Group V Secretory Phospholipase A₂-modified Low Density Lipoprotein Promotes Foam Cell Formation by a SR-A- and CD36-independent Process That Involves Cellular Proteoglycans

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Accumulating evidence indicates that secretory phospholipase A₂ (sPLA₂) enzymes promote atherogenic processes. We have previously showed the presence of Group V sPLA₂ (GV sPLA₂) in human and mouse atherosclerotic lesions, its hydrolysis of low density lipoprotein (LDL) particles, and the ability of GV sPLA₂-modified LDL (GV-LDL) to induce macrophage foam cell formation in vitro. The goal of this study was to investigate the mechanisms involved in macrophage uptake of GV-LDL. Peritoneal macrophages from C57BL/6 mice (wild type (WT)), C57BL/6 mice deficient in LDL receptor (LDLR−/−), or SR-A and CD36 (DKO) were treated with control LDL, GV-LDL, oxidized LDL (ox-LDL) or LDL aggregated by vortexing (vx-LDL). As expected, ox-LDL induced significantly more cholesterol ester accumulation in WT and LDLR−/− compared with DKO macrophages. In contrast, there was no difference in the accumulation of GV-LDL or vx-LDL in the three cell types. ¹²⁵I-ox-LDL exhibited high affinity, saturable binding to WT cells that was significantly reduced in DKO cells. Vx-LDL and GV-LDL showed low affinity, non-saturable binding that was similar for both cell types, and significantly higher compared with control LDL. GV-LDL degradation in WT and DKO cells was similar. Analyses by confocal microscopy indicated a distinct intracellular distribution of Alexa-568-labeled GV-LDL and Alexa-488-labeled ox-LDL. Uptake of GV-LDL (but not ox-LDL or vx-LDL) was significantly reduced in cells preincubated with heparin or NaClO₃, suggesting a role for proteoglycans in GV-LDL uptake. Our data point to a physiological modification of LDL that has the potential to promote macrophage foam cell formation independent of scavenger receptors.

A critical event in early atherogenesis is the formation of lipid-laden macrophages (“foam cells”) (1–4). According to the “response-to-retenion” hypothesis (5), conditions leading to enhanced LDL entrapment in the subendothelium trigger this process. Once retained in the vessel wall, LDL undergoes various modifications in both protein and phospholipid (PL) moieties that promote retention and lead to enhanced macrophage uptake. Several types of modifications of LDL, such as oxidation (6–8), depletion of sphingomyelin by secretory sphingomyelinase (9), hydrolysis of glycerol-PLs by sPLA₂ enzymes (10–12), and aggregation (13–18) have been implicated in lipid accumulation in the vessel wall.

The sPLA₂ family comprises a group of enzymes that hydrolyze the acyl-ester bond at the 2 position (sn-2) of glycerol-PLs. Of the 10 sPLA₂ isoforms that have been described in mammals, three members (Group IIA, Group V, and Group X) have been detected in human and/or mouse atherosclerotic lesions (10, 11, 19). Accumulating evidence indicates that sPLA₂ hydrolysis of LDL-PL results in structural alterations of the particles that promote lipid accumulation in the vessel wall and enhances macrophage uptake. Hydrolysis of LDL by sPLA₂ in vitro results in an increased affinity for proteoglycans, which would be expected to increase retention in the subendothelium (20, 21). Lipolysis of LDL by sPLA₂ leads to conformational changes in apolipoprotein (apo)-100 and reorganization of lipids that induce particle aggregation (22–24). We recently reported that in vitro hydrolysis of LDL by Group V sPLA₂ in the presence of physiological concentrations of albumin leads to spontaneous particle aggregation, and the extent of aggregation is proportional to the degree of LDL hydrolysis (11). When incubated with mouse peritoneal macrophages, this modified LDL induces foam cell formation. Group X sPLA₂ modification of LDL has also been shown to promote macrophage foam cell formation, although extensive modification by Group X sPLA₂ is not reported to induce LDL aggregation (10). The structural alterations of the LDL particles brought about by sPLA₂ hydrolysis that are responsible for enhancing macrophage lipid accumulation and the pathway(s) by which macrophages take up this modified LDL have not been defined.

In the present study, we investigated the mechanisms involved in macrophage uptake of GV sPLA₂-modified LDL. Using mouse peritoneal macrophages deficient in LDLR or SR-A and CD36, we demonstrated that GV-LDL uptake and degradation is not dependent on LDLR or scavenger receptors SR-A and CD36. Analysis by confocal microscopy revealed differences in the rate of uptake and subsequent intracellular trafficking of GV-LDL and ox-LDL by macrophages. Treatments that inhibited apoB-proteoglycan interaction or proteoglycan assembly significantly decreased GV-LDL accumulation by macrophages but had no effect on uptake of ox-LDL or LDL aggregated by vortexing. Taken together, our results indicate that Group V sPLA₂-hydrolyzed LDL promotes macrophage foam cell formation through a pathway that is distinct from other modified forms of LDL and involves cell-surface proteoglycans.
EXPERIMENTAL PROCEDURES

Isolation, Modification, and Labeling of LDL—LDL (density 1.019–1.063) was isolated from the plasma of healthy volunteers by sequential ultracentrifugation and stored at 4 °C under argon gas. LDL was subjected to hydrolysis by GV sPLA₂ as previously described (11). Briefly, LDL particles (1 mg/ml) were incubated in hydrolysis buffer (0.1 M HEPES, 0.1 M NaCl, 1 mM CaCl₂, 10 mg/ml fatty acid-free BSA, and 0.01% butylated hydroxytoluene) under argon gas at 37 °C for 24 h in the presence (GV-LDL) or absence (mock-LDL) of 500 units/ml GV sPLA₂. LDL- PL hydrolysis was quantified by measuring the amount of free fatty acids released in the solution (11). For the experiments described here, incubation with GV sPLA₂ in the absence of EDTA resulted in the hydrolysis of >80% of LDL-PL. For some experiments, a portion of GV-LDL was filtered through a 0.1-μm filter (Whatman, Clifton, NJ), and the total cholesterol content of samples before and after filtration was used to estimate the amount of GV-LDL retained by the filter. We also analyzed hydrolyzed particles before and after filtration by electron microscopy. LDL preparations were stained with 2% uranyl acetate solution and then viewed and photographed in a Philips Tecnai 12 transmission electron microscope at the Electron Microscopy and Imaging Facility, University of Kentucky. The diameters of aggregates were measured from the electron micrographs using Scion Image software (Scion Corporation, Frederick, MD). For other experiments, LDL was incubated with GV sPLA₂ in hydrolysis buffer supplemented with 10 mM EDTA to prevent LDL-PL hydrolysis (25). To prepare oxidized LDL, native LDL was dialyzed against 5 mM CuSO₄ overnight at 4 °C. Oxidation was stopped by adding EDTA at a final concentration of 1 mM. Ox-LDL was then dialyzed against 150 mM saline and stored under argon gas.

Relative electrophoretic mobility was assessed by running the ligands for 90 min at 100 V on 2% agarose gel (26). Mock-LDL, GV-LDL, and ox-LDL were labeled with ¹²⁵I according to Bilheimer’s modifications (27) of the procedure described by Goldstein et al. (1). Native LDL or ¹²⁵I-LDL was aggregated by vortexing at maximal speed (Fisher, vortex model Genie 2) for 1 min. Native LDL and ox-LDL were labeled with Alexa-Fluor 568 and Alexa-Fluor 488, respectively (Molecular Probes, Eugene, OR), according to manufacturer’s instructions. Native LDL was then hydrolyzed with GV-sPLA₂, as already described.

Isolation of Peritoneal Macrophages—C57BL/6 mice and C57BL/6 mice lacking the LDL receptor were originally obtained from The Jackson Laboratory (Bar Harbor, ME). To generate SR-A/CD36-deficient mice, SR-A⁻/⁻ mice (28) backcrossed at least six times in C57BL/6 background were crossed with CD36⁻/⁻ mice (29) backcrossed at least four times in the C57BL/6 strain.

Animals were injected intraperitoneally with a sterile solution (1 ml) of 1% Biogel 100 (Bio-Rad) in phosphate-buffered saline. After 96 h, the animals were anesthetized, and peritoneal macrophages were harvested by lavage with 5 ml of ice-cold phosphate-buffered saline. All procedures were in accordance with the guidelines of the Veterans Affairs Institutional Animal Care and Use Committee. Macrophages were seeded in 12-well dishes at a density of 1×10⁶ cells/well in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, and 25 ng/ml macrophage colony-stimulating factor (Calbiochem) and allowed to attach for 4 h. Non-attached cells were then removed by washing the dishes with phosphate-buffered saline. Macrophages were incubated overnight at 37 °C. When indicated, cells were preincubated for 3 h with 100 units/ml heparin (Sigma) or 5 mg/ml lactoferrin (Sigma) or overnight with 10 mM NaClO₄ prior to the addition of ligands.

Lipid Extraction and Cholesterol Ester (CE) Measurements—After incubation with LDL ligands, cells were solubilized in 0.25 ml of 0.1 N NaOH for 2 h on a shaking platform, and 50-μl aliquots were used for protein determinations. Lipids were extracted from the remaining cell lysate by adding 3 ml of methanol:chloroform 2:1 (v/v) and incubating at 37 °C for 1 h. Phases were separated by adding 2 ml of 0.5% H₂SO₄ followed by vigorous vortexing and centrifugation at 3,000 revolutions/min for 10 min. The organic phase was collected, and 1 ml of 1% Triton X-100 in chloroform was added. The samples were dried in a Freesozy system (Freezone 4.5, Labconco, Kansas City, MO) and dissolved in H₂O. Aliquots were assayed for total and free cholesterol content using a colorimetric kit (Wako, Richmond, VA). CE was calculated as the difference between the total and free cholesterol.

Binding and Degradation Assay—For binding studies, cells were cooled to 4 °C and after washing with ice-cold phosphate-buffered saline, were incubated with ice-cold medium containing the indicated concentrations of ¹²⁵I-labeled ligands. After a 2-h incubation at 4 °C, the medium was removed and cells were washed rapidly three times with washing buffer (50 mM Tris, 150 mM NaCl, and 2 mg/ml fatty acid-free BSA) followed by two washes with washing buffer without BSA. All washes were performed at 4 °C with prechilled solutions. The cells were solubilized in 0.25 ml of 0.1 N NaOH for 2 h on a rotary platform at room temperature. The total radioactivity was measured on a γ counter (Cobra II, Packard Instrument Co., Minneapolis, MN). Kᵥ values were determined by nonlinear regression analysis of receptor-specific cell association values using Prism® software (GraphPad Software, San Diego, CA). For apoB degradation assays, cell-free supernatants were analyzed for trichloroacetic acid-soluble, chloroform-unextractable radioactivity (30). Background for degradation assays was determined by performing ligand incubations in wells containing no cells.

Oil Red O Staining—Peritoneal macrophages from C57BL/6 mice were seeded on glass coverslips in 12-well dishes and incubated for 48 h with 0.2 mg/ml mock-LDL, GV-LDL, or GV-LDL prefiltered through a 0.1-μm filter, as described under “Isolation, Modification, and Labeling of LDL.” After incubations, the cells were fixed for 10 min in 10% formalin, washed with 60% isopropyl alcohol, and then stained with Oil Red O (60% in isopropyl alcohol; Sigma) for 30 min. After washing twice with 60% isopropyl alcohol, the cells were counterstained with hematoxylin and mounted on slides for light microscopy.

Confocal Microscopy—Peritoneal macrophages from WT or DKO mice were seeded on glass coverslips in 12-well dishes and incubated with 0.2 mg/ml Alexa-fluor 488-labeled ox-LDL and Alexa-fluor 568-labeled GV-LDL. After incubations, the cells were mounted on slides using fluorescence protecting medium ( Vectashield, Vector Laboratories, Burlingame, CA). Confocal microscopy was performed at the University of Kentucky Imaging Facility using a Leica laser scanning confocal microscope with argon (488 nm) and krypton (568 nm) lasers.

Statistical Analysis—Data are expressed as mean ± S.E. Results were analyzed by Student’s t test and one-way analysis of variance followed by Bonferroni’s post-test. Values of p < 0.05 were considered statistically significant.

RESULTS

Hydrolysis by GV sPLA₂ Promotes Macrophage LDL Uptake—We reported previously that LDL hydrolyzed by GV sPLA₂ (GV-LDL) is susceptible to aggregation and promotes the accumulation of neutral lipids in mouse peritoneal macrophages (11) (Fig. 1B). In the present study, we investigated the mechanisms involved in macrophage uptake of GV-LDL. Because aggregated LDL is a potent inducer of foam cell formation (16), we considered the possibility that GV-LDL is taken up by macrophages because of its highly aggregated state. To determine the size of the aggregates formed after LDL hydrolysis, we analyzed mock-LDL and GV-LDL by electron microscopy (Fig. 1A). Our data revealed that the mean size of the aggregates was 123 ± 19 nm. To remove these
aggregates from GV-LDL preparations, we filtered GV-LDL through a 0.1-μm filter, which appeared to efficiently remove the large aggregates (Fig. 1A). Based on the cholesterol content of the filtered fraction, >75% of the hydrolyzed LDL was recovered in the flow-through. We then assessed the ability of the filtered fraction to induce foam cell formation. Mouse peritoneal macrophages were incubated for 48 h with equivalent amounts of GV-LDL or GV-LDL-filtered fraction. For comparison, cells were also incubated with a control LDL, which was treated similarly to GV-LDL, except that the enzyme was omitted from the reaction mixture (mock-LDL). Compared with mock-LDL, incubation with GV-LDL did not lead to increased cellular free cholesterol content in macrophages (Fig. 1C), whereas CE accumulation in macrophages treated with GV-LDL was significantly higher compared with cells treated with mock-LDL (Fig. 1, B and C). Our data indicated a slight and insignificant decrease in CE accumulation in cells incubated with the filtered fraction compared with cells treated with unfiltered GV-LDL. Thus, increased CE accumulation in macrophages incubated with GV-LDL is not dependent on large (>0.1 μm) LDL aggregates produced as a result of GV sPLA₂ hydrolysis.

We considered the possibility that GV sPLA₂ mediates LDL uptake through a non-enzymatic function, whereby the enzyme itself promotes the interaction of the particle with the cell through a mechanism that does not involve LDL hydrolysis. To investigate this, we used the fact that hydrolytic activity of GV sPLA₂, similar to other members of the sPLA₂ family, is dependent on millimolar concentrations of Ca²⁺ (25). Thus, to inhibit hydrolysis, LDL was incubated with GV sPLA₂ in the presence of 10 mM EDTA. Quantification of the amount of free fatty acids released in the reaction mixture confirmed that LDL-PL hydrolysis was completely inhibited under these reaction conditions (data not shown). Incubation of macrophages with LDL treated with GV sPLA₂ in the presence of EDTA failed to induce foam cell formation (Fig. 1C). Thus, particle hydrolysis appears to be a prerequisite for increased uptake of GV-LDL by macrophages, suggesting that GV sPLA₂ promotes foam cell formation by altering the structure of the LDL particle.

LDL Receptor, SR-A, and CD36 Are Not Involved in the Uptake of GV-LDL—We next examined the role of LDLR and scavenger receptors SR-A and CD36 in GV-LDL-mediated foam cell formation. To assess the role of these receptors in GV-LDL uptake, our approach was to compare CE accumulation in peritoneal macrophages isolated from C57BL/6 (WT), LDLR⁻/⁻, and SR-A/CD36⁻/⁻ (DKO) mice after incubations with LDL hydrolyzed with GV sPLA₂ in the presence of BSA (1%). For comparison, WT, LDLR⁻/⁻, and DKO cells were also incubated with mock-LDL, ox-LDL, or ox-LDL hydrolyzed in the presence of BSA (1%) and CD36 (2.5 μg of LDL protein/lane) was assessed by agarose gel electrophoresis (8).

Because recognition by scavenger receptors is believed to be mediated through interactions with polyanionic ligands, to explain our findings it was important to determine whether GV sPLA₂ hydrolysis results in an altered charge of the LDL particle. To assess whether GV sPLA₂ hydrolysis alters LDL charge similarly to oxidation, the relative electro-
Macrophage Uptake of Group V sPLA$_2$-modified LDL

GV-LDL Degradation Is Independent of SR-A and CD36—To determine the extent of GV-LDL internalization and degradation by macrophages, WT and DKO cells were incubated at 37 °C with 0.2 mg/ml $^{125}$I-labeled mock-LDL, GV-LDL, ox-LDL, and vx-LDL, and the amount of trichloroacetic acid-soluble, chloroform-unextractable radioactivity in the medium was quantified at selected intervals. In the case of ox-LDL, there was a time-dependent increase in the degradation of apoB in WT macrophages that was significantly higher compared with DKO cells (Fig. 4A). In contrast, for the other three ligands, there was no significant difference in apoB degradation in WT and DKO macrophages. In both vx-LDL- and GV-LDL-treated cells, the amount of degraded apoB was significantly higher compared with cells treated with mock-LDL (Fig. 4, B–D). These findings confirmed our previous data that GV sPLA$_2$ hydrolysis promotes macrophage LDL uptake and that SR-A and CD36 are not involved in the internalization of such modified LDL. Although it appeared that macrophages degraded significantly more vx-LDL compared with the other modified LDL ligands, our data indicate that only 2% of the total amount of vx-LDL associated with cells was degraded after 8 h. This contrasts to ox-LDL and GV-LDL, where ~40 and 19%, respectively, of the total amount of ligand taken up was degraded after 8 h (data not shown).

GV-LDL and ox-LDL Internalized by Macrophages Have Distinct Intracellular Localization—As another approach to comparing macrophage uptake of GV-LDL and ox-LDL, we incubated WT and DKO cells simultaneously with 0.2 mg/ml Alexa-fluor 568-labeled GV-LDL (Fig. 5, red) and Alexa-fluor 488-labeled ox-LDL (green) and then visualized the cells at selected intervals by confocal microscopy. The analysis of DKO macrophages after 10 min of incubation confirmed the finding that macrophage binding of GV-LDL (red) does not require CD36 or SR-A expression (Fig. 5A), unlike ox-LDL (green) (Fig. 5, B versus A). Interestingly, after 10 min of incubation, the majority of the cell-associated GV-LDL appeared to be at or near the cell surface of both WT and DKO cells (Fig. 5, A and B). This contrast to ox-LDL, where a large amount of ligand internalization by WT cells was evident after a 10-min incuba-
tion. The analysis of WT cells at later time points showed that, although GV-LDL was eventually taken up, its intracellular distribution was markedly distinct from ox-LDL (Fig. 5, C and D). Taken together, these data indicate that the pathways for uptake and/or intracellular trafficking of ox-LDL and GV-LDL are distinct.

**GV-LDL Uptake by Macrophages Is Inhibited by Heparin and NaClO₃**—One possible mechanism for GV-LDL uptake by macrophages is via an interaction with cellular proteoglycans. We investigated this possibility using two approaches. For the first approach, we incubated WT macrophages with ligands in the presence or absence of 100 μg/ml heparin. At this concentration, heparin specifically blocks interactions with proteoglycans without interfering with lipoprotein binding to lipoprotein receptors (32). For the second approach, we blocked sulfation of cellular proteoglycans by preincubating cells for 18 h at 37 °C in 10 mM sodium chlorate, an inhibitor of sulfate adenyltransferase (33). Compared with untreated cells, the accumulation of GV-LDL was significantly reduced in macrophage cells treated with either heparin or sodium chloride (Fig. 6). Neither heparin nor sodium chloride significantly altered CE accumulation in cells incubated with mock-LDL, ox-LDL, or vx-LDL. We conclude from these results that macrophage uptake of GV-LDL (and not ox-LDL or vx-LDL) is mediated by interactions with cell surface proteoglycans.

Several possibilities for proteoglycan-mediated uptake of GV-LDL exist. One is that proteoglycan binding of GV-LDL may be followed by actions with cell surface proteoglycans. We investigated this possibility using two approaches. For the first approach, we incubated WT macrophages with ligands in the presence or absence of 100 μg/ml heparin. At this concentration, heparin specifically blocks interactions with proteoglycans without interfering with lipoprotein binding to lipoprotein receptors (32). For the second approach, we blocked sulfation of cellular proteoglycans by preincubating cells for 18 h at 37 °C in 10 mM sodium chlorate, an inhibitor of sulfate adenyltransferase (33). Compared with untreated cells, the accumulation of GV-LDL was significantly reduced in macrophage cells treated with either heparin or sodium chloride (Fig. 6). Neither heparin nor sodium chloride significantly altered CE accumulation in cells incubated with mock-LDL, ox-LDL, or vx-LDL. We conclude from these results that macrophage uptake of GV-LDL (and not ox-LDL or vx-LDL) is mediated by interactions with cell surface proteoglycans.

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**DISCUSSION**

A critical event in early atherosclerosis is the accumulation of LDL in the subendothelial extracellular matrix. One pathological consequence of this retained lipoprotein is the cholesterol ester loading of macrophages (foam cell formation). Notably, native LDL, whose level in plasma strongly correlates with atherogenesis, does not readily induce macrophage foam cells. This suggests that LDL must undergo modification to promote lipid accumulation. We have focused on modification of LDL by Group V sPLA₂, a member of the secretory phospholipase A₂ family of enzymes shown by others to have potent activity toward phosphatidylcholine-containing substrates (40, 41). We recently reported that GV sPLA₂ effectively hydrolyzes LDL *in vitro* to generate modified LDL particles capable of promoting CE accumulation in cultured mouse peritoneal macrophages (11). These data, along with evidence that GV sPLA₂ is present in regions of atherosclerotic lipid accumulation in human and mouse lesions (11), suggests a role for this enzyme in atherogenesis. In the current study, we investigated the mechanism of uptake of GV sPLA₂-modified LDL by macrophages. The major finding of this study is that macrophage accumulation of GV-LDL is not dependent on SR-A and CD36, scavenger receptors known to be involved in the uptake of other forms of modified LDL. Our data are consistent with the possibility that GV sPLA₂ hydrolysis of LDL brings about structural changes in the particles that promote interaction with cellular proteoglycans and subsequent internalization.

Our studies using peritoneal macrophages deficient in LDLR or CD36 and SR-A unequivocally show that the binding, internalization, and accumulation of GV-LDL is independent of these receptors. The lack of interaction of GV-LDL with CD36 or SR-A was not totally unexpected, because all known scavenger receptor ligands are polyanionic molecules (42). We have confirmed the finding of Kleinman et al. (22) that sPLA₂ modification of LDL in the presence of physiologic concentrations of albumin does not increase the negative charge of the particle. Although sPLA₂ hydrolysis might be expected to promote oxidative modification of LDL *in vivo* (43, 44), and consequently, scavenger receptor-mediated uptake, we have taken steps in our *in vitro* studies to minimize oxidation of GV-LDL. Under these conditions, we have been able to identify a novel physiological pathway leading to foam cell formation.

We reported previously that one consequence of GV sPLA₂ hydrolysis is the generation of LDL particles with increased propensity to aggregate (11). Because aggregated LDL is known to be a potent inducer of macrophage foam cells (16), we considered the possibility that GV-LDL is taken up because it is highly aggregated. To address this, based
on our electron microscopy analysis of the aggregates, we filtered GV-LDL to remove aggregates with diameters larger than 0.1 \( \mu \text{m} \), which comprised <1/4 of the LDL after hydrolysis. When vx-LDL was subjected to filtration, we were not able to recover any vx-LDL in the filtered fraction, indicating that virtually all of the LDL particles after vortexing were in aggregates >0.1 \( \mu \text{m} \). Incubation of macrophages with equal concentrations of filtered and non-filtered GV-LDL revealed that both fractions were equally potent in promoting foam cell formation, indicating that either monomeric GV-LDL or microaggregates are internalized by macrophages at an enhanced rate compared with native LDL.

As expected, LDL particles were not hydrolyzed by GV sPLA\(_2\) when EDTA (10 \( \mu \text{M} \)) was added to the incubation mixtures. The finding that LDL incubated with GV sPLA\(_2\) in the presence of EDTA failed to induce foam cell formation indicates that PL hydrolysis is a prerequisite for macrophage uptake of GV-LDL. We interpret this finding to suggest that GV sPLA\(_2\) does not appear to be serving a mere “bridging” function that has been described for other lipoprotein-modifying enzymes, such as lipoprotein lipase, endothelial lipase, and hepatic lipase (32, 45, 46). An alternative possibility that we cannot rule out is that enzymatic hydrolysis of LDL-PL is required for GV sPLA\(_2\) to perform a bridging function promoting the interaction of the hydrolyzed LDL with macrophages.

Our data indicate that GV sPLA\(_2\) alters the structure of the LDL particle in a manner that enhances its uptake by macrophages. Previous studies have shown that lipolysis of LDL with sPLA\(_2\) results in an increased affinity for proteoglycans secreted by human arterial smooth muscle cells (47) and proteoglycans isolated from human aorta (20). In a recent study, Flood et al. (24) identified the molecular basis for this altered interaction. The direct interaction between apoB-containing lipoproteins and negatively charged proteoglycans involves positively charged amino acids in apoB (48). Previous analyses of apoB-100 identified eight separate domains that have the capacity to bind proteoglycans. One of these domains (“site B”, corresponding to residues 3359–3369) appears to be the primary proteoglycan-binding site in native LDL (49). Modification of LDL by Group IIA sPLA\(_2\), leads to a conformational change in apoB-100, thereby exposing a cryptic site (“site A”, residues 3148–3158), which then acts cooperatively with site B to increase proteoglycan-binding affinity (24). Our current results showed that treatments that would be expected to disrupt the interaction of GV-LDL with cellular proteoglycans significantly reduce macrophage GV-LDL uptake.

The mechanism by which macrophages take up GV-LDL after proteoglycan binding has not been delineated. One possibility is that uptake of GV-LDL occurs through a multistep process beginning with an initial binding to cell-surface heparan sulfate proteoglycans (HSPG), followed by their uptake into cells by a receptor-mediated process that utilizes members of the low density lipoprotein receptor family (34–36). Several studies have shown that LRP binds apoE-enriched particles (50, 51) and that the clearance of apoE-containing particles, such as chylomicron remnants and \( \beta \)-very low density lipoprotein, is inhibited by lactoferrin (38, 52, 53), a molecule that also binds to LRP (37). We tested the possible involvement of LRP in proteoglycan-mediated uptake of GV-LDL using lactoferrin. Our data indicate that lactoferrin does not interfere with the accumulation of GV-LDL in macrophages, indicating an uptake pathway different from that described for apoE-rich lipoproteins (54).

Accumulating evidence indicates that cell surface proteoglycans, in particular syndecan and perlecian, directly mediate endocytic uptake of ligands, including lipoproteins (32, 55). In the case of syndecan HSPGs, ligand uptake is triggered by clustering of the transmembrane and cytoplasmic domains of the core protein followed by internalization via a non-clathrin-coated pit pathway (32, 55). Internalization via perlecian appears to be kinetically and biochemically distinct from both coated pits or syndecan-mediated endocytosis (31, 55). Previous investigations of HSPG-mediated lipoprotein uptake have been performed in the presence of lipoprotein lipase, which serve as a bridge to mediate binding of the particle to cell-surface HSPGs. In the case of GV-LDL, we envision that altered apoB conformation brought about by phospholipid hydrolysis is sufficient for promoting LDL/HSPG interaction.

An interesting aspect of our results is that foam cell formation induced by ox-LDL, vx-LDL, and GV-LDL appeared to be mediated through different pathways. After uptake, GV-LDL was degraded more efficiently than vx-LDL. Interestingly, GV-LDL had a slower rate of degradation compared with ox-LDL, which was 40% at 8 h for the ox-LDL and 19% at 8 h for GV-LDL. Analysis by confocal microscopy showed striking differences in the trafficking of ox-LDL and GV-LDL. The internalization of GV-LDL appeared to be markedly delayed compared with ox-LDL, and once taken up, a considerable portion of intracellular GV-LDL and ox-LDL did not co-localize. These observations are consistent with previous findings that receptor-mediated and HSPG-mediated endocytosis are biochemically and kinetically distinct (32). Interestingly, unlike GV-LDL, macrophage accumulation of vx-LDL was not dependent on an interaction with proteoglycans. This result indicates that the highly aggregated state induced by vortexing promotes an uptake mechanism that is different from GV-LDL uptake. This is also suggested by the finding that only a small portion of vx-LDL was degraded by WT macrophages (<2% in 8 h), whereas 19% of GV-LDL was degraded in the same time period. This difference in the extent of degradation is consistent with the conclusion that the uptake and/or internalization pathways for the two modified forms of LDL may be distinct.

In summary, we have shown that modification of LDL by GV sPLA\(_2\), an enzyme known to be present in atherosclerotic lesions (11), promotes macrophage foam cell formation through a mechanism that is independent of scavenger receptors SR-A and CD36. Evidence also suggests that uptake of GV-LDL occurs via a pathway that is distinct from the pathway used for vx-LDL. The finding that cell-surface proteoglycans are involved in the uptake of GV-LDL suggests the intriguing possibility that sPLA\(_2\)-induced alterations in apoB conformation increases the atherogenicity of LDL not only by enhancing retention to proteoglycans of the extracellular matrix (12, 19, 21) but also by directly promoting macrophage binding and uptake.

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