Sphingomyelinase Induces Aggregation and Fusion of Small Very Low-Density Lipoprotein and Intermediate-Density Lipoprotein Particles and Increases Their Retention to Human Arterial Proteoglycans

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Objectives—Infiltration of low-density lipoprotein (LDL) into subendothelial space is an early step in atherosclerosis. In addition to LDL particles, small very low–density lipoprotein (sVLDL) and intermediate-density lipoprotein (IDL) particles are also able to enter the arterial intima and be retained within the subendothelial extracellular matrix. Here we compared how proteolysis with α-chymotrypsin and phospholipid hydrolysis with phospholipase A₂ or sphingomyelinase (SMase) of sVLDL, IDL, and LDL particles can influence their aggregation, fusion, and binding to human arterial proteoglycans in vitro.

Methods and Results—In each of the 3 lipoprotein classes, the particles became only slightly aggregated with α-chymotrypsin or phospholipase A₂. However, the particles strongly aggregated when treated with SMase. The aggregated/fused particles were found to bind to proteoglycans in proteoglycan affinity chromatography more tightly than the native-sized counterparts. In addition, in a microtiter well assay, the binding of SMase-treated lipoproteins was enhanced: the amounts of proteoglycan-bound SMase-treated LDL, IDL, and sVLDL were 4-, 5-, and 20-fold higher, respectively, than the amounts of proteoglycan-bound native lipoproteins.

Conclusion—These results imply a specific role for SMase as an sVLDL- and IDL-modifying enzyme and also suggest a novel mechanism of lipid accumulation in atherogenesis, namely enhanced retention of atherogenic triglyceride-rich lipoprotein particles in intimal areas expressing extracellular SMase activity. (Arterioscler Thromb Vasc Biol. 2005; 25:1678-1683.)

Key Words: VLDL ■ IDL ■ proteolysis ■ lipolysis

Cholesterol in atherosclerotic lesions is derived from lipoproteins that have entered the arterial intima from the bloodstream. Most of the cholesterol in the bloodstream is carried in low-density lipoprotein (LDL) particles (diameter 20 to 25 nm) and, therefore, most of the cholesterol in the arterial wall is thought to be derived from LDL particles. However, because lipoproteins with a diameter <75 nm can enter the arterial wall via transcytosis,1 very low–density lipoprotein (VLDL) (diameter 30 to 80 nm) and intermediate-density lipoprotein (IDL); diameter 25 to 35 nm) particles can also potentially contribute to lipid accumulation.2,3 In fact, the larger lipoprotein particles have a slower rate of efflux from the arteries when compared with the LDL-sized particles.4 Indeed, triglyceride-enriched apolipoprotein B (apoB)–containing lipoproteins have been isolated from human atherosclerotic lesions.5 Moreover, in studies in which measurements of lipoprotein subfractions have been carried out, plasma levels of IDL have been more predictive of atherosclerosis progression than plasma levels of LDL, as assessed by coronary artery angiography or carotid artery ultrasonography.6

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The concentration of the lipoproteins within the arterial intima depends on their selective retention in the intima.7–9 Thus, compared with high-density lipoprotein or other plasma proteins, such as albumin, the apoB-100–containing lipoprotein particles (VLDL, IDL, and LDL) are selectively retained in the intima, and their local concentrations are relatively high.10 Moreover, VLDL and IDL particles are suggested to be trapped to a greater extent than LDL particles by the dense, proteoglycan-rich extracellular matrix of the arterial intima.1 Proteoglycans have the potential to bind apoB-100–containing lipoproteins,11–14 and the importance of the initial lipoprotein–proteoglycan interaction has been directly shown by the use of transgenic mice expressing proteoglycan-binding–deficient human apoB-100.15,16 The extracellular arterial intima contains proteolytic and lipolytic enzymes capable of modifying the retained lipopro-
tein particles. Thus, chymase and tryptase of mast cells and matrix metalloproteinases and cathepsins of macrophages and smooth muscle cells are found particularly at intimal sites affected by atherosclerotic changes. Similarly, 2 types of secretory phospholipases, sphingomyelinase (SMase) and phospholipase A₂ (PLA₂), are found in human atherosclerotic lesions.

Treatment of LDL with either proteolytic or phospholipolytic enzymes can lead to aggregation and fusion of the modified LDL particles. These aggregated and fused lipid particles resemble the lipid particles that have been isolated from atherosclerotic arteries. Like the LDL particles, the arterial VLDL and IDL particles may also be attacked by proteolytic and lipolytic enzymes. We have now treated VLDL and IDL particles isolated from human plasma with α-chymotrypsin, and with 2 phospholipolytic enzymes: SMase and PLA₂. The effects of proteolysis and phospholipolysis on the integrity of the lipoprotein particles and on the binding of the particles to human arterial proteoglycans were then examined and compared with those found with similarly treated LDL particles.

Methods
An online Methods section is available at http://atvb.ahajournals.org.

Results
Aggregation of Modified sVLDL, IDL, and LDL Particles
To study the effect of proteolysis and phospholipolysis on the aggregation of lipoprotein particles, sVLDL, IDL, and LDL particles were first incubated with α-chymotrypsin, SMase, or PLA₂, and the turbidity of the lipoprotein particles was measured at various time points. Turbidity measurement is suitable for detection of large aggregates. As shown in Figure 1A, proteolysis by α-chymotrypsin produced only a minor increase in the turbidity of LDL but increased the turbidity of IDL and VLDL from 0.02 to 0.25. The degree of proteolysis was measured by determining the quantities of radioactive peptides that were released from the lipoprotein particles (Figure 1A, inset), and the measurements showed a progressive increase in the degradation of the apolipoproteins. After incubation for 24 hours, ~23% of the radioactivity was released from the particles. SDS-PAGE analysis of the lipoproteins proteolyzed for 24 hours showed that none of the apolipoproteins in sVLDL, IDL, and LDL had remained intact (ie, they been degraded into smaller peptides; data not shown). Modification of the lipoproteins by PLA₂ increased the turbidity of the lipoprotein samples only slightly (Figure 1B), whereas the turbidity of the lipoproteins was significantly increased by treatment with SMase (Figure 1C), indicating that SMase had induced formation of large lipoprotein aggregates. The degrees of lipolysis are shown in the insets. Lipid analyses of the modified lipoproteins showed that treatment of the lipoproteins for 24 hours with PLA₂ or SMase led to hydrolysis of ~90% of the phosphatidyl choline or sphingomyelin, respectively, in the lipoproteins (Figure I, available online at http://atvb.ahajournals.org).

Size Distribution of Modified sVLDL, IDL, and LDL Particles
Next, the modified lipoprotein particles were analyzed by transmission electron microscopy. The size distribution of native lipoproteins and lipoproteins incubated with α-chymotrypsin, SMase, or PLA₂ was determined from negatively stained samples, and the morphology of the lipoproteins was studied by thin-section electron microscopy (Figure 2). In all 3 lipoprotein classes, proteolytic degradation and lipolysis with SMase induced lipoprotein fusion (increase in the size of individual lipoprotein particles), whereas PLA₂ led to formation of slightly smaller lipoprotein particles. The changes in the lipoprotein sizes are also seen in the thin-section electron micrographs of modified lipoprotein particles (Figure 2, insets). In addition, the thin-section electron micrographs show the aggregated lipoproteins in the proteolyzed and particularly in the SMase-treated lipoprotein samples.

Binding of Modified sVLDL, IDL, and LDL Particles to Proteoglycans
Aggregation and fusion of LDL particles increase the binding strength of LDL to proteoglycans. To examine whether the studied proteolytic and lipolytic modifications would have an effect on the binding of sVLDL and IDL particles to proteoglycans, native lipoproteins or lipoproteins incubated for 16 hours with α-chymotrypsin, PLA₂, or SMase were applied to a proteoglycan affinity column and eluted with a linear NaCl gradient (Figure II, available online at http://atvb.ahajournals.org). LDL and IDL eluted from the proteoglycan column with ~70 mmol/L NaCl, whereas sVLDL eluted with a slightly lower salt concentration (50 mmol/L NaCl), indicating a weaker binding strength to proteoglycans. In accord with previously published results, proteolysis and
lipolysis of LDL induced formation of tight-binding LDL particles that eluted from the proteoglycan column at a higher salt concentration than native LDL particles. In contrast, PLA2 treatment decreased the binding strength of IDL and sVLDL to proteoglycans, although a small fraction of the PLA2-treated IDL particles eluted with a higher salt concentration than native IDL. Proteolyzed and SMase-treated IDL and sVLDL particles eluted from the proteoglycan column in 2 (overlapping) peaks, the first peak at a similar or slightly lower salt concentration than the native lipoproteins and the second peak at a higher salt concentration. The peak fractions (Figure Ila and IIb) were then analyzed using rate zonal ultracentrifugation, where increased flotation velocity of the particles in an ultracentrifugal field indicates an increase in particle size, either through aggregation or fusion. It was found that the fraction of the lipoprotein particles that bound to proteoglycans tightly (fractions b; “high affinity”) floated faster than fractions showing lower binding strength to proteoglycans (fractions a; “low affinity” [Figure 3]). Thus, in each case, the proteolyzed and lipolyzed lipoprotein particles having an increased binding strength to proteoglycans were aggregated/fused.

Finally, the binding of modified lipoprotein particles to proteoglycans was analyzed on microtiter wells coated with human aortic proteoglycans (Figure 4). Treatment with α-chymotrypsin enhanced only slightly the binding of the lipoproteins to proteoglycans. In contrast, the PLA2-induced lipolysis increased dramatically the binding of LDL and, to a smaller extent, the binding of IDL, but did not significantly increase the binding of sVLDL. Finally, lipolysis with SMase enhanced significantly the binding of all 3 classes of lipoproteins to the proteoglycans. Thus, SMase, which was the only enzyme with a powerful capacity to induce aggregation and fusion of all 3 classes of lipoproteins, was also able to enhance their binding to proteoglycans.

Discussion
In this study, we compared the effects of proteolysis and phospholipid hydrolysis by PLA2 and SMase on the interaction between 3 classes of apoB-100—containing lipoproteins (sVLDL, IDL, and LDL) and human aortic proteoglycans. The main finding was that lipolysis by SMase induced fusion and extensive aggregation of all 3 classes of lipoproteins, and
that such modification increased the ability of the lipoproteins to bind to the proteoglycans.

Proteolysis and phospholipid hydrolysis of the 3 lipoprotein classes caused variable changes in their physicochemical characteristics (aggregation or fusion). The lipoproteins have a hydrophobic core filled with nonpolar lipids that is surrounded by a monolayer shell composed of phosphatidyl choline, sphingomyelin, unesterified cholesterol, and apolipoproteins. It has been shown previously that changes in the surface layer of LDL particles can lead to aggregation or fusion of the modified particles.30,31 Thus, extensive proteolysis induces fusion of LDL particles,31 whereas their lipolysis with SMase leads to fusion of LDL particles and also to their extensive aggregation.32 Lipolysis with PLA2 induces also LDL aggregation.29 However, these aggregates are smaller than those formed by treatment with SMase. In addition, PLA2 treatment decreases the size of the individual lipoprotein particles. Because the basic structure of the sVLDL and LDL particles is similar to that of LDL particles, it is likely that their enzymatic modifications lead to changes similar to those in LDL particles. This appeared to be true, despite slight differences in the relative magnitudes of the observed changes after the hydrolytic treatments. The degree of fusion was analyzed by electron microscopy and the degree of aggregation by determining sample turbidity. It should be noted that the turbidity of the samples increases only if large aggregates are formed. An increase in the size of the individual particles (eg, particle fusion without aggregation) does not increase the turbidity of the samples significantly. Indeed, the absorbance of native sVLDL particles (44±7.1 nm) is only ≈0.02 absorbance units higher than the absorbance of native LDL particles (23±1.4 nm). Based on our findings, the following general pattern of physical changes among the variously hydrolyzed particles can be envisioned.

The proteolyzed sVLDL and LDL particles fused but became only slightly aggregated, the SMase-treated lipoprotein particles became fused and extensively aggregated, and the PLA2-treated lipoprotein particles became smaller in size and a fraction of the particles aggregated. Moreover, PLA2-treated LDL particles appeared to aggregate more readily than similarly treated LDL and especially sVLDL particles.

To evaluate the capability of the modified lipoproteins to bind to proteoglycans, we used 2 different analytical systems, which differed markedly in their capacities to bind the ligands. Thus, the proteoglycan affinity column contained ≈700 μg of proteoglycans, whereas the microtiter wells contained only ≈1 μg, and yet, the amounts of the lipoproteins applied to the systems were about equal (10 μg and 1 to 25 μg, respectively). Because the first system is nonsaturable, it allows binding of virtually all of the applied particles. In contrast, in the latter system, only a minor fraction (<1%) of the particles present in the incubation medium were able to bind to the proteoglycans. Therefore, it is likely that the particles, which had bound to the proteoglycans, were those with highest affinity for the proteoglycans (ie, the multivalent aggregated/fused particles). This led us to propose an overall model in which the physicochemical changes result in higher binding of lipoproteins to the proteoglycans, which accords with previous in vitro data using LDL and also supports the current notion of atherosclerosis being a lipid storage disease in which various lipoproteins contribute to the accumulation by binding to proteoglycans.

As discussed in the introduction, the arterial intima contains several proteases and phospholipases. Of these en-
zymes, mast cell chymase$^{26,33}$ and cathepsin F$^{20}$ are able to induce LDL fusion. Similarly, these enzymes should be able to attack apоБ-100 in IDL and sVLDL particles. Group II and group V secretory PLAc8 can hydrolyze phospholipids in LDL particles and induce similar changes in LDL particles as bee venom PLAc25,34,35. The activity PLAc8 depends on the surface properties (eg, the fluidity) of the lipid membrane. The surface of VLDL particles is more fluid than that of LDL particles,36 which should make VLDL an even better substrate than LDL for the arterial PLAc8. Native lipoproteins at neutral pH are not good substrates for secretory SMase.$^{37,38}$ However, secretory SMase has been shown to hydrolyze PLAc2-treated lipoproteins at neutral pH.$^{37}$ This was suggested to depend on the increased sphingomyelin/phosphatidyl choline ratio of the PLAc2-treated particles, which activates the secretory SMase. Similarly, secretory SMase could degrade PLAc2-modified IDL and sVLDL particles in the arterial intima. This idea is further supported by the finding that apoC-III, which is present in IDL and sVLDL, activates the secretory SMase.$^{39}$ A third type of lipase, lipoprotein lipase, which is also known to be present in the arterial intima, can degrade triglycerides of the sVLDL and IDL particles.$^{40}$ Interestingly, lipoprotein lipase can bind LDL, and especially modified LDL, and link the particles tightly to proteoglycans. Similarly, the enzyme should be able to bind (modified) IDL and sVLDL particles in the arterial intima and so enhance their retention in the arterial wall.

Clinical studies performed within the past 50 years have implicated elevated concentrations of VLDL and IDL plasma as causative agents for the development of atherosclerosis.$^{6,41,42}$ The present data suggest one mechanism by which these lipoproteins may contribute to lipid accumulation during the development of atherosclerotic lesions. Thus, although the affinity of IDL and especially of sVLDL for proteoglycans is lower than that of native LDL, the present results demonstrate that treatment of the lipoproteins with SMase considerably enhances their binding to the proteoglycans. If this reflects the situation in the arterial intima, the increased capacity of the proteoglycans to bind the modified lipoproteins may lead to their accumulation within the extracellular matrix of the intima. Therefore, we suggest that SMase-induced aggregation and fusion of sVLDL and IDL particles within the arterial wall may have an important role in the retention of these lipoprotein particles and so could contribute to the development of atherosclerotic lesions, especially if the plasma triglyceride levels are elevated.

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Fig. I. Lipid composition of unmodified, SMase- and PLA2-treated sVLDL, IDL, and LDL particles. Lipid composition of the unmodified lipoproteins and lipoproteins incubated for 24 h in the presence of 25 mU/ml of SMase or 15 mU/ml of PLA2 was analyzed with HPTLC. Data shown are means of three individual experiments performed with three different lipoprotein preparations ± SD.
Figure II. Binding of α-chymotrypsin, SMase- and PLA₂-treated sVLDL, IDL, and LDL to human aortic proteoglycans. ³H-labeled lipoproteins (0.5 mg/ml) were incubated with 50 µg/ml of α-chymotrypsin, 25 mU/ml of SMase, or 15 mU/ml of PLA₂ for 20 h. After incubation, the lipoprotein samples were applied to a proteoglycan affinity column and eluted with a linear NaCl gradient. Elution was monitored by collecting fractions and determining their radioactivity. The results are representative of four individual experiments. The gray area in each panel represents the elution profile of native LDL, IDL, or sVLDL.
Methods

Preparation and labeling of small VLDL, IDL, and LDL

Human VLDL ($d < 1.006$ g/ml), IDL ($d = 1.006-1.019$ g/ml), and LDL ($d = 1.019-1.050$ g/ml) were isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mM EDTA. In this study, lipoproteins isolated from eight individual donors were used and each experiment was performed with 2-5 different lipoprotein preparations. Small VLDL (sVLDL) ($S_r 20-175$) was isolated from the VLDL fraction by discontinuous density gradient ultracentrifugation. [$^3$H]Cholesteryl linoleate lipoproteins and $^{35}$S-labeled lipoproteins were prepared as described previously. The amounts of sVLDL, IDL, and LDL are expressed in terms of their protein concentration, which was determined by the method of Lowry et al., with bovine serum albumin as standard.

Treatment of sVLDL, IDL and LDL with $\alpha$-Chymotrypsin, PLA$_2$ and SMase

$^{35}$S/$^3$H-labeled lipoproteins (0.5 mg/ml) were incubated with 50 $\mu$g/ml of $\alpha$-chymotrypsin (from bovine pancreas), 50 ng/ml (25 mU/ml) of SMase (from Bacillus cereus) or with 25 ng/ml (15 mU/ml) of PLA$_2$ (from bee venom) in buffer A (10 mM Tris-HCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 140 mM NaCl, pH 7.4). When PLA$_2$ was used, 5% (w/v) fatty acid-free bovine serum albumin (BSA) was added to the incubation mixtures. After incubation at 37°C for the times indicated, proteolysis was stopped by addition of trypsin inhibitor to give a final concentration of 0.5 mg/ml and lipolysis was stopped by addition of EDTA to give a final concentration of 10 mM.

Analysis of Modified Lipoproteins

The turbidity of the modified lipoproteins (100 $\mu$l) was measured at 430 nm, using a microplate reader. The degree of proteolysis was determined by measuring the amount of trichloroacetic acid–soluble $^{35}$S-radioactivity produced. The degree of lipolysis with SMase was determined as the amount of phosphocholine generated and the degree of lipolysis with PLA$_2$ was determined by measuring the amount of free fatty acids generated using a commercial kit (Nefa-C-kit, Wako Chemicals). Thin layer chromatography of native and modified lipoproteins
was performed as described earlier. The degree of aggregation/fusion was determined by rate zonal ultracentrifugation of 3H-labeled lipoproteins as described previously.

**Electron Microscopy of the Modified Lipoproteins**

For thin-section transmission electron microscopy, the lipoprotein samples were cast in agarose, and fixed, and stained with the osmium-tannic acid-paraphenylenediamine technique as described. For negative staining electron microscopy, samples (3 µl) were dried on carbon-coated grids, after which 3 µl of 1% potassium phosphotungstate, pH 7.4, was added and also dried on the grids. The samples were viewed and photographed in a JEOL 1200EX electron microscope at the Institute for Biotechnology, Department of Electron Microscopy, Helsinki, Finland. For determination of the size distribution of the lipoprotein particles, the diameters of 100 randomly selected particles were measured from the electron micrographs.

**Preparation and Characterization of Aortic Proteoglycans**

Proteoglycans from the intima-media of human aortas were obtained at autopsy within 48 h of accidental death and were prepared essentially by the method of Hurt-Camejo et al., as described previously. The amounts of the proteoglycans are expressed in terms of their glycosaminoglycan content, determined by the method of Bartold and Page. Three different proteoglycan preparations were used during this study and they contained 35-40% chondroitin-4-sulfate, 27-34% dermatan sulfate, 18-25% chondroitin-6-sulfate, and less than 10% heparan sulfate. For affinity chromatography, proteoglycans (1 mg) were coupled to an NHS-activated HiTrap column (1 mL) (Amersham Biosciences) according to the manufacturer’s instructions.

**Affinity Chromatography of Modified Lipoprotein Particles**

Aliquots (10 µg) of the 35S/3H-labeled modified lipoprotein samples were applied to a proteoglycan affinity column, and eluted with a linear NaCl gradient (20–250 mM) in buffer B (10 mM HEPES, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.0) at a flow rate of 0.5 ml/min. Fractions (250 µl) were collected and their radioactivities were determined by liquid scintillation counting.

**Binding of the lipoproteins to proteoglycans in a microtiter well assay**

The wells in polystyrene 96-well plates were coated with 100 µl of human aortic proteoglycans (50 µg/ml) or BSA (5 mg/ml) and blocked as described earlier. The lipoproteins
were modified as described above and aliquots of the incubation mixtures or native lipoproteins were added to proteoglycan-coated wells in buffer A containing 2% BSA and the plate was incubated for 1 h at 37°C. The wells were washed three times with buffer A containing 50 mM NaCl, and the bound lipoproteins were detected by a commercial cholesterol kit (Aplex Red, Molecular Probes). Specific binding to proteoglycans was calculated by subtracting the amounts of the lipoproteins bound to the BSA-coated wells from the amounts of lipoproteins bound to the proteoglycan-coated wells.

References


