Group V sPLA₂ Hydrolysis of Low-Density Lipoprotein Results in Spontaneous Particle Aggregation and Promotes Macrophage Foam Cell Formation

C. Ruth Wooton-Kee, Boris B. Boyanovsky, Munira S. Nasser, Willem J.S. de Villiers, Nancy R. Webb

**Objectives**—Secretory phospholipase A₂ (sPLA₂) enzymes hydrolyze the sn-2 fatty acyl ester bond of phospholipids to produce a free fatty acid and a lysophospholipid. Group V sPLA₂ is expressed in cultured macrophage cells and has high affinity for phosphatidyl choline-containing substrates. The present study assesses the presence of group V sPLA₂ in human and mouse atherosclerotic lesions and its activity toward low-density lipoprotein (LDL) particles.

**Methods and Results**—Group V sPLA₂ was detected in human and mouse atherosclerotic lesions by immunohistochemical staining. Electron microscopic analysis showed that mouse group V sPLA₂-modified LDL is significantly smaller (mean diameter±SEM = 25.3±0.25 nm) than native LDL (mean diameter±SEM = 27.7±0.29 nm). Hydrolysis by group V sPLA₂ induced spontaneous particle aggregation; the extent of aggregation was directly proportional to the degree of LDL hydrolysis. Group V sPLA₂ modification of LDL led to enhanced lipid accumulation in cultured mouse peritoneal macrophage cells.

**Conclusions**—Group V sPLA₂ may play an important role in promoting atherosclerotic lesion development by modifying LDL particles in the arterial wall, thereby enhancing particle aggregation, retention, and macrophage uptake. (Arterioscler Thromb Vasc Biol. 2004;24:762-767.)

**Key Words:** atherosclerosis ■ group V secretory phospholipase A₂ ■ LDL aggregation ■ macrophages
promote atherosclerotic lipid accumulation by modifying LDL particles retained in arterial tissues. In the current study, we establish that group V sPLA2 can be detected in human and mouse atherosclerotic lesions. We also demonstrate that LDL particles hydrolyzed by group V sPLA2 are significantly smaller compared with native LDL and are susceptible to spontaneous aggregation. These group V sPLA2-modified particles promote foam cell formation in cultured mouse peritoneal macrophages.

Methods

Immunohistochemistry

Mouse anti-human group V sPLA2 was purchased from Cayman Chemicals (Ann Arbor, Mich). Rabbit anti-mouse group V sPLA2 was raised against residues 9 to 113 expressed in bacteria using the pET system (Novagen, Madison, Wisc). Chicken anti-mouse group V sPLA2 was raised against the synthetic peptide ELKSMIEKVTRKNAFKNY, a region of group V sPLA2 that has no sequence homology with group X sPLA2. For immunostaining, the rabbit and chicken antibodies were eluted from an affinity column conjugated to the corresponding antigen. Anti-human CD68 (macrophage marker) antibody and mouse macrophage-specific antisera were obtained from Dako and Accurate, respectively. Human pathological samples were obtained from subjects in studies approved by the University of Kentucky Institutional Review Board and with informed consent. Formalin-fixed human aortic tissue was embedded in paraffin for sectioning. Sections on glass slides were deparfynized using EZ-Dewax solution (Biogenex, San Ramon, Calif). Antigen retrieval was performed using an antigen retrieval solution (Dako) and blocked for 30 minutes with 10% mouse serum. Hearts were obtained from female apoE-deficient or male LDL receptor-deficient mice (C57BL/6 background, Jackson Laboratory) fed a diet deficient in LDL particles retained in arterial tissues. In the current study, we establish that group V sPLA2 can be detected in human and mouse atherosclerotic lesions. We also demonstrate that LDL particles hydrolyzed by group V sPLA2 are significantly smaller compared with native LDL and are susceptible to spontaneous aggregation. These group V sPLA2-modified particles promote foam cell formation in cultured mouse peritoneal macrophages.

Expression of Mouse Group V sPLA2 by Adenoviral Vector

A replication-defective adenoviral vector encoding mouse group V sPLA2 (AdmGV) was generated using the AdEasys system. To express group V sPLA2 in vitro, COS-7 cells were incubated with AdmGV at a multiplicity of infection of 4000 particles per cell. To prepare partially purified enzyme, conditioned media was collected 48 hours after infection and sequentially chromatographed on Hi-Trap SP ion exchange and Hi-Trap heparin columns (Amersham Biosciences) as previously described for group IIa sPLA2.23

Phospholipase Assays Using Synthetic Micelles as Substrate

Phospholipase activity was measured using a colorimetric assay. Mixed micelles were prepared by warming to 37 °C and vortexing at maximum speed for 1 minute. Peritoneal macrophage fluorescent bead preparations were stained with 2% uranyl acetate solution. Electron micrographs were taken at lower magnifications. Group V sPLA2 was clearly seen in human and mouse atherosclerotic lesions. In this study, we assessed whether group V sPLA2 could also be detected in human and mouse lesions. Immunohistochemical analysis of advanced human atherosclerotic aortic lesions and aortic root sections from apoE-deficient mice fed an atherogenic diet demonstrated the presence of group V sPLA2 (Figure 1B and 1D). The sections stained with control antibodies (mouse- and rabbit-irrelevant IgG) were negative (Figure 1A and 1C). Immunostaining of apoE-deficient mouse lesions with affinity-purified chicken anti-mouse group V sPLA2 IgY produced results similar to those depicted in Figure 1D (data not shown). To more clearly see the distribution of group V sPLA2 in human lesions, please see Figure I (available online at http://atvb.ahajournals.org). Which shows staining of the sections with antisera specific for smooth muscle cell actin, macrophages, or group V sPLA2 at lower magnifications. Group V sPLA2 was also detected in lesions of LDL receptor-deficient mice stained with affinity-purified chicken anti-mouse group V sPLA2 (Figure II, available online at http://atvb.ahajournals.org).
Expression of Group V sPLA₂ in Cultured Cells

To study group V sPLA₂ activity in vitro, a replication-defective adenoviral vector encoding mouse group V sPLA₂ (AdmGV) was used to express the enzyme in COS-7 cells. Western blot analysis showed group V sPLA₂ expression in cells 48 hours after treatment with AdmGV but not cells treated with a control adenoviral vector (Figure 2A). A single immunoreactive band that migrated similarly with an apparent molecular weight of ≈14 kDa was also detected in supernatants from AdmGV-treated, but not control, COS-7 cells (not shown). Phospholipase activity secreted by AdmGV-treated cells was quantified using mixed micelles containing either POPC or POPG as substrate. In these assays, phospholipase activity in supernatants collected from control COS-7 cells was <5% of the activity present in supernatants from AdmGV-treated cells. The results from this analysis showed ~2-fold difference in specific activity toward these 2 phospholipid substrates (Figure 2B). This contrasts to group Ia sPLA₂, which has been shown to be more than 100-fold more active in hydrolyzing PG compared with PC.¹⁸

Group V sPLA₂ Hydrolysis of LDL

The ability of group V sPLA₂ to hydrolyze PC is notable, given that PC is the major phospholipid component of LDL. Thus, it was of interest to determine whether mouse group V sPLA₂-expressing cells have the capacity to hydrolyze LDL. Accordingly, COS-7 cells were treated with AdmGV or control virus, Adnull. Twenty-four hours after adenovirus treatments, cells were incubated for an additional 24 hours with media containing 0.2 mg/mL LDL (0.4 μmol/L), and the extent of LDL hydrolysis was assessed by measuring the FFA content of media. As shown in Figure 3A, a significant amount of FFA was liberated when LDL was added to group V sPLA₂-expressing cells but not control cells. The data indicates that ~85 molecules of FFA were generated per particle of LDL added. Group V sPLA₂ hydrolysis of LDL particles was further analyzed using partially purified enzyme, prepared as described in Methods. At maximal hydrolysis, group V sPLA₂ generated ≈1 nmol FFA per μg LDL (Figure 3B). Assuming 1.4 nmol phospholipid per μg LDL, this represents ~70% hydrolysis by group V sPLA₂.

To assess whether modification by group V sPLA₂ alters LDL particle size, hydrolyzed particles were analyzed by electron microscopy after negative staining. As shown in Figure 4, moderately hydrolyzed LDL particles (~38% phospholipids hydrolyzed) were significantly smaller (mean diameter ±SEM = 25.3 ± 0.25 nm) compared with native LDL (mean diameter ±SEM = 27.7 ± 0.29 nm). This finding is significant, because small dense LDL particles have increased

**Figure 1.** Immunohistochemical detection of group V sPLA₂ in atherosclerotic lesions. Sections from a human aortic plaque were stained with mouse anti-human IgG (A) or mouse anti-human group V sPLA₂ (B). Sections from the aortic sinus region of an atherogenic diet-fed apoE⁻⁻ mouse were stained with rabbit anti-mouse IgG (C) or rabbit anti-mouse group V sPLA₂ (D). Immunoreactivity was visualized using the brown chromogen diaminobenzidine tetrahydrochloride (human) or the red chromogen aminomethyl carbazole (mouse). Sections were counterstained with hematoxylin. Magnification ×100 (human) or ×200 (mouse).

**Figure 2.** Expression of mouse group V sPLA₂ in COS-7 cells by adenoviral vector. Cells and media were collected 48 hours after addition of AdmGV or control virus, Adnull. A, Aliquots of cell lysates (10 μg protein) were separated by SDS-PAGE (15% acrylamide), immunoblotted with rabbit anti-mouse group V sPLA₂, and visualized by chemiluminescence detection. The migration of molecular weight standards and 13.6-kDa group V sPLA₂ is indicated. B, The indicated amount of partially purified mouse group V sPLA₂ was incubated with mixed micelles containing either POPC or POPG. After 4-hour incubations at 37°C, the amount of FFA released was quantified. The results shown are the mean of triplicate determinations (±SD) and are representative of 3 separate experiments.

**Figure 3.** Hydrolysis of LDL by mouse group V sPLA₂. A, Twenty-four hours after treatment with AdmGV or Adnull, COS-7 cells were incubated an additional 24 hours in the absence or presence of 0.2 mg/mL LDL (0.4 μmol/L), and the FFA content of media was determined. Values shown represent the mean (±SD) of 4 to 5 different experiments. The phospholipase activity in the media at the end of the experiments was 500 to 750 U/mL. B, LDL (300 μg) was incubated for 20 hours at 37°C with the indicated amount of partially purified mouse group V sPLA₂, and the amount of FFA liberated in the 600 μL reactions was determined. The data shown are the mean of duplicate determinants and are representative of 3 experiments.

AdmGV-treated cells was quantified using mixed micelles containing either POPC or POPG as substrate. In these assays, phospholipase activity in supernatants collected from control COS-7 cells was <5% of the activity present in supernatants from AdmGV-treated cells. The results from this analysis showed ~2-fold difference in specific activity toward these 2 phospholipid substrates (Figure 2B). This contrasts to group Ia sPLA₂, which has been shown to be more than 100-fold more active in hydrolyzing PG compared with PC.¹⁸
affinity for proteoglycans and may be more effectively retained in the vessel wall subendothelium.26,27

Interestingly, electron microscopic analysis of extensively hydrolyzed LDL particles (68% phospholipids hydrolyzed) showed evidence of particle aggregation (Figure 5A, inset). To analyze the extent of aggregation quantitatively, group V sPLA2-modified LDLs were separated by size exclusion chromatography. Aggregated/fused particles elute in the void volume of a Superose 6 gel filtration column. The analysis of maximally modified LDL showed that a large portion (>50%) of the modified LDL was in an aggregated form. To examine the relationship between phospholipid hydrolysis and LDL aggregation, LDLs hydrolyzed to various extents were separated by gel filtration, and the percent of particles that had undergone spontaneous aggregation (defined as the amount of lipoprotein cholesterol eluting in the void volume relative to the total amount of cholesterol eluting from the column) was determined. Correlation analysis showed a significant relationship (r=0.75, P<0.001) between the degree of particle aggregation and the percent of phospholipids hydrolyzed (Figure 5B). Analysis of group V sPLA2-modified LDLs (48% hydrolyzed, 50% aggregated) by SDS-PAGE showed no evidence of apoB-100 degradation (data not shown).

Macrophage Uptake of Group V sPLA2-Modified LDL

Previous studies have shown that aggregated LDL is a potent inducer of macrophage foam cells.4,5 Thus, it was of interest to determine whether group V sPLA2-modified LDL particles that are susceptible to spontaneous aggregation promote foam cell formation in vitro. LDL was incubated at 37°C in the presence or absence of group V sPLA2. Peritoneal macrophages from C57BL/6 mice were incubated with the group V sPLA2-modified LDL (>70% hydrolyzed) or “mock” hydrolyzed LDL as described in Methods. For comparison, cells were also incubated with aggregated LDL produced by vortexing. After 48-hour incubations, cells were stained with Oil Red O and analyzed by light microscopy. This analysis clearly showed that cells incubated with vortexed LDL or group V sPLA2-modified LDL accumulated more neutral lipid compared with control cells (Figure 6).

Discussion

In humans, circulating levels of group IIa sPLA2 are an independent risk factor for coronary artery disease and a predictor of cardiovascular events.24 The possibility that sPLA2 plays a role in the pathophysiology of atherosclerosis...
(and is not merely a predictor of events) is substantiated by the fact that transgenic C57BL/6 mice expressing human group IIa sPLA₂ have significantly larger aortic lesions compared with non-transgenic littermates. In a recent study using a bone marrow transplantation approach, we determined that macrophage expression of human group IIa sPLA₂ significantly enhances atherosclerotic lesion formation in LDL receptor-deficient mice. This finding points to a local pro-atherogenic effect of sPLA₂ in the microenvironment of a developing lesion. It is now recognized that other related sPLA₂ isozymes in addition to group IIa sPLA₂ may play a role in cardiovascular disease. In this study, we investigated another member of the sPLA₂ family, group V sPLA₂. Our data show that this enzyme is present in human and mouse atherosclerotic lesions in regions of lipid accumulation. The source of group V sPLA₂ in lesions has not been determined. Group V sPLA₂ is expressed by a murine macrophage cell line, and it is possible that lesional macrophage cells produce group V sPLA₂. We cannot rule out the possibility that other cell types within the vessel wall produce and secrete group V sPLA₂. Alternatively, sPLA₂ associated with lipoprotein particles may enter the subendothelium and be deposited in the vessel wall at sites of lipid accumulation. Irrespective of the cellular source of group V sPLA₂, our data indicate that hydrolysis of LDL by group V sPLA₂ promotes spontaneous particle aggregation, a modification that has been associated with atherogenic lipid accumulation in the artery wall. In assays in vitro, we demonstrate that group V sPLA₂ modification of LDL leads to enhanced lipid accumulation in macrophages. Taken together, our data suggest that group V sPLA₂ in the arterial wall may lead to the localized production of aggregated lipoprotein particles, which consequently leads to foam cell formation and enhanced atherosclerosis.

Our analysis of recombinant mouse group V sPLA₂ demonstrates that this enzyme, like human group V sPLA₂, hydrolyzes PC-containing substrates, including human LDL. Additional studies have demonstrated that mouse group V sPLA₂ is similarly potent in hydrolyzing phospholipids on LDL isolated from LDL receptor-deficient mice and very-low-density lipoprotein isolated from apoE-deficient mice (data not shown). Group V sPLA₂ was approximately 2-fold more potent in hydrolyzing PG compared with PC. In contrast, group IIa sPLA₂ has been shown to be more than 100-fold more active in hydrolyzing PG compared with PC and has relatively low enzymatic activity toward LDL. This difference in activity between the 2 enzymes has been attributed to the presence of tryptophan residues in the interfacial binding region of group V sPLA₂ that are absent in group IIa.11

Our data indicate that group V sPLA₂-modified LDL undergoes structural alterations that could lead to atherogenic lipid accumulation in the vessel wall. Moderately hydrolyzed LDL particles were significantly smaller compared with native LDL. Because small dense LDL particles have increased affinity for proteoglycans,16,27 group V sPLA₂ modification could enhance LDL retention in the vessel wall subendothelium. We also show that hydrolysis of 15% to 20% of the phospholipids on the LDL particle is sufficient to induce particle aggregation, and the extent of aggregation is proportional to the degree of LDL hydrolysis. We are not aware of any other published report showing that hydrolysis by a mammalian sPLA₂ leads directly to LDL aggregation. Although human group IIa sPLA₂ has been implicated in LDL aggregation/fusion, this activity appears to require LDL binding to proteoglycans, which may itself promote LDL aggregation.32 In a recent study, human group X sPLA₂ was shown to have the capacity to hydrolyze virtually all PC molecules on LDL.33 Interestingly, extensive modification by group X sPLA₂ was not reported to induce LDL aggregation. This may be caused by the fact that LDL hydrolysis was performed in the presence of 0.0125% BSA. In the absence of lipid-binding proteins, such as albumin, LDL hydrolysis by sPLA₂ generates lyso-PC and FFA that accumulate in the LDL particle. In the presence of physiological albumin concentrations, such as in our study, most of the FFA and some of the lyso-PC molecules are transferred from LDL to albumin.33 This can lead to conformational changes in apoB-100 and reorganization of lipids that induce particle aggregation.33

Analysis by electron microscopy indicated that group V sPLA₂-modified LDL does not undergo a substantial amount of particle fusion. It is possible, however, that within the microenvironment of the vessel wall, group V sPLA₂ lipolysis could promote subsequent LDL modification leading to fusion. The extracellular matrix (ECM) may play an important role, both by mediating the retention of LDL particles and by co-localizing group V sPLA₂ and its substrate LDL, as has been suggested for group IIa sPLA₂.32 Group V sPLA₂ exhibits high-affinity binding to proteoglycans that is mediated by a cluster of cationic residues near the C-terminal end.33 Group V sPLA₂ may also act in concert with secretory sphingomyelinase (s-SMase) in the vessel wall to produce pro-atherogenic LDL. Treatment of LDL with SMase in vitro induces aggregation and fusion of particles and enhances binding to human aortic proteoglycans.36,37 s-SMase has been detected by immunocytochemistry in human atherosclerotic lesions in association with the ECM.38 Although native LDL in plasma is not hydrolyzed by s-SMase at neutral pH, group IIa sPLA₂ hydrolysis of LDL can confer susceptibility to this enzyme at neutral pH.39 Interestingly, in vitro data suggest that SMase modification can alter the susceptibility of LDL particles to group V sPLA₂ because progressive depletion of LDL sphingomyelin results in a proportional increase in phospholipid hydrolysis by group V sPLA₂.19 Thus, both group IIa and group V sPLA₂, together with s-SMase and ECM, may synergistically promote atherosclerosis by enhancing LDL retention, LDL aggregation/fusion, and, ultimately, foam cell formation.

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Fig II

M

GV

sPLA$_2$

x 100  x 200