Lipolysis of LDL by Human Secretory Phospholipase A2 Induces Particle Fusion and Enhances the Retention of LDL to Human Aortic Proteoglycans

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Abstract—The first morphological sign of atherogenesis is the accumulation of extracellular lipid droplets in the proteoglycan-rich subendothelial layer of the arterial intima. Secretory nonpancreatic phospholipase A2 (snpPLA2), an enzyme capable of lipolyzing LDL particles, is found in the arterial extracellular matrix and in contact with the extracellular lipid droplets. We have recently shown that in the presence of heparin, lipolysis of LDL with bee venom PLA2 induces aggregation and fusion of the particles. Here, we studied the effect of human snpPLA2 on the integrity of LDL particles and on their interaction with human aortic proteoglycans. In addition, the capacity of the proteoglycans to retain PLA2-lipolyzed LDL particles was tested in a microtiter well assay. We found that lipolysis of LDL induced fusion of proteoglycan-bound LDL particles, which increased their binding strength to the proteoglycans. Moreover, lipolysis of LDL with snpPLA2 under physiological salt and albumin concentrations induced a 3-fold increase in the amount of LDL bound to proteoglycans. The results imply a role for PLA2 in the retention and accumulation of LDL to the proteoglycan matrix in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2001;21:1053-1058.)

Key Words: phospholipases ■ LDL ■ fusion ■ retention ■ proteoglycans

I nitiation of atherosclerosis in both humans and experimental animals is characterized by the appearance of extracellular lipid droplets in the proteoglycan (PG)-rich subendothelial layer. Experimental models have shown that similar droplets can be formed directly from LDL particles both in situ and in vivo. Moreover, both chemical analyses and measurements of the size of the extracellular lipid droplets in human arterial lesions suggest that the majority of the droplets originate from LDL particles. Because native LDL particles do not fuse into such lipid droplets, the LDL particles must undergo modification in the arterial intima. Indeed, the lipid droplets isolated from the arterial intima have features suggesting that they may have been derived from plasma LDL by extensive modification (reviewed by Öörni et al). Modification of LDL in vitro by proteolytic enzymes, by oxidative compounds, or by lipolytic enzymes such as sphingomyelinase, phospholipase C, or phospholipase A2 (PLA2) has been shown to induce aggregation, or both aggregation and fusion of the particles.

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Phospholipase A2s are enzymes that catalyze the hydrolysis of the sn-2 fatty acyl ester bond in phospholipids, yielding a free fatty acid and a lysophospholipid. Recently, type II secretory nonpancreatic phospholipase A2 (snpPLA2), which is capable of lipolyzing LDL, has been shown to be located in both atherosclerotic and nonatherosclerotic arterial intima and to be associated with extracellular matrix structures and lipid droplets. Interestingly, lipolysis of LDL by bee venom PLA2 reduces the size of the LDL particles in the absence of glycosaminoglycans (GAGs) but increases their size in the presence of heparin-GAG.

Retention of LDL in the arterial intima has been suggested to be a prerequisite for the development of atherosclerotic lesions. Physical trapping of LDL and its direct binding to the various components of the extracellular matrix, especially to the PGs, increase the retention time of LDL particles in the arterial intima and so increase their susceptibility to modification. We have previously shown that aggregation and fusion of modified LDL particles increase the binding strength of the particles to PGs. We have now studied the effect of snpPLA2 on the aggregation/fusion of LDL particles in the presence of human aortic PGs. We found that lipolysis of LDL by snpPLA2 induced fusion of the PG-bound particles, increased the binding strength of the fused particles to PGs, and, most importantly, enhanced the retention of LDL to human aortic PG matrix.

Methods

Materials

Essentially fatty acid–free BSA, PLA2 (from bee venom), and all the lipids (lysophosphatidylcholine, phosphatidylcholine [PC], sphingo-
myelin, cholesterol, oleic acid, triolein, and cholesteryl linoleate) were from Sigma Chemical Co. t-Butoxy carbonyl-L-<sup>3</sup>H)methionine N-hydroxysuccinimidyl ester (the 'H-labeling reagent), HiTrap Hep- arin Sepharose columns, HiTrap NHS-activated Sepharose HP col- umns (1 mL), and PD-10 columns were from Amersham Pharmacia Biotech. The NEFA C kit was from Waco Chemicals. Microtiter plates (Combiplate 8, enhanced binding) were from Labsystems.

**Preparation and Labeling of LDL**

Human LDL (d=1.019 to 1.050 g/mL) was isolated from plasma of healthy volunteers by sequential ultracentrifugation in the presence of 3 mmol/L EDTA. Apolipoprotein (apo) B-100 of LDL was labeled with 'H-labeling reagent by the Bolton-Hunter procedure as described previously. When unlabeled and 'H-labeled LDL were lipolyzed with bee venom PLA<sub>2</sub>, it was found that the degrees of lipolysis and of aggregation/fusion in the 2 samples were similar, ie, labeling of LDL did not affect LDL lipolysis or the tendency of LDL to aggregate/fuse. For some experiments, radiolabeled LDL was used. The amount of LDL is expressed in terms of its protein concentration, which was determined by the method of Lowry with BSA as standard.

**Isolation and Characterization of Human Aortic PGs and Preparation of PG Affinity Columns**

PGs from the intima and media of human aortas obtained at autopsy were isolated essentially by the method of Hurt-Camejo et al as described previously. The PG preparation used contained 49% PGD<sub>3</sub>, 34% chondroitin 6-sulfate, 34% chondroitin 4-sulfate, and 17% dermatan sulfate, determined by high-performance liquid chromatography. The amounts of the PGs are expressed in terms of their GAG content, determined by the method of Bartold and Page. For lipolysis experiments and affinity chromatography, PGs (1 mg) were coupled to an NHS-activated HiTrap column (PG-Sepharose) according to the manufacturer’s instructions.

**Lipolysis of LDL With snpPLA<sub>2</sub> in the Presence of Human Aortic PGs**

Human snpPLA<sub>2</sub> was isolated as described from snpPLA<sub>2</sub>-transfected Chinese hamster ovary cells. The PG-Sepharose was removed from the columns and resuspended in buffer A (10 mmol/L Tris-HCl, 10 mmol/L CaCl<sub>2</sub>, 2 mmol/L MgCl<sub>2</sub>, pH 7.4). The PG-Sepharose beads were first incubated without (control) or with 7.5 μg snpPLA<sub>2</sub> in microcentrifuge tubes (to allow snpPLA<sub>2</sub> to bind to the PGs). After incubation for 30 minutes at room temperature, 300 μg of [‘H]LDL (1 mg/mL) in buffer A containing 20 mmol/L NaCl, 10 μmol/L BHT, and 2% BSA was added to the reaction tube and gently resuspended. The PG-Sepharose-[‘H]LDL mixture was then incubated for the indicated times at 37°C. PG-Sepharose-[‘H]LDL complexes were sedimented by centrifugation at 10000g, and the supernatant was taken for quantification of free fatty acids with a NEFA C kit. To characterize the PG-Sepharose-bound LDL particles, the Sepharose beads were first washed once with buffer A, and the bound LDL particles were released with buffer A containing 0.5 mol/L NaCl.

**Characterization of Aggregation and Fusion of LDL Particles**

Aggregated/fused LDL particles were separated from monomeric native-size LDL by rate-zonal flotation. Briefly, NaBr was added to 500 μL of modified ['H]LDL to yield 40% NaBr (wt/vol), and a linear NaBr gradient (d=1.006 to 1.10 g/mL) was layered on top of each sample and centrifuged at 33 000 rpm in an SW 4 Ti rotor (Beckman) (194 000g<sub>rot</sub>) for 1 hour at 20°C. The gradient was then fractionated into 500-μL aliquots, the radioactivities of which were determined.

Fusion of LDL particles was confirmed by transmission electron microscopy. For this purpose, LDL preparations were negatively stained as described previously. The stained samples were viewed and photographed in a JEOL 1200EX electron microscope at the Institute for Biotechnology, Electron Microscopy, University of Helsinki, Helsinki, Finland. For determination of the size distribution of the LDL particles, the diameters of 200 randomly selected lipoprotein particles were measured from the electron micrographs.

**Affinity Chromatography**

Aliquots (20 to 30 μg) of the PLA<sub>2</sub>-modified LDL samples were dialyzed against buffer B (10 mmol/L HEPES, 5 mmol/L CaCl<sub>2</sub>, 2 mmol/L MgCl<sub>2</sub>, pH 7.4) containing 20 mmol/L NaCl, applied to PG affinity columns, and eluted with a linear NaCl gradient (20 to 250 mmol/L) in buffer B at a flow rate of 0.5 mL/min. Elution was monitored by UV absorbance at 280 nm. Fractions (200 μL) were collected and their radioactivities determined by liquid scintillation counting. The chromatographic apparatus was SMART (Amersham Pharmacia Biotech).

**Binding of LDL to PGs in a Microtiter Well Assay**

The wells in polystyrene 96-well plates were coated with 100 μL of human aortic PGs (50 μg/mL) or BSA (5 mg/mL) by incubation at 4°C overnight. The wells were blocked for 1 hour at 25°C with 250 μL of 3% BSA/1% fat-free milk powder in buffer A, then washed once with 0.1% BSA/0.02% Tween-20 in buffer A, and reincubated without (control) or with 0.5 μg snpPLA<sub>2</sub> for 1 hour at 25°C. ['H]LDL (5 μg in reaction buffer (3% BSA, 20 μmol/L BHT, 150 mmol/L NaCl in buffer A) was added to each well, and the plate was incubated for 6 hours at 37°C. The supernatants were removed, and the degrees of lipolysis were measured from the supernatants with the NEFA C kit. The wells were then washed 3 times with 250 μL of buffer A containing 50 mmol/L NaCl and 0.02% Tween-20, and the radioactivity bound to the wells was measured. Specific binding to PGs was calculated by subtracting the amount of LDL bound to the BSA-coated wells from the amount of LDL bound to the PG-coated wells.

**Other Assays**

The degrees of LDL oxidation and apoB-100 degradation were determined by measuring the amounts of thioarbituric acid–reactive substances and trichloroacetic acid–soluble apoB-100 radioactivity, respectively. The lipid compositions of the lipid extracts of various LDL preparations were analyzed by high-performance thin-layer chromatography using chloroform/methanol/concentrated acetic acid/H<sub>2</sub>O (50:30:8:3.5, vol/vol/vol/vol) for phospholipids and hexane/diethylether/concentrated acetic acid/H<sub>2</sub>O (130:30:2:0.5, vol/vol/vol/vol) for neutral lipids. Individual lipid classes were visualized by dipping the thin-layer chromatography plate into CuSO<sub>4</sub> (3%/H<sub>2</sub>PO<sub>4</sub> (8%)) and then heating the plate for 20 minutes at 150°C. The spots were scanned with an automatic plate scanner (CAMAG TLC Scanner No. 3).

**Results**

We have previously observed that phospholipolysis of LDL with bee venom PLA<sub>2</sub> induces fusion of LDL particles in the presence, but not in the absence, of heparin GAGs. We have now studied the effect of human secretory nonpancreatic PLA<sub>2</sub> on the aggregation/fusion of LDL in the presence of human aortic PGs. For this purpose, 300 μg of ['H]LDL and 5 μg snpPLA<sub>2</sub> were incubated for 48 hours at 37°C with immobilized PGs (PG-Sepharose) in buffer A containing 2% BSA, 20 mmol/L NaCl, and 10 μmol/L BHT. Control LDL was incubated with immobilized PGs in the absence of snpPLA<sub>2</sub>. After incubation, the degrees of lipolysis were quantified by determining the amounts of free fatty acids in the supernatants, and the PG-bound LDL was detached with buffer A containing 0.5 mol/L NaCl. Under these conditions, 140 mol fatty acids/mol LDL were released from the particles (Figure 1A), corresponding to hydrolysis of ~30% of PC molecules of LDL. To confirm the purity of both the enzyme and the PG preparation, parallel LDL samples were analyzed for markers of oxidation (thioarbituric acid–reactive substances) and proteolysis (trichloroacetic acid–soluble radio-
activity), and the lipid composition of the lipolyzed LDL was
determined. The samples showed no signs of oxidation or
proteolysis, and the lipolyzed LDL showed a decrease only in
the amount of LDL PC (not shown).

Next, the size of the lipolyzed LDL was analyzed by
rate-zonal flotation. The lipolyzed LDL and control LDL
were subjected to ultracentrifugation in a linear NaBr gradi-
ent. As shown in Figure 1B, lipolysis of LDL particles led to
formation of fast-floating LDL particles in addition to the
fraction floating at the rate of control LDL. An increase in
particle size through either aggregation or fusion increases
the flotation velocity of the particles in a centrifugal field.39
Thus, as shown in Figure 1B, the majority of the lipolyzed
LDL particles had been aggregated and/or fused.

To distinguish between aggregation and fusion of the
snpPLA₂-lipolyzed LDL particles, their morphology was
examined by electron microscopy. PG-bound LDL was first
incubated with snpPLA₂ as described above. After incubation,
lipolyzed LDL and control LDL were detached from PG-
Sepharose, and the sizes of the individual particles were
analyzed by negative-staining electron microscopy (insets in
Figure 2A through 2C). The size distribution of the control
LDL (23 ± 3 nm, median 22 nm), which was incubated in the
absence of snpPLA₂, resembled that of native LDL (22 ± 4
nm, median 22 nm). In contrast, the particles treated with
snpPLA₂ were larger (32 ± 6 nm, median 32 nm), the largest
particles having diameters of 62 nm. Thus, almost all of
the lipolyzed LDL particles were enlarged, i.e., they had fused.

Next, the binding strength of the snpPLA₂-treated LDL
particles to human aortic PGs was determined. For this
purpose, PG-Sepharose was preincubated with or without
(control) 5 μg of snpPLA₂ for 30 minutes at room tempera-
ture, then incubated with [³H]LDL for 36 hours at 37°C. The
PG-bound [³H]LDL particles were released with buffer A
containing 0.5 mol/L NaCl, and the binding strengths of the
particles were analyzed in a PG affinity column (Figure 3A).
The control LDL eluted at 80 mmol/L NaCl, whereas the
lipolyzed LDL eluted at 140 mmol/L NaCl, showing that

Figure 1. Degree of lipolysis and rate-zonal flotation of
snpPLA₂-lipolyzed LDL. [³H]-labeled LDL was modified by
snpPLA₂ as described under Methods. After incubation for 48
hours, the degree of phospholipid hydrolysis was determined
from the supernatants (A). The PG-Sepharose–bound LDL parti-
cles were detached with buffer A containing 0.5 mol/L NaCl and
then analyzed by rate-zonal flotation. The gradient was fraction-
ated into 500-mL aliquots, whose radioactivities were measured
(B). The results are representative of 3 independent exper-
iments. FFA indicates free fatty acids.

Figure 2. Size distribution of modified LDL. [³H]LDL was modi-
fied by snpPLA₂ as described under Methods. The bound LDL
particles were detached from the PG-Sepharose and examined
in the electron microscope after negative staining of the sam-
ples (insets in A through C). The diameters of 200 particles were
measured from the electron micrographs (A through C). The results are representative of 2 independent experiments.
lipolysis had increased the strength of binding of LDL to PGs. Analysis of the LDL peak fractions by rate-zonal flotation revealed that the particles with high affinity for the PGs were aggregated and/or fused (Figure 3B). In a parallel experiment, LDL was lipolyzed with snpPLA2 for 36 hours at 37°C, and before application to the PG column, the lipolyzed LDL particles were separated into aggregated/fused and native-size particles by rate-zonal flotation. It was found that the native-size LDL particles and the control LDL eluted from the PG column at a similar NaCl concentration (80 mmol/L). In contrast, the aggregated/fused LDL particles eluted at a much higher concentration of NaCl (140 mmol/L), indicating that their binding strength to PGs was increased. When the phospholipid compositions of the native-size and aggregated/fused LDL particles were analyzed, it was found that the PC/sphingomyelin weight ratio in both native-size particles and the control LDL eluted from the PG column, the lipolyzed LDL particles were separated into aggregated/fused and native-size LDL particles by rate-zonal flotation. It was found that the native-size LDL particles and the control LDL eluted from the PG column at a similar NaCl concentration (80 mmol/L). In contrast, the aggregated/fused LDL particles eluted at a much higher concentration of NaCl (140 mmol/L), indicating that their binding strength to PGs was increased. When the phospholipid compositions of the native-size and aggregated/fused LDL particles were analyzed, it was found that the PC/sphingomyelin weight ratio in both native-size particles and aggregated/fused particles was decreased compared with native LDL (0.7, 0.2, and 2.3, respectively). Thus, hydrolysis of PC alone did not appear to affect the binding strength of LDL to the PGs. Rather, aggregation/fusion of the lipolyzed LDL particles was required for the increase in binding strength to the PGs.

Surprisingly, a small fraction of the LDL particles in the control samples appeared to float faster than native LDL (see Figure 1B) and to have an increased binding strength to PGs (see Figure 3A). No aggregation/fusion or increase in the binding strength was observed in LDL that had not been incubated with PGs (not shown). The control LDL showed no signs of lipolysis, proteolysis, oroxidation. To confirm that the changes in the LDL particles did not depend on our PG preparation, LDL was bound to commercial heparin-Sepharose beads and incubated under similar conditions for various periods of time. In accordance with the results with PGs, after incubation for 36 hours, a small fraction of the heparin-bound particles were aggregated/fused and had increased binding strength to PGs (not shown).

In this study, we have shown that lipolysis of LDL by snpPLA2 in the presence of PGs induces fusion of the PG-bound particles and increases the binding strength of the particles to PGs. This could lead to increased accumulation of LDL-derived lipid on the extracellular PGs of the arterial intima. We tested this hypothesis by studying whether lipolysis of LDL with snpPLA2 would increase the capacity of the PGs to bind LDL. Microtiter wells coated with human aortic PGs or BSA were incubated without (control) or with snpPLA2 for 1 hour at room temperature. LDL was added to each well, and the plate was incubated at 37°C for 6 hours. Thereafter, the amounts of free fatty acids were measured from the supernatants. Under these conditions, lipolysis of LDL with snpPLA2 released 14 mol free fatty acids/mol LDL (Figure 4A) and, importantly, induced a 3-fold increase in the capacity of the PG matrix to bind LDL (Figure 4B). Thus, even the very low degree of lipolysis achieved with snpPLA2 under the above conditions led to increased deposition of LDL on the PG matrix.

Discussion

The present results show that lipolysis of LDL phospholipids with secretory pancreatic PLA2 induces fusion of LDL particles, increases the binding strength of the fused particles to human aortic PGs, and thus leads to increased deposition of LDL particles on a PG matrix. Because the arterial intima has been shown to contain sPLA2,22–26 and both LDL-like particles and lipid droplets isolated from atherosclerotic lesions show signs of phospholipid hydrolysis, we suggest that the present results can be extrapolated to the actual events in the arterial intima.

snpPLA2 is found in the intimas of even early atherosclerotic lesions24–26 and is occasionally found in nonatherosclerotic intimas containing considerable macrophage infiltration.28 The enzyme is able to bind to arterial PGs21 and was found in the arterial intima in association with extracellular matrix components.24 Most importantly, sPLA2 has also been found in contact with extracellular lipid droplets.24 At present, there is convincing evidence that such extracellular lipid droplets are formed from LDL particles by modification, for instance by hydrolytic enzymes, of the arterial intima.11 As shown in this study, one of these enzymes may be sPLA2.

Interestingly, interaction of LDL with GAGs is a prerequisite for PLA2-induced fusion of LDL particles.20 This interaction has been shown in vitro to induce irreversible changes in the conformation of apoB-10044 and in the
Figure 4. Capacity of PGs to bind lipolyzed LDL. [3H]LDL was modified in either human PG or BSA-coated microtiter wells as described under Methods. After incubation for 6 hours at 37°C, the degree of LDL lipolysis was determined from the supernatants (A). The radioactivities of the wells were determined and used to calculate the amount of LDL bound to the PGs (B). Values are mean±SEM of 2 independent experiments, each performed in quadruplicate.

organization of LDL lipids. Surprisingly, as shown in this study, after prolonged incubation (36 hours), mere binding of LDL to PGs can induce aggregation of a fraction of the LDL particles. This finding is supported by similar observations by Camejo et al and Tirziu et al, who showed that complexing of LDL by human aortic PGs or GAGs produces aggregated/fused particles resembling those found in the arterial intima.

Binding of LDL particles to intimal PGs in atherosclerosis-prone areas leads to retention of LDL within the arterial wall. The present results show that lipolysis of PG-bound LDL particles by snpPLA₂ increases their binding strength to PGs by inducing particle fusion. In addition, with a sensitive gel mobility shift assay, Sartipy et al have shown that even unfused PLA₂-treated LDL particles have a higher affinity for PGs than does native LDL. Taken together, lipolysis of LDL with PLA₂ is likely to increase retention of LDL in the PG-rich extracellular matrix.

Finally, in addition to increasing the retention of LDL, snpPLA₂ is also likely to lead to progressive deposition of lipid within the extracellular matrix by inducing aggregation and fusion of LDL. In fact, in this study we found that lipolysis of LDL with snpPLA₂ induced a 3-fold increase in the capacity of PGs to bind LDL. Similar results were obtained when bee venom PLA₂ or decorin PG was used (unpublished data, 2000). Interestingly, with bee venom PLA₂, it appeared that the higher the degree of LDL lipolysis (and aggregation/fusion), the larger was the amount of LDL bound to PGs (unpublished data, 2000). Thus, it is likely that the increased deposition of PLA₂-treated LDL on a PG matrix depends on the aggregation/fusion of the lipolyzed LDL particles.

Taken together, our previous and present in vitro studies have shown that lipolysis of LDL with PLA₂ increases the binding strength of LDL to PGs by inducing aggregation and fusion of LDL particles and thus enhances the retention of modified LDL to human aortic PGs. Because the subendothelial retention of LDL particles and the appearance of aggregated and fused lipid droplets and vesicles in the subendothelial region of the arterial intima are early key events in athrogenesis and snpPLA₂ is an enzyme that has been found in association with extracellular matrix components and with lipid droplets in the human arterial intima, PLA₂-induced aggregation and fusion of LDL may be one mechanism leading to an increase in the initial retention of LDL and accumulation of LDL-derived lipid droplets in the subendothelial PG-rich area of the arterial intima during the early stages of atherosclerosis.

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