Secretory Phospholipase A\textsubscript{2} Group V 
Lesion Distribution, Activation by Arterial Proteoglycans, 
and Induction in Aorta by a Western Diet

Birgitta Rosengren, Helena Peilot, Mia Umaerus, Ann-Cathrine Jönsson-Rylander, 
Lillemor Mattsson-Hultén, Carina Hallberg, Philippe Cronet, Mariam Rodriguez-Lee, Eva Hurt-Camejo

Objective—To study the distribution of group V secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) in human and mouse lesions and compare its expression by human vascular cells, its activity toward lipoproteins, and the interaction with arterial proteoglycans (proteoglycans) with those of sPLA\textsubscript{2}-IIA. In addition, we also investigated the effect of a Western diet and lipopolysaccharide challenge on the aortic expression of these enzymes in mouse models.

Methods and Results—Immunohistochemistry showed sPLA\textsubscript{2}-V in human and mouse lesions to be associated with smooth muscle cells and also surrounding foam cells in lipid core areas. mRNA of the enzyme was expressed in human lesions and human vascular cells, supporting the immunohistochemistry data. sPLA\textsubscript{2}-V but not sPLA\textsubscript{2}-IIA was active on lipoproteins in human serum. The association with proteoglycans enhanced 2- to 3-fold sPLA\textsubscript{2}-V activity toward low-density lipoproteins but not that of the group IIA enzyme. Experiments in mouse models showed that treatment with a Western diet induced expression of sPLA\textsubscript{2}-V but not that of sPLA\textsubscript{2}-IIA in aorta. On the other hand, lipopolysaccharide-induced acute inflammation augmented the expression of sPLA\textsubscript{2}-IIA but not that of sPLA\textsubscript{2}-V.

Conclusions—These results indicate that these phospholipases could have different roles in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:1579-1585.)

Key Words: phospholipase \textbullet} atherogenesis \textbullet} inflammation \textbullet} lipoprotein-retention \textbullet} proteoglycans

During atherosclerosis development, there is a progressive decrease of the lesion phospholipid content and enrichment in cholesterol.\textsuperscript{1} Furthermore, apolipoprotein B (apoB) lipoproteins isolated from human and rabbit lesions contain less phosphatidyicholine (PC) and more sphingomyelin than circulating lipoproteins.\textsuperscript{2,3} Therefore, lipoproteins trapped in the intima appear to be hydrolyzed by secretory phospholipases.\textsuperscript{4} These enzymes may contribute to atherosclerosis by hydrolysis of low-density lipoprotein (LDL) phospholipids that induce fusion and binding of cholesterol-rich particles to intima proteoglycans, triggering further modifications.\textsuperscript{5-8} In addition, phospholipase(s) A\textsubscript{2} contribute to local release of lyso-phospholipids and nonesterified fatty acids, which have proinflammatory properties in arterial cells.\textsuperscript{9-12}

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Secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) group IIA is present in human atherosclerotic lesions, and experimental and clinical evidence suggest its involvement in atherosclerosis and cardiovascular disease.\textsuperscript{13-17} sPLA\textsubscript{2}-IIA and the more recently cloned sPLA\textsubscript{2}-V are members of a family of enzymes that hydrolyze the fatty acids at the sn-2 position of glycerophospholipids. Both enzymes have low molecular weight (14 kDa), are histidine and calcium dependent, rich in disulfide bonds, are basic, and share structure similarities.\textsuperscript{18} Several of these properties stabilize and enhance their activity in the extracellular milieu. The genes of sPLA\textsubscript{2}-IIA and sPLA\textsubscript{2}-V enzymes are located at close positions in homologous regions in mouse chromosome 4 and human chromosome 1 and share the same promoter.\textsuperscript{19} This region was identified as an atherosclerosis susceptibility locus in the LDL receptor-deficient mouse and is considered a human candidate locus.\textsuperscript{20} The C57BL/6 mouse strain is a natural knockout of sPLA\textsubscript{2}-II because a frame shift mutation in exon 3 blocks gene translation.\textsuperscript{21} Therefore, either sPLA\textsubscript{2}-IIA does not contribute to atherogenesis in this mouse strain, or other(s) sPLA\textsubscript{2} compensates its absence. On the other hand, the human sPLA\textsubscript{2}-IIA transgenic mouse is more susceptible to atherosclerosis than its littermates that only express the group V.\textsuperscript{13,16} This suggests that the expression of both enzymes may contribute to lesion formation. The group V sPLA\textsubscript{2} is also present in human atherosclerotic lesions, but the cell source and regulation of sPLA\textsubscript{2}-V activity in lesions are unknown.\textsuperscript{22} Here, we report on the immunohistochemical localization and cell association of sPLA\textsubscript{2}-V in human and mouse lesions. We
compared the mRNA expression in human lesions and vascular cells, the activities on lipoproteins, and modulation by extracellular arterial proteoglycans of sPLA₂-V and sPLA₂-IIA. In addition, we investigated in mouse models the effect of a Western diet and acute inflammation on the aortic expression of the enzymes.

**Materials and Methods**

The Materials and Methods section is available online at http://atvb.ahajournals.org.

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**Results**

**Immunohistochemical Staining of sPLA₂-V in Human Lesions**

The specificity of all used antibodies was evaluated by Western blotting, and no cross-reactivity of the antibodies against the 2 enzymes was detected (supplemental Figure I, available online at http://atvb.ahajournals.org). The antibodies against sPLA₂-IIA that we used in our previous studies⁵,²³ were also specific and did not cross-react with sPLA₂-V. Figure 1 shows serial sections of 3 human atherosclerotic
lesions. Figure 1A, 1D, 1G, 1J, 1N, and 1O are from an advanced lesion characterized by a thick intima and a well-formed necrotic lipid core. The second column (Figure 1B, 1E, 1H, 1K, 1L, 1P, and 1Q) shows sections from an intermediate type of aortic lesion characterized by a thick neointima with foam cells and a defined media, still with no tissue damage. The third column (Figure 1C, 1F, 1I, 1M, and 1R) shows sections from an advanced type of lesion in coronary artery characterized by a confluent mass of extracellular lipids (lipid core) and a structural altered intima. Actin-positive smooth muscle cells (SMCs) were present in all 3 lesion types (Figure 1A through 1C, arrows). CD68-positive macrophages were present only in the intima and lipid core of the lesions (Figure 1D through 1F, arrows). Immunostained sPLA2-V (Figure 1G through 1I, double arrows; 1N and 1O) was associated with SMCs colocalizing with actin in the media in intermediate lesions and the neointima of more advanced lesions. sPLA2-V was detected only extracellularly surrounding macrophages (MΦ) foam cell–rich areas and cholesterol crystals (Figure 1G through 1J and 1L and 1M, arrows). sPLA2-V was also detected in the endothelium of advanced lesions (Figure 1S and 1T, arrow) colocalizing with the marker for endothelial cells, von Willebrand factor (vWF), in serial consecutive sections (Figure 1U, arrow). vWF-positive endothelial cells in the vasa vasorum also showed positive immunostaining with sPLA2-V (Figure 1V and 1Z, respectively, arrows). sPLA2-V was not present in the adventitia, where we have previously shown sPLA2-IIA to be prominent4 (Figure 1B and 1H; supplemental Figure II). All the respective negative controls were devoid of staining (Figure 1P through 1R and 1W and 1Y).

Immunohistochemical Staining of sPLA2-V in Mouse Lesions

Figure 2 shows serial sections from lesions of 2 individual apolipoprotein E and low-density lipoprotein receptor (apoE/LDLr) double deficient mice (Figure 2A through 2E and 2H). There were similarities in the immunohistochemical distribution of sPLA2-V and that described above for human lesions, with the exception that the enzyme was not detected in mice endothelium (supplemental Figure III). This could have been caused by endothelium disruption during perfusion. Immunostaining showed shared areas positive for sPLA2-V (Figure 2A and 2E, arrows) and apoB (Figure 2B and 2F, arrows) using serial consecutive sections. More extensive colocalization was observed between macrophage-rich areas (Figure 2D and 2H) and sPLA2-V (Figure 2A and 2E) indicated with stars. Colocalization of sPLA2-V (Figure 2E) with actin-positive cells was also observed (Figure 2G, arrowheads).

mRNA Expression of sPLA2-V and sPLA2-IIA in Human Vascular Cells, Human Lesions, and CD14-Positive Macrophages From Carotid Artery Lesions

The levels of sPLA2-IIA mRNA were 1000-fold higher than those of sPLA2-V in all 3 different sources of SMCs in culture, but the relative order of expression for different cells was similar (Figure 3). However, no expression of sPLA2-IIA was detected in MΦ, MΦ-loaded with lipids by incubation with acetylated LDL, or in arterial endothelial cells, whereas low levels of sPLA2-V mRNA were consistently present in these cells. The expression levels for both enzymes in human fibrotic lesion samples and CD14-positive lesion macrophages were different between samples (donors) but always ≥2-fold higher for sPLA2-IIA than sPLA2-V (supplemental Figure IV). Expression levels of the housekeeping gene, 36B4, were similar between the different tissue samples and cultured cells (data not shown).

Activity of Recombinant sPLA2-IIA and sPLA2-V on Human Serum and Lipoproteins

Both enzymes were equally active hydrolyzing phosphoethanolamine micelles and showed substrate specificity (supple-
Interaction of sPLA₂ With SMC Proteoglycans

sPLA₂-IIA resides in the extracellular intima associated to proteoglycans, a situation that facilitates its action on lipoproteins also bound to the sulfated polysaccharides. The immunohistochemical results discussed above indicate that this could also occur with the group V enzyme (Figure 1). Gel mobility shift assay with metabolically labeled chondroitin-6-sulfate proteoglycans synthesized by human aortic SMCs showed that sPLA₂-IIA was bound to proteoglycans with an apparent affinity constant ($k_d$) of 19 nmol/L, whereas sPLA₂-V bound with a lower-affinity $k_d$ of 951 nmol/L (Figure 5A). To study the effect of binding to proteoglycans on the enzymatic activity, LDL was incubated with increasing concentrations of free or proteoglycans-bound sPLA₂-IIA and sPLA₂-V. These experiments indicated that LDL is a better substrate for sPLA₂-V than for sPLA₂-IIA, corroborating results shown in supplemental Figure VI. More important, the data in printed Figure 5B demonstrate that when sPLA₂-V was bound to proteoglycans, the hydrolysis of LDL phospholipids increased significantly. This upregulation of enzymatic activity on LDL phospholipids by binding to proteoglycans was not observed for sPLA₂-IIA under similar conditions (Figure 4B).

Induction of sPLA₂-V and sPLA₂-IIA Expression in Mouse Aorta

After 4 weeks on a Western diet (0.15% cholesterol, 21% cacao fat), sPLA₂-V mRNA and protein were induced significantly in the aortas of C57BL/6 mice compared with animals on chow diet (Figure 5A). Furthermore, the apoE×LDL receptor double-deficient mice, which develop hyperlipidemia and spontaneous atherosclerosis without a Western diet, showed elevated sPLA₂-V protein expression in aorta similar to that of C57BL/6 on the Western diet (Figure 5B). There was no effect of the Western diet treatment on the spontaneous high levels of enzyme expression in the double knockout mice. On the other hand, acute inflammation triggered by an intraperitoneal injection with lipopolysaccharide (5 mg/kg) lipopolysaccharide did not change sPLA₂-V expression in the aorta of untreated mice (data not shown). In contrast, sPLA₂-IIA mRNA and protein were induced significantly in the aortas of C57BL/6 mice treated with lipopolysaccharide (5 mg/kg) intraperitoneal injection (Figure 5B). These results suggested that sPLA₂-IIA expression was induced by acute inflammation as well as by Western diet treatment.

Figure 4. Binding of sPLA₂-IIA and sPLA₂-V to arterial proteoglycans. A, Increasing amounts of sPLA₂-IIA and sPLA₂-V were added to 32P-labeled proteoglycans (2,000 cpm, 0.030 μg) purified from human arterial SMCs. Apparent affinity dissociation constants were determined by 1-site hyperbola binding curves fitted to the data. Added sPLA₂-IIA and sPLA₂-V are indicated in the x axis. B, LDL (20 μg apoB, 1.38 μmol/L of phospholipids) was added to free or proteoglycans-bound sPLA₂-IIA and sPLA₂-V samples (shown in Figure 4A). After 2-hour incubation at 37°C, enzymatic activity was monitored by measuring the free fatty acids content. sPLA₂-IIA proteoglycans-bound (●) and -free (□); sPLA₂-V proteoglycans-bound (●) and -free (□). Values are means of duplicate values and are representative of 3 separate experiments.
Expression of different phospholipase A2 enzymes in arteries may represent redundant activities because of similar properties. However, our results indicate that group II A and V sPLA2 have dissimilar characteristics that could differentially modulate their postulated contribution to atherosclerosis. We found strong immunostaining of sPLA2-V extracellularly surrounding macrophage-like foam cells and in lipid-rich core areas. sPLA2-V was also present in SMCs in the media and neointima of intermediate and advanced lesions. However, only endothelial cells of advanced lesions showed positive immunostaining for sPLA2-V colocalizing with vWF. sPLA2-V was not detected in adventitia (Figure 1; supplemental Figure II). This tissue distribution of sPLA2-V in atherosclerotic lesions differs in some aspects from that described previously for group II A sPLA2 by our laboratory and other laboratories.4,23,25–28 These apparent differences should be confirmed using common serial sections of the same tissue samples immunostained with antibodies specific against each enzyme. Levels of sPLA2-IIA and sPLA2-V mRNA in human lesions and in vitro human cell cultures indicate that cells present in lesions can produce the enzymes (supplemental Figure IV; Figure 3). We have previously shown that SMCs are the main source of sPLA2-IIA in vivo in arteries and in cell cultures.4,29 The present data indicate that nonproliferating human coronary SMCs and aortic SMCs have a higher mRNA expression for sPLA2-IIA and sPLA2-V than those of uterine SMCs, aortic endothelial cells, Mφ, and lipid-loaded Mφ (Figure 3). There are large differences in expression levels of the 2 enzymes in vascular cells and human lesions (Figure 3; supplemental Figure IV) that could be caused by dissimilar transcription levels or differences in mRNA stability. Interestingly and in accord with the immunohistochemistry data, sPLA2-V mRNA but not sPLA2-IIA mRNA was detected in human endothelial cells and Mφ and lipid-loaded Mφ (Figure 3). sPLA2-IIA mRNA was detected in CD14-positive Mφ from human lesions; however, no

C57BL/6 mice but induced significantly the expression of sPLA2-IIA in the aorta of the mice transgenic for this human enzyme. Treatment with the Western diet did not affect the aortic expression of sPLA2-IIA in the transgenic mice, but similar to the C57BL/6 mice, it increased the expression of sPLA2-V (data not shown).

### Discussion

Expression of different phospholipase A2 enzymes in arteries may represent redundant activities because of similar properties. However, our results indicate that group II A and V sPLA2 have dissimilar characteristics that could differentially modulate their postulated contribution to atherosclerosis. We found strong immunostaining of sPLA2-V extracellularly surrounding macrophage-like foam cells and in lipid-rich core areas. sPLA2-V was also present in SMCs in the media and neointima of intermediate and advanced lesions. However, only endothelial cells of advanced lesions showed positive immunostaining for sPLA2-V colocalizing with vWF. sPLA2-V was not detected in adventitia (Figure 1; supplemental Figure II). This tissue distribution of sPLA2-V in atherosclerotic lesions differs in some aspects from that described previously for group II A sPLA2 by our laboratory and other laboratories.4,23,25–28 These apparent differences should be confirmed using common serial sections of the same tissue samples immunostained with antibodies specific against each enzyme. Levels of sPLA2-IIA and sPLA2-V mRNA in human lesions and in vitro human cell cultures indicate that cells present in lesions can produce the enzymes (supplemental Figure IV; Figure 3). We have previously shown that SMCs are the main source of sPLA2-IIA in vivo in arteries and in cell cultures.4,29 The present data indicate that nonproliferating human coronary SMCs and aortic SMCs have a higher mRNA expression for sPLA2-IIA and sPLA2-V than those of uterine SMCs, aortic endothelial cells, Mφ, and lipid-loaded Mφ (Figure 3). There are large differences in expression levels of the 2 enzymes in vascular cells and human lesions (Figure 3; supplemental Figure IV) that could be caused by dissimilar transcription levels or differences in mRNA stability. Interestingly and in accord with the immunohistochemistry data, sPLA2-V mRNA but not sPLA2-IIA mRNA was detected in human endothelial cells and Mφ and lipid-loaded Mφ (Figure 3). sPLA2-IIA mRNA was detected in CD14-positive Mφ from human lesions; however, no

### Figure 5

Induction of sPLA2-V and sPLA2-IIA expression in mouse aorta after Western diet and lipopolysaccharide (LPS) challenge, respectively. A, RT-PCR analysis of sPLA2-V mRNA from aorta of C57BL/6 mice (n = 5) and human sPLA2-IIA transgenic mice (n = 5). B, Immunoblotting of sPLA2-V (100 μg protein per well) and sPLA2-IIA (35 μg protein per well) protein extracted from aorta of C57BL/6, apoExLDLr double knockout mice and human sPLA2-IIA transgenic mice. C indicates controls on chow diet; LPS, tissue collected 48 hours after an intraperitoneal injection with lipopolysaccharide; WD, tissue from animals on Western diet for 4 weeks. *P < 0.01; ***P < 0.001.
mRNA could be detected in THP-1–derived MΦ in vitro, even when loaded with lipids. This discrepancy between ex vivo and in vitro RT-PCR results suggests that sPLA₂-IIA gene transcription may require both differentiation and local exposure of macrophages to specific stimuli.

In the C57BL/6 mouse, no group IIA enzyme is expressed. However, we found sPLA₂-V expression in the plaques of apoE⁻/⁻LDL receptor double knockout of the same strain (Figure 2; supplemental Figure III). The distribution pattern of sPLA₂-V in mouse lesions was similar to that observed in human lesions. In addition, mouse showed shared areas positive for sPLA₂-V and apoB. This could be caused by sPLA₂-V entering the subendothelial space associated with apoB lipoproteins. However, we could not detect any association of the enzyme with lipoproteins after fractionation of serum in deuterium oxide gradients at physiological salt concentrations (supplemental Figure IX).

How sPLA₂-IIA and sPLA₂-V could modify circulating lipoproteins is not clear. We found that sPLA₂-V but not sPLA₂-IIA hydrolyzed lipoprotein phospholipids in the presence of complete serum. When purified plasma lipoproteins were used as substrates, the sPLA₂-V preferentially hydrolyzed the phospholipid in VLDL (Kₘ = 546 μmol/L), followed by those of HDL (Kₘ = 1.3 mmol/L) and LDL (Kₘ = 3 mmol/L; supplemental Figures VI and VII). Differences in sphingomyelin content on the lipoproteins surface may be responsible in part for the dissimilar activities observed. The group IIA sPLA₂ did not hydrolyze phospholipids of any of the native lipoproteins (supplemental Figure VI). SPLA₂-V has tryptophan residues in the interfacial binding region, which are absent in sPLA₂-IIA. This probably enables sPLA₂-V but not type IIA to penetrate and hydrolyze phospholipid monolayers from and lipoproteins in extracellular fluid.

Treatment with sPLA₂-V increased the association of LDL with arterial proteoglycans (supplemental Figure VIII), thus suggesting that it is better suited than group II sPLA₂ for acting on lipoproteins in the extracellular arterial intima. We speculate that modification of apoB-containing lipoproteins by extracellular sPLA₂-V may contribute to 2 atherogenic mechanisms: the increased entrapment of partially modified LDL and the generation of proinflammatory lipid products. This hypothesis is supported by recent data showing that sPLA₂-V–modified LDL induces foam cell formation by a process that involves proteoglycans. However, in spite of VLDL being a better substrate for the type V enzyme, we found no consistent increase of its binding to proteoglycans, probably because of the much lower affinity of proteoglycans for triglyceride-rich particles. The consequences of HDL hydrolysis by sPLA₂-V are unknown and deserve further investigation because a PC-deficient lipoprotein in the intima could be a poor acceptor of excess cell cholesterol. Proteoglycans in the intima may not only contribute to the extracellular accumulation of the 2 enzymes but also to enhancing its activity. sPLA₂-IIA associated to proteoglycans with higher affinity than sPLA₂-V (Figure 5A). These differences are probably caused by the higher content of basic amino acids in sPLA₂-IIA. However, the binding of sPLA₂-V to proteoglycans resulted in a more significant increase in its capacity to hydrolyze LDL phospholipids than for sPLA₂-IIA (Figure 5B). Mutational studies indicate that in human sPLA₂-V, the interfacial-binding surface is separated from its glycosaminoglycan-binding surface, but in sPLA₂-IIA, these areas partially overlap, and the glycosaminoglycan-binding surface is more diffuse. Such differences suggest that in sPLA₂-V, binding to proteoglycans may increase the exposure of the interfacial catalytic domain, thus facilitating the interaction with the substrate. In contrast, the binding of sPLA₂-IIA to proteoglycans may block the interfacial domain and, as a consequence, impair association with the substrate.

Despite group IIA and V sPLA₂ enzymes being structurally similar, we found that a hyperlipidemic high-fat diet upregulates the expression in aorta of group V but not that of the group IIA. On the other hand, an acute inflammatory stimulus increased group IIA but not group V aortic expression (Figure 5). The response of sPLA₂-IIA to inflammatory stimuli is well known. However, we believe that our finding of the effect of dyslipidemia on the type V enzyme is novel. We speculate that this phenomenon may be related to the described low-level inflammation associated with hyperlipidemia.

In conclusion, our results showed clear differences between the 2 enzymes in the expression levels in vascular cells and in their ability to use human lipoproteins in serum as substrates. They also differ in the functional response to associations with arterial proteoglycans. Interestingly, in the mice models used, the enzymes differ in their response to high-fat diet and inflammatory challenge. The described properties of these enzymes suggest that they can affect atherosclerosis by different pathways.

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References


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MATERIALS and METHODS

Human Tissue Samples

Atherosclerotic vascular tissue was collected from patients undergoing carotid endarterectomy and coronary bypass surgery. Arterial tissues were obtained with ethical approval. In this study we used artery samples from 22 different donors. Immunostaining was performed in six atherosclerotic tissue samples; CD14-positive macrophages for mRNA analysis were isolated from nine atherosclerotic carotid arteries, and seven atherosclerotic samples (aortic and coronary combined) were used for RNA extraction.

Animal Experiments

Male apoE-deficient and LDL-receptor-deficient double knockout on a C57Bl/6 background, C57Bl/6J mice (wild type) and female human group IIA sPLA₂ transgenic mice were purchased from M&B A/S, Denmark. Procedures for perfusion, fixation and evaluation of atherosclerosis were carried out as described. The aortas were removed and stored at −80 °C. Total RNA and protein were extracted as described in the online Material and Methods. The local Ethics Review Committee on Animal Experiments, Göteborg Region, Sweden approved the experiments.

Immunohistochemistry

The following primary antibodies were used in serial consecutive sections of human lesions: monoclonal antibody against human sPLA₂-V (Cayman Chemicals, Ann Arbour MI USA) 1:2000 or 1:500; two rabbit anti-human sPLA₂-V antibodies against the synthetic peptides: CNIRTQSYKYRFAYGR (antibody “A”) and CLKRNLSYNQYQY (antibody “B”) diluted 1:300 and 1:50 respectively; monoclonal antibody against α-actin, 1:2000 (Cedarlane Labs, Hornby, Ontario, Canada) to identify smooth muscle cells, and antibody against CD68, clone KIM6 (DakoCytomation, Denmark) 1:100 to identify macrophages and foam cells, and rabbit polyclonal antibody A082 against von willebrand factor 1:30 000.
In serial consecutive sections of mouse lesions: polyclonal antibody against murine sPLA$_2$-V (Cayman Chemicals, Ann Arbour MI USA) 1:1000; polyclonal antibody Mac2, clone M3/38 (Cederland, Canada) 1:14.000; rat monoclonal antibody against alfa-actin, clone asm (Cederland, Canada) 1:2000; rabbit polyclonal against rat apoprotein B (kind gift from Dr. Jan Oscarsson, Wallenberg Laboratory, Göteborg, 3) 1:3000. Secondary biotinylated donkey-anti-mouse, donkey-anti-rat and donkey-anti-rabbit (Jackson IR Labs, West Baltimore Pike, Pennsylvania, USA) were diluted 1:1000. Controls included omission of the primary antibody, IgG isotype controls and pre-absorption with the corresponding peptide sequence. Sections from paraffin-embedded human and mouse arterial samples were dehydrated through decreasing concentrations of alcohol, ending in a phosphate buffered saline solution (PBS). The sections were then either subjected to heat-induced antigen retrieval for 10 min in citrate buffer at 100°C (when staining for macrophages and smooth muscle cells) or trypsin treated (when staining for sPLA$_2$-V). The immunohistochemistry was carried out in a Techmate immunostainer from Daco, following the manufacturer’s suggestions. The primary antibodies were incubated on the sections for 15-16 hours. Endogenous peroxidase activity was blocked with a kit from Dakocytomation for HP-blockage. Sections were incubated for 30 minutes with secondary antibodies followed by HRP for another 30 minutes, washed and color visualized using an AEC chromogen Kit which give a red color precipitation on the sections or DAB chromogen which give a brown color precipitation (Dakocytomation). The sections were counterstained in hematoxylin, mounted in Kaisers gelatin glycerine, and examined by light microscopy. Images of the immunoreactive staining were captured using a Sony RGB video camera and stored in a SQL database utilizing Image Pro analysis (Euromed Networks, Stockholm, Sweden) 4.

**Cell culture**

Penicillin/streptomycin, non-essential amino acids, sodium pyruvate, glutamine, Trypsin-EDTA, fetal bovine serum (FBS), Dulbecco’s phosphate buffered saline (DPBS) with and without calcium and magnesium and culture vessels were from Bio Whithaker Europe, (Verviers, Belgium).
Isolation and purification of human recombinant sPLA\(_2\)-IIA

A pCDNA3 vector (invitrogen) containing the coding sequence for the human sPLA\(_2\)-IIA was transfected into CHO cells. A cloned cell line expressing human sPLA\(_2\)-IIA at a level of 0.6mgs/L culture was selected and expanded to provide 7L of culture media containing active hsPLA\(_2\)-IIa. The growth medium was centrifuged at 1200 x g for 5 min and adjusted to pH 6.7 using 1 M acetic acid and loaded at 20 ml/min to an 80 ml SP sepharose column equilibrated with 10 mmol/L Tris-HCl pH 6.7 (Buffer A). After sample application the column was washed with Buffer A+ 0.3 mol/L NaCl until a stable baseline was obtained. Elution was then carried out using Buffer A + 5% glycerol and a step gradient with 0.8 and 1.2 mol/L NaCl. sPLA\(_2\)-IIA was found in the 0.8 mol/L fraction. This fraction was diluted 10 times using 10 mmol/L Tris-HCl pH 6.7 and loaded onto a HiTrap Heparin sepharose column. Elution was carried out using 10 mmol/L Tris-HCl pH 6.7, 5% glycerol and step gradients of 0.2, 0.4, 0.8 and 1.2 mol/L NaCl. sPLA\(_2\)-IIa was eluted with the 0.8 mol/L NaCl fraction. A final gel filtration on a Superdex 200 26/60 column was made using buffer: 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 5% Glycerol pH 7.1

Isolation and purification of human recombinant sPLA\(_2\)-V

The cDNA of the human sPLA\(_2\)-V was cloned and a fragment coding for the following sequence was cloned into a pET24a vector (Novagen) between the Ndel and BamHI site:

\[\text{GLDLKKMSIKKMTGFYGCGWGRPTKPTDDWCCWAHDCYGRLEKGC NIRTQSYKRFAGVVTCEPSPFCHVNLCADRLYKLVCLKNLRSYNPQYYPNICS.}\]

LB growth medium (with 20 \(\mu\)g/ ml Canamycin) was inoculated with an overnight culture of BL21(DE3) and grown at 37deg to an OD600= 0.8. Expression was induced with 0.4 mmol/L IPTG. Four hours later, the cells were harvested (6000xg, 10 minutes) and frozen at -80°C. The frozen cell pellet was resuspended in 100 ml lysis buffer (50 mmol/L Tris/HCl, pH 8.0, 50 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L PEFAblock SC, 1 % Triton X-100) and homogenized by using an Ultra-Turrax (T-25 basic) and then sonication (58 % power, 4x 20 sec). After a
centrifugation step (8000xg, 60 minutes at 4°C) the pellets were resuspended in lysis buffer (50 mmol/L Tris/HCl, pH 8.0, 50 mmol/L NaCl, 2 mmol/L EDTA, 1 % Triton X-100) without protease inhibitor, Ultra-Turrax, sonicated (58 % power, 2x20 sec) and centrifuged as above. The inclusion bodies were dissolved in 100 ml sulfonation buffer (20 mmol/L Boric acid, pH 8.5, 6 mol/L Guanidine/HCl, 0.3 mol/L Sodium sulphite) and 0.05 volume of Thannhauser reagents was added. The sulfonation was left for 1 h at room temperature and centrifuged (10000xg, 60 minutes) to remove insoluble material. hsPLA₂ type V was precipitated with the addition of 400 ml water and incubated overnight at 4°C. The precipitated material was spun down by centrifugation (10000xg, 60 minutes) and resuspended in 100 ml Guanidine buffer (20 mmol/L Boric acid, pH 8.5, 6 mol/L Guanidine/HCl). The protein was diluted to a final concentration of 0.05 mg/ml with refolding buffer (20 mmol/L Boric acid, pH 8.5, 1 mol/L Guanidine/HCl 1 mmol/L Glutathione red 0.1 mmol/L Glutathione ox) and incubated at 4°C for 2 days. The precipitated material was removed by centrifugation (10000xg, 60 minutes) and the supernatant was dialyzed (MWCO 3.5 kDa) against 10 volumes of dialysis buffer (40 mmol/L Sodium Acetate, pH 4.0) at 4°C for 3 days, with a buffer renewal after 2 days. The dialyzed sample was centrifuged at 10000xg for 60 minutes and the clear solution was applied to a 6.5 ml Source 15 RPC column (HR 10/10) at 5ml/min. After washing to baseline with 0.1 % Trifluoroacetic acid (TFA) in water, the protein was eluted in a gradient of 0-100 % Acetonitrile (AcN) for 44 minutes at 3 ml/min. 3 ml fractions were collected and the hsPLA₂ type V eluates at ≈ 40 % AcN. To each fraction containing hsPLA₂ type V 100 μL of 2 mol/L Tris/HCl, pH 8 and 15 μmol/L 1 M CaCl₂ was added and the AcN was removed by vacuum induced evaporation in a speedvac for 35-40 minutes.

**Enzymatic activity of recombinant purified human sPLA₂-IIA and sPLA₂-V**

The kinetics of enzyme activity for the recombinant human enzymes were analyzed using specific phospholipid substrates as described.[5] The purity of the recombinant enzyme preparations was analyzed by peptide mass fingerprinting and amino acid sequence in a...
Both enzymes showed similar enzymatic activity when using 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine as substrate. SPLA₂-V was better for hydrolyzing L-α-phosphatidylcholine-β-oleoyl-γ-palmitoyl than sPLA₂-IIA (online Figure 5).

**Assay of sPLA₂ activity on human serum and lipoproteins**

VLDL, LDL and HDL were isolated by differential ultracentrifugation from pooled sera from healthy subjects. The isolated lipoproteins were equilibrated in buffer Hepes 10 mmol/L, NaCl 140 mmol/L, CaCl₂ 5 mmol/L, 150 μmol/L albumin and 10 μmol/L BHT and incubated with different concentrations of human recombinant sPLA₂-V and sPLA₂-IIA at 37°C as indicated in each figure. At different time points, the reaction was stopped with 10 mmol/L EDTA. In the experiments for measuring LDL association with PGs the reaction was stopped with 400 μmol/L of the specific inhibitor LY315920 to avoid the use of EDTA that interferes with the LDL-PGs interaction. The enzymatic activity was monitored by measuring the formation of free fatty acids (FFA) (Wako Chemicals GmbH, Neuss Germany). Lipid class analysis of chloroform: methanol extracts of the lipoproteins were done by high performance liquid chromatography (HPLC) using light scattering mass detection as described below.

**Lipid analysis**

The organic phase was evaporated under nitrogen and dissolved in nine volumes Heptane/Tetrahydrofuran 99:1 (v/v) and one volume Aceton/Dichlorometane 2:1 (v/v). Extracted lipids were analyzed on reversed phase HPLC, column temperature 45°C, Column: 4.6X100mm, Waters spherisorb 5μm Silica no. 830112 with a light scattering detector from Polymer Laboratories, PL-ELS 1000, with the following settings: Evaporator: 110, Nebuliser: 90, Transfer Line: 45, Gas flow rate: 1.5.
Binding affinity of sPLA$_2$-IIA and sPLA$_2$-V to arterial proteoglycan.

Human arterial smooth muscle cell-derived $^{35}$SO$_4$-labeled chondroitin-sulfate proteoglycan (PGs) was isolated as described. Affinity binding of sPLA$_2$-V and sPLA$_2$-IIA to PGs was measured by electrophoretic mobility shift assay. The effect of PGs-binding on enzymatic activity towards LDL or L-α-phosphatidylcoline-β-oleoyl-γ-palmitoyl (PC) was evaluated as described by measuring the FFA released after 2h at $37^\circ$C.

RNA preparation from human arterial tissues, mice aorta and cell cultured in vitro

CD-14 positive macrophages were isolated from human carotid atherosclerotic lesions and the RNA extracted as described below. Fresh human and mice tissue samples were put in RNAlater solution and RNA isolation was done using an RNeasy mini kit, according to the manufacturer's instructions. Total RNA from HASMC, HAEC and THP-1 cells was isolated using an RNeasy mini kit following the instructions. RNAlater and RNeasy mini kits were purchased from Qiagen, (Hilden, Germany). RNA quality was evaluated by electrophoresis before further use.

Real Time-PCR quantification of sPLA$_2$-IIA and sPLA$_2$-V

First-strand cDNA was transcribed using 10ng/ul of total RNA and TaqMan Reverse Transcription Reagents. PCR was performed on an Applied Biosystems RealTime PCR 7700 instrument, using TaqMan Universal PCR Mastermix. Each sample was run in triplicate (30ng cDNA/reaction), and related to the housekeeping gene 36B4. Data were analyzed using the comparative C$_T$ method according to the manufacturer's instructions. The following primers and probes were used for human: sPLA$_2$-V, upper: 5´CAGTCCTACAATACAGTTGCAG T 3´, lower: 5´ CACAGAGGTTCCATGGCAGA 3´, probe: 5´CCGGGTCCGAGGTTGCAG A 3´, cPLA$_2$-IV, upper: 5´AGAATAGTGAAGGTTG T´3, lower: 5´CCCCCACCTGACCCAAT AT 3´, probe: 5´GCTACCACAGGCACATCACGTGCA 3´. 36B4, upper:
5´AGTCACTGCAGATGGATGATTAATGGT 3´, lower: 5´CTGCAATACCTGGCTTTTCTC 3´; and Mouse 36B4, upper:
5´GAGGAATCAGATGAGGATATGGGA 3´; lower: 5´AAGCAGGCTGACTTGTTGC 3´; and probe: 5´TCGGTCTCTTCGACTAATCCCGCAA3´.

The following primers and probes were used for mouse: sPLA2-IIA, upper:
5´GGCAAAGGATTCCCCCAA 3´; lower: 5´GGCGCTTGAGCAACAGTCA 3´; probe:
5´CTTGCAGTGTGCTGCAGTCCCAG 3´. SPLA2-V, upper:
5´TCACACTGGCTTGTTCCCTG 3´; lower: 5´CAATCATGGACTTGAGTTCA 3´; probe: 5´CTTGCAGTGTGCCTGCAGTCCCAG 3´. 36B4, upper: 5´GAGGAAATCATGGATATGGGA 3´; lower: 5´AAGCAGGCTGACTTGTTGC 3´; probe: 5´(VIC) TCGGATTCTCATTGGCCCA (TAMRA)-3´.

Binding of sPLA2-modified LDL to arterial proteoglycans

Analysis of interaction between sPLA2-modified LDL and arterial proteoglycan (PGs) isolated from porcine aorta was done by measuring the amount of insoluble complex formed as described 10.

Immunoblotting

Aorta protein extraction was performed as described 11. Immunoblotting of tissue extracts and recombinant enzymes was performed in reducing and non-reducing conditions for detection sPLA2-V and sPLA2-IIA respectively in NOVEX NUPAGE Gels according to the manufacturer’s protocols. Monoclonal antibody against sPLA2-IIA and polyclonal antibody against sPLA2-V were purchased from Cayman Chemicals and diluted 1:2000.

Expression of sPLA2-IIA and sPLA2-V mRNA by cells in vitro

Human uterine, aortic, coronary smooth muscle cells (uSMC, aSMC, cSMC), human arterial endothelial cells (aEC) and the specific culture medium were purchased from
Cambrex (NJ, USA). The monocytic cell line THP-1 (ATCC) was cultured in RPMI 1640 (PAA Laboratories GmbH) supplemented with 10% FBS, 100 Units/ml Penicillin, 100μg/ml streptomycin. RNA isolation method, sequence of primers and probes, and Real Time-PCR quantification protocol are described in the online supplementary information.

**Data analysis.**

Results are expressed as mean ± SD (n = 3 - 5). Each experiment was performed independently at least 3 times in duplicate. Differences between means of paired samples related to control values were evaluated by student's t-test (P-values < 0.05 (*)< 0.01 (**) < 0.001 (***)).

**References**


Online supplemented Figures

**Figure I. Immunospecificity of antibodies.** Western blot analysis of human recombinant sPLA$_2$-IIA (II) and sPLA$_2$-V (V) (50 ng/well) with the antibodies used in human immunohistochemistry. Upper three blots show commercial monoclonal antibodies: a) Upstate Biotechnology Inc. monoclonal antibody against human sPLA$_2$-IIA (used in our previous immunohistochemistry detection of sPLA$_2$-IIA in human lesions $^{12}$; b) Cayman monoclonal antibody against human sPLA$_2$-IIA; c) Cayman monoclonal antibody against human sPLA$_2$-V. Lower two blots show rabbit polyclonal antibodies against human sPLA$_2$-V: d)antibody (A) and e) antibody (B).

**Figure II. Immunohistochemistry of human atherosclerotic lesions.** Sections from a human aortic atherosclerotic lesion showing sPLA$_2$-V positive immunostaining (red color) in the intima (I) and media (M). Absence of positive immunostaining in the adventitia (A). L = Lumen. The frame indicates areas with high magnification shown at the right. Blue color corresponds to hematoxilin staining.

**Figure III. Immunohistochemistry of mouse atherosclerotic lesion.** Positive immunostaining (red color) of sPLA$_2$-V of an atherosclerotic lesion of the brachiocephalic artery in apoE x LDL receptor double knockout mouse. Lumen (L), Intima (I), Media (M) and Adventitia (A) are indicated in the image with 10 X objective magnification.

**Figure IV. RT-PCR measurement of sPLA$_2$-IIA and sPLA$_2$-V mRNA in arterial lesions CD14-positive macrophages and human fibrotic lesions.** Upper two figures: expression levels in CD14-positive macrophages isolated from carotid arteries. (A) sPLA$_2$-IIA and (B) sPLA$_2$-V. Lower two figures: expression levels from five individual human fibrotic lesions obtained from 3 aorta and 2 coronary arteries. (C)sPLA$_2$-IIA and (D) sPLA$_2$-V. Each bar
corresponds to an individual donor. Expression levels were normalized against the housekeeping gene 36B4. Each bar shows the average values of determinations in triplicate.

Figure V. Enzymatic activity of sPLA$_2$-IIA and sPLA$_2$-V recombinant proteins on different phospholipid micelles as substrates. Increasing amounts of recombinant enzyme were incubated with micelles of (A) L-$\alpha$-phosphatidylcholine-$\beta$$\gamma$-palmitoyl (Sigma P3017) or (B) 2-Oleoyl-1-palmitoyl-sn-glycerol-3-phosphoethanolamine (Sigma P5203). 10 mg of substrates were dissolved in 4% Nonidet P40, 2% Deoxycholic acid, in Tris-HCl buffer pH 8.0, containing 12 mmol/L CaCl$_2$ and 0.1 mmol/L EDTA. After 20 minutes incubation at 37°C, the amount of FFA released was measured as described in material and methods. The data show the mean of duplicate values and are representative of 3 experiments.

Figure VI. Enzymatic activity of sPLA$_2$-IIA and sPLA$_2$-V in total human sera and lipoproteins as substrates. A) Pooled sera from three donors were incubated with 500 ng/ml (35 nmol/L) sPLA$_2$-V (●), and sPLA$_2$-IIA (O) and without enzymes (Control) (X) at 37°C. VLDL (●) and LDL (■) (150 µg apoB/ml) and HDL (◆) (150 µg apoA/ml) equilibrated in buffer Hepes 10 mmol/L, NaCl 140 mmol/L, CaCl$_2$ 5 mmol/L, 150 µmol/L albumin and 10 µmol/L BHT were incubated with 14 nmol/L (200ng/ml) of sPLA$_2$-V (B) and sPLA$_2$-IIA (C). After different incubations times, the enzymatic reaction was stopped with 10 mmol/L EDTA and the free fatty acids content was measured. The data shown are the means of duplicate determinants and are representative of 3 separate experiments.

Figure VII. Analysis of sPLA$_2$-V enzyme activity using lipoproteins as substrates. Human recombinant sPLA$_2$-V (35.8 nmol/L or 500ng/ml) was incubated with increasing concentrations of VLDL, LDL and HDL expressed as lipoprotein phospholipid concentrations as indicated. After 10 minutes incubation at 37 °C, the amount of FFA released was measured as described in material and methods.
**Figure VIII.** The effect of hydrolysis of LDL phospholipids by the 2 enzymes on complex formation between the lipoprotein and PGs (A) LDL was incubated with sPLA$_{2}$-IIA and sPLA$_{2}$-V as described in Figure VII. After the indicated times with and without enzymes (controls), LDL (500μg/ml) was incubated with porcine arterial PGs (10 μg/ml) for 1h and the cholesterol content in the complex was measured. The data shown are averages ± SD ($n = 3$) from 3 individual incubations experiments.

**Figure IX.** Lipoprotein profile of total human plasma with and without recombinant sPLA$_{2}$-V enzyme. Samples of 2 ml pooled normal human serum from three donors were run in a preformed gradient of buffers containing 140 mM NaCl, in mixtures of deuterium oxide and water$^{13}$. Profile A shows control serum and Profile B is serum with 1.5μg of human recombinant sPLA$_{2}$-V. The number of fractions collected is indicated in the X axis. Lines show the protein absorption at 280 nm (●) (left axis) and sPLA$_{2}$-V activity (♦) (right axis).
CD-14 Positive Macrophages

Human Fibrotic Atherosclerotic Lesions

Online Figure IV
Online Figure V

A

![Graph A](image)

B

![Graph B](image)
Online Figure VII

**VLDL**

![VLDL plot](image)

- $V_{\text{max}} = 237.2 \mu\text{mol/L}$
- $K_m = 546.2 \mu\text{mol/L}$

**LDL**

![LDL plot](image)

- $V_{\text{max}} = 348.2 \mu\text{mol/L}$
- $K_m = 2974 \mu\text{mol/L}$

**HDL**

![HDL plot](image)

- $V_{\text{max}} = 442 \mu\text{mol/L}$
- $K_m = 1320 \mu\text{mol/L}$
Online Figure VIII

LDL-PG Complex Cholesterol μmol/L

sPLA₂ II

sPLA₂ V

0h 4h 6h 24h 24h+inhib.

0h 4h 6h 24h 24h+inhib.