPERIMENTAL PROCEDURES**

**Materials**—Essentially fatty acid-free bovine serum albumin (BSA), e-aminocaproic acid, PLA$_2$, (from bee venom), immobilized PLA$_2$, (from bee venom), and sphinogomyelinase (from B. cereus) were from Sigma. t-Butoxycarbonyl-$t$-$\text{H}$methylamino-3-$\text{H}$methionine N-$\text{H}$ydroxysuccinimidyl ester (the $\text{H}$-labeling reagent) was from Amersham Pharmacia Biotech. Phenylmethylsulfonyl fluoride was from Boehringer Mannheim. Chondroitinase ABC and AC and the unsaturated chondro-disaccharide kit for high performance liquid chromatography were from Seikagaku Kogyo. [1$^\text{14}$C]Formaldehyde (99% isotope enrichment) as a 20% solution in water was from Isotec Inc., and [1$^\text{14}$C]formaldehyde was from DuPont. NaCNBH$_3$ from Sigma was purified by recrystallization from dichlo-romethane before use (21). Superose 6 HR 10/30 column and HitTrap NHS activated (1 ml) column were from Amersham Pharmacia Biotech. Bio-Gel A-5m gel filtration media was from Bio-Rad, and the 5-$\mu$m NH$_2$ (0.3 x 25 cm) column was from Spherisorb.

**Preparation and Labeling of LDL**—Human LDL ($\mu$ = 1.019–1.050 g/ml) was isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mM EDTA (22, 23). $\text{H}$-LDL was prepared by the protein component of LDL by the Bolton-Hunter procedure (24) with a $\text{H}$-labeling reagent, as described previously (25). For each experiment, labeled LDL was diluted with unlabeled LDL to give the specific radioactivities indicated in the figure legends. The amount of LDL is expressed in terms of its nitrogen concentration.

**Treatment of LDL with Phospholipase A$_2$ and Sphingomyelinase**—LDL (1 mg/ml) was incubated with 95 ng/ml (50 milliunits/ml) SMase in 5 mM HEPES, 2 mM CaCl$_2$, 5 mM MgCl$_2$, and 140 mM NaCl, pH 7.4, or with 50 ng/ml (60 milliunits/ml) PLA$_2$, or 16$^\mu$m fatty acid-free BSA in 5 mM HEPES, 5 mM CaCl$_2$, 2 mM MgCl$_2$, and 140 mM NaCl, pH 7.4. After incubation at 37 °C for the indicated times, lipolysis was stopped by addition of EDTA to give a final concentration of 20 mM. Then the immobilized PLA$_2$ or LDL, the incubation mixtures were centrifuged at 2000 $\times$ g for 5 min. The normal and active lysines peak at 42.8 and 43.2 ppm, respectively.

**Analysis of Lipolyzed LDL**—The turbidity of modified LDL (250 $\mu$m) was measured at 430 nm, using a cuvette with a 1-cm light path with a Biochrom 4060 spectrophotometer (Amersham Pharmacia Biotech). The degree of lipolysis with SMase was determined as the amount of phosphocholine generated (26), and the degree of lipolysis with PLA$_2$ was determined by measuring the amount of free fatty acids generated (26). The phospholipid subclasses were visualized by dipping the TLC plate into methanol/acetic acid/H$_2$O (50:30:8, v/v). Individual phospholipid classes were detected with an automatic plate scanner (CAMAG TLC Scanner 3). Protein was determined by the method of Lowry et al. (28), with bovine serum albumin as standard.

**Isolation of Aggregated/Fused LDL**—The degree of aggregation and/or fusion of lipolyzed $\text{H}$-LDL was determined by gel filtration chromatography. Aliquots corresponding to 100–400$\mu$m of LDL in 100–400$\mu$l were run through a Superose 6 HR 10/30 column in 5 mM Tris-HCl, 1 mM EDTA, and 150 mM NaCl, pH 7.4. The flow rate was 0.5 ml/min, and 500–$\mu$l fractions were collected. The degree of aggregation/fusion of the $\text{H}$-LDL (eluting in the void volume of the column) was determined by calculating the ratio of the radioactivity in fractions 14–19 (void volume peak) to the total eluted radioactivity (fractions 14–30). The degree of aggregation/fusion is expressed in percent (radioactivity in void volume/total eluted radioactivity $\times$ 100).

**Preparation and Characterization of Aortic Proteoglycans**—Proteoglycans from 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. (29) as described previously (7). The disaccharide composition of the proteoglycans was analyzed after treatment with chondroitinase ABC and AC by high performance liquid chromatography using a 5-$\mu$m NH$_2$ column (30). The proteoglycan preparation used here contained 36% of chondroitin 6-sulfate, 52% of chondroitin 4-sulfate, and 12% of dermatan sulfate. Glycosaminoglycans were determined by the method of Bartold and Page (31), and the amounts of the proteoglycans are expressed in terms of their glycosami-glycan content.

**Affinity Chromatography of Lipolyzed LDL on a Proteoglycan Affinity Column**—Human arterial proteoglycans were coupled to an NHS-activated HiTrap column (1 ml) according to the manufacturer’s instructions. For this purpose, 1.0 mg of proteoglycans in 0.2 M NaHCO$_3$ and 0.5 M NaCl, pH 8.3, were coupled to the column at 25 °C for 2 h. The column was then blocked with 0.5 M ethanolamine, pH 8.3, containing 0.5 M NaCl. Under these conditions, 0.7 mg of the proteoglycans was found to be coupled to the column. The columns were equilibrated with buffer A (50 mM HEPES, 2 mM CaCl$_2$, and 2 mM MgCl$_2$, pH 7.4) before use. LDL was treated with SMase or PLA$_2$, as described above and separated into aggregated/fused and native-sized monomeric lipolyzed particles by gel filtration chromatography on a Superose 6 HR 10/30 gel filtration column. Aliquots (30 $\mu$l) of the incubation mixtures and the peak fractions were analyzed on a HitTrap proteoglycan column by eluting with a linear NaCl gradient (0–250 mM NaCl in 10 min) in buffer A. Chromatography was performed at a flow rate of 1.0 ml/min. Elution was monitored by UV absorbance at 280 nm and by collecting 500–$\mu$l fractions and determining their radioactivity. The chromatographic apparatus was the Smart system (Amersham Pharmacia Biotech).

**Modification of Lipolyzed LDL**—LDL (2 mg) was lipolyzed for 12 h with SMase or PLA$_2$, as described above, and after lipolysis, the lysine residues of apoB-100 were modified by treatment of 1 mg of the lipolyzed LDL samples with acetic anhydride (32).

**Preparation of [1$^\text{3}$C]-Labeled LDL and [1$^\text{14}$C]-Labeled LDL for NMR Spectroscopy**—LDL (20–40 mg, 2 mg/ml) was lipolyzed with PLA$_2$ or SMase as described above. After incubation at 37 °C for 8 h, the samples were applied to a SepPak C$_18$ cartridge (Waters, 4$\times$70 cm) equilibrated in saline (150 mM NaCl, 1 mM EDTA, pH 7.4) to remove the aggregated/fused particles from the monomers. For NMR analysis, the amino groups of lysine residues (lysine-NH$_2$) in the samples and, as a control, in untreated LDL, were reductively methylated with [1$^\text{3}$C]formaldehyde to give lysine-NH$_3$CH$_3$ as described by Lund-Katz et al. (33). First, 0.2 M NaCNBH$_3$ was added to untreated LDL, monomeric PLA$_2$-lipolyzed LDL, aggregated PLA$_2$-lipolyzed LDL, and aggregated/fused SMase-lipolyzed LDL (1 mg/ml in saline) to give 20 mM. Then the immobilized PLA$_2$ (1 mg/ml) was added to the samples, and these were incubated at 4 °C for 18 h. The reactions were stopped by extensive dialysis against saline at 4 °C. The labeled LDL solutions were concentrated in Amicon 50 concentrators with a 50-kDa cut-off membrane. The [1$^\text{3}$C]formaldehyde was doped with a trace of [1$^\text{14}$C]formaldehyde to give a known specific radioactivity. The degree of reductive methylation was calculated by measuring the amount of [1$^\text{14}$C]radioactivity incorporated into the lysine residues of apoB-100.

**NMR Spectroscopy**—Broad- and proton-decoupled [1$^\text{3}$C]$\times$1$^\text{14}$C NMR-free induction decay signals were measured from LDL samples comprising 2–10 mg of protein/ml in a solution containing 150 mM NaCl, 1 mM EDTA, and 0.02% NaN$_3$, pH 7.4, and 10% of D$_2$O for the spectrometer field-lock. The [1$^\text{14}$C]$\times$NMR data were obtained at 37 ± 0.5 °C. In all experiments, the relaxation delay was 5$\mu$s, and pulse length was 8$\mu$s (34). The spectral width of 200 ppm and the free induction decay was recorded for 0.273 s. The total acquisition time varied from 6 to 16 h, depending on the LDL concentration of the sample. Proton decoupling was performed with a GARP-1 sequence (35). Each measured free induction decay was zero-filled twice prior to the Fourier transformation to the frequency domain spectrum. No apodization was used.

The normal and active lysines peak at 42.8 and 43.2 ppm, respectively, and the cholesterol backbone 1$^\text{3}$C$_{13}$ peaks at 42.2 ppm were subjected to line shape fitting analysis to accurately resolve the areas of these partly overlapping peaks. Each peak was modeled with a Lorentzian basis line shape, but for each spectrum the shape was corrected for a Gaussian contribution. In addition, the phase and the baseline level were adjusted during the analysis. The total line shape optimization of the spectrum was performed with Resovach software. The model analysis resulted in a good correspondence with the experimental resonances in every case and the model parameters obtained could be used to derive the areas of the normal and active lysine peaks.

**Electron Microscopy of LDL**—Samples (3 $\mu$l) were dried on carbon-coated grids, after which 3 $\mu$l of 1% potassium phosphotungstate, pH 7.4, was also dried on the grids (37). The negatively stained samples were viewed and photographed in a JEOL 1200EX electron microscope at the Institute for Biotechnology, Department of Electron Microscopy, Helsinki, Finland. The diameters of 200 randomly selected lipoprotein particles were measured from the electron micrographs.
PLA2 in the presence of 2% BSA for 1 h at 37 °C. The degrees of lipolysis were determined with gel filtration chromatography on a Superose 6 HR 10/30 column. SMase induced substantial aggregation/fusion of lipolyzed LDL, whereas PLA2 treated LDL remained monomeric. Recovery of the particles, whether eluting in peak I (void volume) or peak II, was quantified by expressing the amount of radioactivity eluted at the void volume of the column in percent of the total eluted radioactivity. These calculations showed that 45% of the SMase-treated LDL and 44% of the PLA2-treated LDL were aggregated/fused.

To analyze the morphology of the SMase- and PLA2-treated LDL particles, native LDL and aliquots of the peak fractions from the gel filtration chromatography were analyzed with electron microscopy after negative staining of the particles. The size distribution of 200 particles was determined from the electron micrographs (Fig. 2, A—E). Native LDL had a mean diameter of 21.1 nm (±1.4 nm, median 21.4 nm). The SMase-treated particles eluting in peak II resembled native LDL, and the PLA2-treated particles eluting in peak II were slightly smaller than native LDL. Their mean diameters were 20.7 (±2.0 nm, median 21.4 nm) and 19.5 (±2.2 nm, median 20.0 nm), respectively. The mean diameter of the SMase-treated particles eluting in peak I was 24.7 nm (±6.2 nm, median 21.4 nm). About 35% of the particles were larger than any of the particles in native LDL; the largest particles had diameters of 47 nm. In contrast, the PLA2-treated particles eluting in peak I resembled native LDL; their mean diameter was 20.4 nm (±2.4 nm, median 21.4 nm). Since elution of the native-sized LDL particles from the gel filtration column at the void volume reveals particle aggregation, we conclude that PLA2 induced aggregation of LDL, whereas SMase induced both aggregation and fusion of the lipolyzed LDL particles.

We next studied the effect of the degree of LDL lipolysis on the degree of aggregation and fusion. For this purpose, 3H-LDL was incubated with 50 milliunits/ml SMase or with 60 milliunits/ml PLAD and 2% BSA for various times. At the indicated time points, lipolysis was stopped with EDTA, and the degrees of lipolysis (Fig. 3A) and aggregation/fusion (Fig. 3B) were determined. Under the above conditions, lipolysis of LDL with both SMase and PLA2 reached a maximum after incubation for 2 h, when 0.51 nmol of PC or 0.36 nmol of SM/µg LDL was hydrolyzed. The degree of aggregation/fusion was determined with gel filtration chromatography on a Superose 6 HR 10/30 column. SMase induced substantial aggregation/fusion of LDL after 1 h, when the degree of lipolysis was 85% of the maximum. In a parallel experiment, the degree of SM hydrolysis was found to be different in the two particle populations; 97% of SM in the aggregated/fused particles and 74% of SM in the monomeric particles were degraded. With prolonged incubation, the SMase-induced aggregation/fusion continued, and after incubation for 8 h, 90% of the radioactivity eluted in the void volume of the gel filtration column. After incubation for 24 h, the recovery of SMase-treated LDL from the gel filtration column was less than 50%, and the degree of aggregation/fusion could not be determined accurately. About 50% of the particles in this sample formed large aggregates that could be sedimented by centrifugation at 14,000 g, and electron microscopic examination of the sample revealed that about 50% of the particles were enlarged (not shown). PLA2-induced aggregation of LDL particles reached a maximum of about 40% after incubation for as little as 2 h, the degree of lipolysis then being maximal. Even after incubation for 24 h, more than half of the PLA2-treated LDL remained monomeric. Recovery of the sample from the gel filtration column was over 85%, and less than 10% of the radioactivity could be sedimented by centrifugation at 14,000 g, indicating formation of small aggregates. The particles, whether eluting in peak I (void volume) or peak II, and whether the incubation time was 1 h or 24 h, were slightly smaller than native LDL (not shown). Phospholipid analysis of the peak fractions showed that, after incubation for 24 h, all of the PC in both the aggregated and monomeric PLA2-treated particles was hydrolyzed. When the above experiments were repeated with LDL from various donors, it was found that the degree of PLA2-induced aggregation varied between 16 and

![Fig. 1. Aggregation/fusion of SMase- or PLA2-treated LDL.](http://www.jbc.org/)

To study the effect of SM and PC hydrolysis of LDL particles, 1 mg/ml 3H-LDL was first treated with 95 ng/ml (50 milliunits/ml) SMase for 1 h at 37 °C or with 50 ng/ml (60 milliunits/ml) PLA2 in the presence of 2% BSA for 1 h at 37 °C. The degrees of lipolysis produced by SMase and PLA2 were determined from the respective amounts of phosphocholine and free fatty acid molecules formed. After incubation for 1 h, 0.28 nmol of SM/µg LDL and 0.45 nmol of PC/µg LDL were hydrolyzed. The aggregation and fusion of the LDL particles were determined from the turbidity of the lipolyzed samples, as measured by their absorbance at 430 nm. The absorbance of the SMase-treated LDL was markedly increased, whereas that of PLA2-treated LDL was not. The absorbances of native LDL, SMase-treated LDL, and PLA2-treated LDL were 0.325, 0.685, and 0.363, respectively. The increased turbidity of the SMase-treated LDL reflects aggregation/fusion of LDL and is in accord with previously reported results (19).

The degree of aggregation/fusion of the lipolyzed LDL samples was determined on a Superose 6 HR 10/30 gel filtration column, whose exclusion limit is 4 × 10^6. Native LDL eluted from the gel filtration column in a single peak at 11 ml (Fig. 1A). Surprisingly, both SMase- and PLA2-treated LDL eluted from the column in two peaks (Figs. 1, B and C, respectively); peak I eluted at the void volume of the column and peak II at a position corresponding to the elution volume of native LDL. The degree of aggregation/fusion of the two lipolyzed samples was quantified by expressing the amount of radioactivity eluting at the void volume of the column (peak I) in percent of the total eluted radioactivity. These calculations showed that 45% of the SMase-treated LDL and 44% of the PLA2-treated LDL were aggregated/fused.

RESULTS

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SMase and PL(A2) Strengthen LDL Binding to Proteoglycans

FIG. 2. Electron microscopic analysis of SMase- and PL(A2)-treated LDL particles. LDL (1 mg/ml) was incubated at 37°C for 1 h with 50 milliunits/ml SMase or with 60 milliunits/ml PL(A2) and 2% BSA and separated into aggregated/fused (peak I) and monomeric (peak II) particles. The LDL particles in these different peak fractions were examined by electron microscopy after negative staining (insets in each panel), and the size distributions of the negatively stained particles were determined as described under "Experimental Procedures." A, native LDL; B, SMase-treated LDL in peak I; C, SMase-treated LDL in peak II; D, PL(A2)-treated LDL in peak I; E, PL(A2)-treated LDL in peak II. The bar under the inset in A represents 100 nm.

FIG. 3. Degrees of lipolysis and aggregation/fusion of LDL induced by incubation with SMase or PL(A2). 3H-LDL (300 μg; 100 dpm/μg protein) was incubated with 50 milliunits/ml SMase or 60 milliunits/ml PL(A2) and 2% BSA at 37°C. At the indicated time points, lipolysis was stopped with EDTA. The degrees of lipolysis were determined as the amounts of phosphocholines or free fatty acids, respectively (4). The degree of aggregation/fusion (% of total) was determined as the amount of LDL radioactivity eluting in the void volume of a Superose 6 HR 10/30 gel filtration column, as described under "Experimental Procedures."

44%. SMase-induced aggregation, on the other hand, always led to aggregation/fusion of virtually all of the lipolyzed LDL particles. Taken together, SMase induced substantial aggregation/fusion of LDL only after most of the SM molecules on the particles were hydrolyzed, but once this had taken place, all of the SMase-treated particles became aggregated and/or fused. In contrast, PL(A2)-induced aggregation of LDL began without any delay, but, even after prolonged incubation times, only a fraction of the PL(A2)-treated particles became aggregated without ensuing particle fusion.

Recently, human secretory SMase (s-SMase), secreted by arterial cells, was shown to be unable to act on native LDL at neutral pH (15). In contrast, LDL pretreated with human secretory PL(A2) was hydrolyzed by the enzyme even at neutral pH. In preliminary experiments, we found that when LDL is treated with PL(A2) in the absence of albumin, the lipolytic products of PL(A2) activity accumulate in the LDL particles; the negatively charged free fatty acids increase the net negative charge of the PL(A2)-treated particles, and they do not aggregate (not shown). However, intimal fluid contains about 2% albumin (38), and if LDL (1 mg/ml) is treated with PL(A2) in the presence of 2% BSA, the net charge of the particles is not increased, indicating that the negatively charged free fatty acids have been released from the particles (not shown). Therefore, to study the effect of PL(A2) pretreatment on SMase-induced aggregation/fusion of LDL, we treated LDL with immobilized PL(A2) in the presence and absence of 2% BSA for various times. At the indicated time points, the PL(A2) beads were sedimented and removed, and 50 milliunits/ml SMase was added to the supernatants. After incubation for 30 min with SMase, EDTA was added to stop the lipolysis, and incubation was continued for an additional 4 h at 37°C. The turbidity of the samples at 430 nm and the degrees of PC and SM hydrolysis were then determined. In all the samples, the degree of SM hydrolysis was similar (130 nmol ± 22 nmol/μg LDL), indicating that pretreatment of LDL with PL(A2) did not affect the activity of SMase on LDL. Treatment of LDL with PL(A2) in either the presence or absence of BSA did not change the turbidity of the samples, even though treatment for 30 min in the presence of albumin induced aggregation of about 20% of the LDL particles. In the absence of albumin, PL(A2) pretreatment decreased the degree of SMase-induced aggregation/fusion of LDL particles (Fig. 4, white columns). In contrast, in the presence of albumin PL(A2) pretreatment promoted SMase-induced aggregation/fusion of LDL; the higher the degree of PC hydrolysis, the higher was the turbidity of the SMase-treated LDL particles (Fig. 4, black columns). Thus, in physiological albumin concentration, pretreatment of LDL with PL(A2) appeared to facilitate SMase-induced aggregation/fusion of LDL.

To examine the binding of the lipolyzed particles to proteoglycans, we isolated proteoglycans from human aortas and coupled them to NHS-activated HiTrap columns to prepare affinity columns as described under the "Experimental Procedures." LDL was treated with PL(A2) or SMase for 1 h, and lipolysis was stopped with EDTA. The lipolyzed samples were separated into aggregated/fused and monomeric particles by gel filtration chromatography. Aliquots (30 μl) of the lipolyzed LDL samples, aggregated/fused and monomeric particles, or, as a control, native LDL, were then applied to the proteoglycan column and eluted with a linear NaCl gradient (0–250 mM in 10 min). Elution was monitored with UV absorbance at 280 nm and by collecting 500-μl fractions and determining their radioactivities. Of the applied native LDL (60 μg), 95% bound to the column and eluted as a single peak at 60 mM NaCl (Fig. 5A).
The SMase-treated sample, on the other hand, eluted from the proteoglycan column in two peaks, the first peak at 60 mM NaCl and the second peak at 100 mM NaCl (Fig. 5B). The PLA₂- treated sample eluted from the column in two overlapping peaks at 55 and 75 mM NaCl (Fig. 5C). The arrows in Fig. 5 show the elution volumes of the aggregated/fused and monomeric particles; both PLA₁ and SMase-treated aggregated/fused particles eluted at higher salt concentration than either native LDL or the lipolyzed LDL monomers. Thus, lipolysis alone did not change the strength of binding of LDL to proteoglycans, but the aggregated/fused particles bound more tightly than native LDL to proteoglycans. Furthermore, the strength of binding of aggregated and fused SMase-treated particles was still higher than that of aggregated PLA₁-treated particles.

Binding of LDL to proteoglycans is governed by ionic interactions between the positively charged lysine and arginine residues of apoB-100 and the negatively charged sulfate and carboxyl groups of the proteoglycans (39–42). Binding of native LDL to proteoglycans can be blocked by removing the positive charge of the lysine or arginine residues in the apoB-100 (43). Similarly, when SMase- or PLA₂-treated LDL particles were treated with acetic anhydride to remove the positive charge of the lysine residues, the lipolyzed LDL particles did not bind to the proteoglycan column (not shown). The lysine residues on apoB-100 are of two forms having different pKₐ values, and it is the active lysines having a pKₐ of 8.9 that are thought to be involved in the binding of LDL to proteoglycans (7, 9, 33). The two lysine populations can be quantified with ¹³C NMR spectroscopy, after attachment of two ¹³C methyl groups to the lysine residues. We used this NMR method to gain information about the effects of SM and PC hydrolysis on the two lysine populations. For this purpose, 20 or 40 mg of LDL (2 mg/ml) was incubated with SMase or PLA₁, respectively, for 8 h at 37 °C. After incubation, lipolysis was stopped by addition of EDTA, and the samples were concentrated with Amicon 100 microconcentrators and applied to a Bio-Gel A-5m (1 × 50) gel filtration column. Most of the SMase-lipolyzed LDL eluted at the void volume of the column, and PLA₂-lipolyzed LDL eluted as two peaks as follows: the aggregated particles (peak I at the void volume of the column) and the monomeric particles (peak II). The three lipolyzed samples, namely SMase-treated aggregated/fused, PLA₁-treated aggregated, and PLA₂-treated monomeric LDL particles, were labeled with [¹³C]formaldehyde to add [¹³C]methyl groups to the lysine residues of apoB-100. As a control, native LDL was also labeled with [¹³C]formaldehyde. Determination of the size distribution of the samples with electron microscopy revealed that the diameters of native LDL and both aggregated and monomeric PLA₂-lipolyzed LDL were similar (Table I). Approximately 50% of the SMase-treated particles were enlarged. The largest particles had diameters of 45 nm.

The NMR spectrum of the [¹³C]-labeled LDL showed lipid resonances and, at 43.2 and 42.8 ppm, [¹³C]dimethyl lysine resonances (Fig. 6), in accord with the results of Lund-Katz et al. (33), who showed that these resonances were those of active and normal lysine residues, respectively. The NMR spectra of [¹³C]-labeled SMase-lipolyzed LDL, aggregated PLA₁-lipolyzed LDL, and monomeric PLA₂-lipolyzed LDL showed similar resonances. Fig. 6, A–D, shows enlargements of the [¹³C]dimethyl lysine resonances. The areas of these two resonances give the percentages of active and normal lysine residues in the samples; these are expressed in relation to the amount of protein (Table I). When calculated per apoB-100 moiety, the number of active lysine residues in native LDL was 11. The numbers of active lysine residues in the monomeric and the aggregated PLA₂-lipolyzed LDL were 11 and 10, respectively. The number of active lysine residues per apoB-100 in the SMase-treated aggregated/fused particles was 40. Each aggregate contains several LDL particles and consequently several copies of apoB-100. Even when some of the lipolyzed LDL particles are buried within the aggregate and so cannot interact with proteoglycans, each aggregate contains several apoB-100 moieties that can take part in the binding of the aggregate to proteoglycans. Accordingly, the number of active lysine residues available for the binding is higher per aggregate than per monomeric particle. However, since we lack a method for determining the number of LDL particles per aggregate, the number of active lysines per aggregate cannot be estimated. In contrast, the effect of particle fusion can be estimated. During fusion, apoB-100 moieties of several LDL particles accumulate on the surface of the fusing particle. The average diameters of native LDL and SMase-lipolyzed LDL were 20.4 and 25.7 nm, respectively (Table I). Of the SMase-treated particles, 50% were larger than any of the native LDL particles, and their mean diameter was 30.5 nm. If the diameter of a spherical particle increases from 20.4 to 30.5 nm, its volume increases 3.3-fold. Thus, one fused particle with a diameter of 30.5 nm results from coalescence of an average of 3.3 native-sized particles and contains 3.3 apoB-100 molecules per fused particle. Accordingly, the number of active lysine residues in such a fused particle must also be 3.3 times as high, i.e. 3.3 × 40 = 132. Thus, the above calculations revealed that, in the SMase-treated fused particles, the number of active lysine residues is increased by approximately 12-fold in comparison to native LDL.

**DISCUSSION**

Interactions between native LDL particles do not lead to aggregation or fusion of the particles. If the structure of LDL is modified, however, particle interactions may favor aggregation, which may then lead to particle fusion. The lipolytic modifications studied in this work induced substantial changes in the composition and surface structure of LDL particles; SMase cleaved SM into phosphocholine and ceramide molecules, and PLA₂ hydrolyzed the fatty acids in the sn-2 position of PC,
generating free fatty acid and lyso-PC molecules. Both of these lipolytic modifications led to aggregation and/or fusion of LDL, but the aggregation and fusion behavior of the lipolyzed particles was dissimilar, i.e. treatment of LDL with PLA2 induced particle aggregation only, whereas lipolysis with SMase induced aggregation and subsequent fusion of the lipolyzed particles.

Aggregation of SMase-treated LDL particles has been shown to depend on the accumulation of ceramide within the particles and does not require the apoB-100 component (14). In PC bilayers, ceramide is known to induce lateral phase separation into regions of liquid crystalline and gel phases (44). Importantly, ceramide is largely partitioned in the resultant gel phase, and SM molecules do not cause such microdomain formation (44). Since the SM molecules on an LDL cover only approximately 15% of the surface volume of the particle, it is likely that, for the formation of ceramide-enriched microdomains, a substantial proportion of the SM molecules must be hydrolyzed. These microdomains, however, once formed, could act as nonpolar spots at the surface of the particles and lead to particle aggregation through hydrophobic associations between the domains. When the LDL particles become aggregated, the surface lipid environments of the attached particles will become connected, and diffusion of the lipid molecules between the particles can commence. Energetic stabilization can then drive subsequent fusion of the attached particles. This kind of structural reorganization at the surface of SMase-treated LDL particles can thus explain the finding that substantial aggregation/fusion of LDL occurs only after a substantial proportion of the SM molecules of the particles has been hydrolyzed.

Treatment of LDL with SMase at low temperature (15 °C) does not lead to aggregation of the particles even though SM is hydrolyzed. However, if these SMase-treated particles are subsequently incubated at 37 °C, they aggregate and fuse. This is understood by the interactions between the core and surface lipids of the LDL particles. It is known that a few percent of the core lipids penetrate to the surface in native LDL (45). At 15 °C the core lipids of LDL are in a radially ordered liquid crystalline phase and at 37 °C in a liquid-like state. Therefore, at the lower temperature, the oriented rigid core lipids interacting with the surface monolayer of the particles can substantially

**FIG. 5.** Strength of binding of lipolyzed LDL to human aortic proteoglycans. ^3H-LDL (400 μg; 250 dpm/μg protein) was incubated with 30 milliunits of SMase or 36 milliunits of PLA2 and 2% BSA for 1 h at 37 °C. Lipolysis was stopped with EDTA, and a fraction of the lipolyzed samples was separated into aggregated/fused and monomeric particles by gel filtration chromatography on a Superose 6 HR 10/30 column (see Fig. 1). Aliquots (30-μl) of native LDL, lipolyzed samples, or peak fractions of the gel filtration chromatography were applied to an NHS-activated HiTrap (1 ml) column to which human aortic proteoglycans had been coupled. The strengths of binding of the samples to the proteoglycans were examined by eluting the LDL at 1 ml/min using a linear NaCl gradient (0–250 mM) in buffer B. Elution was monitored by UV absorbance at 280 nm and by collecting fractions and determining their radioactivity (inset in each panel). The gradient was controlled by measuring the conductivity of the eluent. The elution profiles of native LDL, SMase-treated LDL, and PLA2-treated LDL are shown in A–C, respectively. Arrows in B and C show the elution volumes of aggregated and monomeric lipolyzed LDL particles. The dashed line in each panel shows the NaCl gradient.
TABLE I
The number of active and normal lysines in PLA2- and SMase-treated LDL particles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Particle diameter (nm)</th>
<th>Normal lysine residues</th>
<th>Active lysine residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per apoB-100 moiety</td>
<td>Per particle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Per particle</td>
<td>Per particle</td>
</tr>
<tr>
<td>LDL</td>
<td>20.4 ± 2.0</td>
<td>165 (93.9%)</td>
<td>165</td>
</tr>
<tr>
<td>PLA2-treated monomeric LDL</td>
<td>20.6 ± 1.6</td>
<td>214 (95.5%)</td>
<td>214</td>
</tr>
<tr>
<td>PLA2-treated aggregated LDL</td>
<td>20.8 ± 3.0</td>
<td>293 (96.4%)</td>
<td>293</td>
</tr>
<tr>
<td>SMase-treated aggregated and fused LDL</td>
<td>25.7 ± 4.8</td>
<td>148 (78.6%)</td>
<td>148</td>
</tr>
<tr>
<td>(a) SMase-treated native-sized LDL</td>
<td>21.0 ± 2.0</td>
<td>148</td>
<td>148</td>
</tr>
<tr>
<td>(b) SMase-treated fused LDL</td>
<td>30.5 ± 4.4</td>
<td>503</td>
<td>40</td>
</tr>
</tbody>
</table>

FIG. 6. Proton-decoupled 13C NMR spectra of LDL (A), PLA2-treated monomeric LDL (B), PLA2-treated aggregated LDL (C), and SMase-treated aggregated/fused LDL (D) in which the lysine residues were converted to dimethyl lysines by reductive methylation. LDL was incubated at 37 °C for 8 h with PLA2 or SMase. After incubation, lipolysis was stopped with EDTA, and the samples were applied to a Bio-Gel A5-m gel filtration column. PLA2-treated sample eluted in two peaks (peak I at the void volume of the column) and SMase-treated sample in one peak at the void volume of the column. The peak fractions were pooled and labeled with [13C]formaldehyde to add [13C]methyl groups to the lysine residues of apo-B-100. As a control, native LDL was also labeled with [13C]formaldehyde. The [13C] NMR data (at 150.8 MHz) were recorded at 37 ± 0.5 °C, as described in detail under “Experimental Procedures.” The resonances in the [13C] NMR spectrum of LDL are shown at the bottom. The enlargements illustrate the dimethyl lysine peaks for LDL (A), PLA2-treated monomeric LDL (B), PLA2-treated aggregated LDL (C), and SMase-treated aggregated LDL (D). In LDL 49.1%, i.e. 175 of the total of 357 lysine residues in apo-B-100, were methylated and detectable in the NMR spectra. The corresponding values in PLA2-treated LDL monomers were 62.7% (224), in PLA2-treated aggregated LDL 85.0% (303), and in SMase-treated aggregated/fused LDL 52.8% (188).

Both PLA2- and SMase-treated aggregated/fused particles showed increased strength of binding to proteoglycans. However, mere lipolysis of LDL, with either SMase or PLA2, did not affect the strength of particle binding to proteoglycans. LDL is known to bind to proteoglycans via ionic interactions between the positively charged lysine- and arginine-containing segments of apoB-100 and the negatively charged sulfate and inhibit lateral diffusion of the surface phospholipids (46). Consequently, formation of the ceramide-enriched microdomains should also be at least partly inhibited. Thus, the observed behavior of SMase-treated LDL below and above the transition temperature of the core lipids gives further support to the idea that the initial SMase-induced aggregation takes place via the formation of hydrophobic ceramide-enriched microdomains at the surface of the modified LDL particles.

Hydrolysis of PC by PLAs did not alone trigger aggregation of LDL particles. For particle aggregation to occur, the majority of the fatty acids formed, and some of the lysophospholipid molecules had to be released from the particles. The negatively charged fatty acids are likely to prevent the particles from aggregating by causing repulsions between them. An observation consistent with this idea is that acetylated, negatively charged LDL is resistant to aggregation and fusion when treated with sphingomyelinase (19). In the presence of 2% BSA, which is the concentration of albumin in physiological extracellular fluids, such as the intimal fluid (38), the PLA2-treated LDL particles aggregated. At this albumin concentration, lipolysis of LDL did not change the net charge of the LDL particles, since the negatively charged fatty acids were released from the particles. Treatment of LDL with PLA2 at a low temperature in the presence of 2% BSA (15 °C) did not inhibit particle aggregation.2 Thus, unlike SMase-induced aggregation of LDL particles, PLA2-induced aggregation seems not to depend on surface microdomain formation dependent on lateral lipid diffusion. Interestingly, PLA2-treated aggregation did not lead to particle fusion. An explanation consistent with these findings would be that the apoB-100 moiety of LDL is involved in the PLA2-induced aggregation of LDL. In fact, treatment of LDL with PLA2 has been reported to induce changes in the conformation of apoB-100 (47). Thus, the apoB-100 moieties of the lipolyzed particles could prohibit contact between the lipids of the aggregated particles, preventing lipid diffusion between the particles and thus also subsequent particle fusion.
carboxyl groups of the proteoglycans (39–42). The likely reasons for the observed increase in the binding strength are thus more ionic interactions or stronger ionic interactions between apoB-100 of lipolyzed LDL particles and the proteoglycans. A specific population of lysine residues, active lysines, has been suggested to be involved in the binding of LDL to proteoglycans (33). Therefore, the number of active lysines may reflect the number of potential proteoglycan-binding sites. Indeed, we previously (7, 9) found that the number of active lysines correlated positively with the strength of binding of LDL to human aortic proteoglycans. Similarly, as shown in this study, in the SMase-treated aggregated/fused particles, the number of active lysine residues was increased. In contrast, in the PLA2-aggregated LDL particles the number of active lysines was not increased even though the strength of binding to proteoglycans was enhanced. This apparent discrepancy can be explained by the fact that each aggregate contains several LDL particles and thereby several copies of apoB-100. Accordingly, the number of active lysine residues available for binding is higher per aggregate than per monomeric particle. Similarly, in the case of SMase, both aggregation and fusion of the lipolyzed particles increase the number of apoB-100 moieties per aggregate and per fused particle. Taken together, the increased number of active lysine residues provides a plausible explanation for the observed increase in the strength of binding of both PLA2-treated aggregated and SMase-treated aggregated/fused particles to proteoglycans.

Secretory SMase has recently been extensively studied by Tabas and co-workers (14, 15, 49). This enzyme has an acidic pH optimum and cannot hydrolyze native LDL at neutral pH (15). However, LDL modified with secretory PLA2 or by acid phospholipid analysis. Therefore, the number of active lysines may reflect the specific population of lysine residues, active lysines, has been suggested to be involved in the binding of LDL to proteoglycans. Similarly, as shown in this study, in the SMase-treated aggregated/fused particles, the number of active lysine residues was increased. In contrast, in the PLA2-aggregated LDL particles the number of active lysines was not increased even though the strength of binding to proteoglycans was enhanced. This apparent discrepancy can be explained by the fact that each aggregate contains several LDL particles and thereby several copies of apoB-100. Accordingly, the number of active lysine residues available for binding is higher per aggregate than per monomeric particle. Similarly, in the case of SMase, both aggregation and fusion of the lipolyzed particles increase the number of apoB-100 moieties per aggregate and per fused particle. Taken together, the increased number of active lysine residues provides a plausible explanation for the observed increase in the strength of binding of both PLA2-treated aggregated and SMase-treated aggregated/fused particles to proteoglycans.

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