Lipoprotein-Associated Phospholipase A₂, Platelet-Activating Factor Acethylhydrolase, Is Expressed by Macrophages in Human and Rabbit Atherosclerotic Lesions


Abstract—We studied the expression of lipoprotein-associated phospholipase A₂ (Lp-PLA₂), an enzyme capable of hydrolyzing platelet-activating factor (PAF), PAF-like phospholipids, and polar-modified phosphatidylcholines, in human and rabbit atherosclerotic lesions. Oxidative modification of low-density lipoprotein, which plays an important role in atherogenesis, generates biologically active PAF-like modified phospholipid derivatives with polar fatty acid chains. PAF is known to have a potent proinflammatory activity and is inactivated by its hydrolysis. On the other hand, lysophosphatidylcholine and oxidized fatty acids released from oxidized low-density lipoprotein as a result of Lp-PLA₂ activity are thought to be involved in the progression of atherosclerosis. Using combined in situ hybridization and immunocytochemistry, we detected Lp-PLA₂ mRNA and protein in macrophages in both human and rabbit atherosclerotic lesions. Reverse transcriptase—polymerase chain reaction analysis indicated an increased expression of Lp-PLA₂ mRNA in human atherosclerotic lesions. In addition, δ6-fold higher Lp-PLA₂ activity was detected in atherosclerotic aortas of Watanabe heritable hyperlipidemic rabbits compared with normal aortas from control rabbits. It is concluded that (1) macrophages in both human and rabbit atherosclerotic lesions express Lp-PLA₂, which could cleave any oxidatively modified phosphatidylcholine present in the lesion area, and (2) modulation of Lp-PLA₂ activity could lead to antiatherogenic effects in the vessel wall. (Arterioscler Thromb Vasc Biol. 1999;19:2909-2917.)

Key Words: platelet-activating factor ■ atherogenesis ■ oxidized LDL ■ macrophages ■ real-time fluorescence polymerase chain reaction

Oxidative modification of LDL, monocyte migration into the vessel wall, subsequent macrophage activation, and foam cell formation are key events in the pathogenesis of atherosclerosis. Several studies have demonstrated that one of the earliest events in LDL oxidation is the hydrolysis of oxidatively modified phosphatidylcholines, which generates lysophosphatidylcholine (lyso-PC) and oxidized fatty acids. This hydrolysis of oxidized phosphatidylcholines within LDL is mediated by the lipoprotein-associated phospholipase A₂ (Lp-PLA₂), also known as a platelet-activating factor (PAF) acetylhydrolase, which has been cloned and characterized previously. The arterial wall also contains other types of secreted group II phospholipase A₂, which may play a role in this process. Although lyso-PC itself is a potent biological effecter molecule able to stimulate monocyte and T-lymphocyte chemotaxis, induce adhesion molecules and various growth factors, and impair vascular relaxation, the oxidized fatty acids liberated together with lyso-PC may also possess relevant biological activity. Given its many biological properties, lyso-PC, together with the enzyme responsible for its generation, Lp-PLA₂, has been postulated to play a causal role in inflammation and atherosclerosis. In addition to lyso-PC formation, it has been established that biologically active PAF-like polar phospholipids are formed during the LDL oxidation process. On the other hand, the transient appearance of these PAF-like phospholipids has been postulated to be due to their hydrolysis and subsequent inactivation by Lp-PLA₂. Thus, in the context of atherogenesis, the enzyme Lp-PLA₂ would appear to have a dual role, one that is proinflammatory (generation of lyso-PC) and another that is anti-inflammatory (degradation of PAF-like phospholipids).

To explore further the role of Lp-PLA₂ in atherogenesis, we have investigated whether the enzyme is expressed in human and rabbit atherosclerotic lesions. Previous work has shown that in addition to its being distributed among plasma lipoprotein fractions (predominantly LDL in humans), an important cellular source appears to be mac-

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From A.I. Virtanen Institute (T.H., J.S.L., M.O.H., S.Y.-H.), University of Kuopio, Kuopio, Finland; the Departments of Vascular Biology (C.H.M., K.J.M., L.P.), Gene Expression Sciences (S.Q.R.), and Molecular Recognition (D.G.T.), SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK; and Provincial State Office of Eastern Finland (K.K.), Kuopio, Finland.


Correspondence to Dr Seppo Ylä-Herttuala, MD, PhD, A.I. Virtanen Institute, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland. E-mail Seppo.YlaHerttuala@uku.fi

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The results of the present study show that lesion macrophages express Lp-PLA₂ mRNA and protein and that Lp-PLA₂ enzyme activity is increased in rabbit atherosclerotic lesions.

**Methods**

**Tissue Samples**

Human aortic samples were obtained from 8 medicolegal autopsies (4 males and 4 females, aged 29 to 73 years; cause of death, traffic accidents, suicide, and gun shot wounds) 4 to 12 hours postmortem. Rabbit aortic samples were dissected from aortic arch and thoracic aorta of Watanabe heritable hyperlipidemic (WHHL) rabbits (aged 6 to 12 months) maintained on normal chow and New Zealand White (NZW) rabbits (aged 4 to 6 months) fed a 0.5% cholesterol diet for 8 weeks (Table). Plasma cholesterol levels for the WHHL and NZW rabbits were 20 to 23 and 11 to 17 mmol/L, respectively. The animals were killed under intravenous fentanyl-fluanisone (0.3 mL/kg, Hypnorm, Jansen Pharmaceuticals) and midazolam (1 mg/kg, Dormicum, Hoffman-La Roche) anesthesia. Tissue samples were removed, immersion-fixed for 4 hours in formal sucrose (4% paraformaldehyde and 15% sucrose containing 50 μmol/L BHT and 1 mmol/L EDTA), and rinsed in 15% sucrose/50 μmol/L BHT/1 mmol/L EDTA for 12 hours. Serial paraffin-embedded 7- to 10-μm sections were used for the assays. Atherosclerotic lesions were classified according to Stary et al. into normal areas, type I (initial lesions), type II (fatty streaks), type III (intermediate lesions), type IV (atheroma), and type V (fibroatheroma, calcified and smooth muscle cell–rich plaques) lesions. All human studies were approved by the Ethics Committee of the University of Kuopio, and animal studies were approved by the Experimental Animal Committee of the University of Kuopio.
Whole-length 1.4-kb human Lp-PLA₂ antisense and sense riboprobes were synthesized using T7 and T3 polymerase in the presence of \( ^{33} \text{P} \)UTP (NEN Life Science Products) from a pBluescript II KS plasmid (Stratagene). In situ hybridizations were performed on pretreated tissue sections (1 \( \times \) 10⁶ cpm per section) as described. The final wash was with 0.1 \( \times \) SSC at 53°C for 30 minutes. The slides were dipped in Kodak NTB-2 nuclear track emulsion (Eastman-Kodak) and exposed for 4 weeks. Nonhybridizing sense probes were used as controls. Serial paraffin-embedded sections were used for immunocytochemistry with the following antibodies: mouse monoclonal antibody (mAb) against human macrophages (CD68, dilution 1:150; Dako); mouse mAb against rabbit macrophages (RAM-11, dilution 1:50; Dako), mouse mAb against muscle α- and γ-actin (HHF35, dilution 1:50; Enzo Diagnostics), guinea pig polyclonal antisera against malondialdehyde-modified LDL (MAL-2, dilution 1:1000),

Figure 2. Expression of Lp-PLA₂ in type II human atherosclerotic lesion (fatty streak). In situ hybridization autoradiography and immunostainings of serial sections (panels A to F) are shown. A, In situ hybridization with a \( ^{33} \text{P} \)UTP-labeled antisense riboprobe for Lp-PLA₂ (positive cells showing bright signal). B, Nonhybridizing Lp-PLA₂ sense probe. C, Antibody specific for CD68 (KP1, dilution 1:150). D, Antibody specific for muscle actin (HHF35, dilution 1:50). E, Nonimmune control for the immunostaining. F, Antibody specific for Lp-PLA₂ protein (2C10, dilution 1:50). Positive cells are shown in red. Insert shows a low-magnification view of the section. Hematoxylin counterstain was used. Panels A and B were taken with polarized light epiluminescence. An asterisk identifies the same location of intimal foam cells. Original magnification \( \times 19.9 \) (panels A to E), \( \times 59.7 \) (panel F), and \( \times 9.9 \) (insert in panel F).

**In Situ Hybridization and Immunocytochemistry**

Whole-length 1.4-kb human Lp-PLA₂ antisense and sense riboprobes were synthesized using T7 and T3 polymerase in the presence of \( ^{33} \text{P} \)UTP (NEN Life Science Products) from a pBluescript II KS plasmid (Stratagene). In situ hybridizations were performed on pretreated tissue sections (1 \( \times \) 10⁶ cpm per section) as described. The final wash was with 0.1 \( \times \) SSC at 53°C for 30 minutes. The slides were dipped in Kodak NTB-2 nuclear track emulsion (Eastman-Kodak) and exposed for 4 weeks. Nonhybridizing sense probes were used as controls.

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and highly specific mAbs (2C10 and 3H2, dilution 1:50) against human Lp-PLA₂ purified to homogeneity. The preparation and specificity of these monoclonal antibodies has been described in detail elsewhere. Briefly, no cross-reactivity was noted with 3 different varieties of human recombinant PLA₂: 14-kDa PLA₂, 85-kDa PLA₂, and a recently described related serine-dependent PLA₂. Both 14-kDa PLA₂ and 85-kDa PLA₂ are calcium-dependent arachidonic acid–selective enzymes, whereas the serine-dependent enzyme is calcium independent and has 40% amino acid identity with Lp-PLA₂.

An avidin-biotin-horseradish peroxidase system (Histostain-Plus Kit, Zymed Laboratories) was used for signal detection according to manufacturer’s instructions with either diaminobenzidine or aminoethyl carbazole as color substrates. When immunocytochemistry was
combined with in situ hybridization to facilitate the simultaneous detection of Lp-PLA2 mRNA and protein on the same section, the immunostaining step was performed after the in situ hybridization. Irrelevant class- and species-matched immunoglobulins and immunostaining step was performed after the in situ hybridization

Micrographs were taken by a digital camera (SenSys KAF1400-G2, Photometrics Ltd), processed with digital image–processing software (Image-Pro Plus, Media Cybernetics), and printed using a sublimation printer (Kodak DS 8650, Eastman-Kodak).

**Reverse Transcriptase—Polymerase Chain Reaction**

Human atherosclerotic plaque mRNAs were isolated from pooled human samples consisting of type II lesions, type IV lesions, and type V lesions by use of the Fast Track mRNA isolation kit (Invitrogen) and reverse-transcribed from cDNA, and used for real-time fluorescence RT-PCR analysis as described in Methods. Results from duplicate determinations are expressed in relation to the expression in primary aortic SMCs, whose value was set to 1.

**Analysis of Rabbit Aortic Lp-PLA₂ Activity**

WHHL rabbits with a Half-Lop (H/LOP) background (Foxfield Farms Ltd, Hampshire, UK) were used to investigate Lp-PLA₂ activity in aortic atherosclerotic lesions. Male WHHL rabbits were compared with sex- and age-matched nondiseased control rabbits, which were either H/LOP or NZW rabbits maintained on normal chow. Rabbits were killed with an overdose of anesthetic, and aortas were immediately removed. Aortic samples were washed at 4°C in a phosphate-buffered saline (PBS) solution, cut into 0.5-cm sections at the very beginning of the ascending aorta were removed, frozen in liquid nitrogen, and stored at −70°C until analyzed. To measure aortic PLA₂ activity, each slice of aorta was first homogenized in 1 mL of homogenization buffer by use of a mortar and pestle on ice. The homogenate was then removed to Eppendorf tubes and microfuged for 20 minutes at 4°C. Supernatants (20 μL), which contained all the PLA₂ activity (data not shown), were then assayed using 50 μmol/L PAF as a substrate and were reverse-transcribed from DNase I–treated total RNA by use of Superscript II and random hexamer primers and reverse-transcribed from DNase I (GIBCO BRL)–treated total RNA by use of the Fast Track mRNA isolation kit (Invitrogen) and reverse-transcribed by use of the cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. All other RNAs were isolated by use of Trizol reagent (GIBCO BRL) and were reverse-transcribed from DNase I (GIBCO BRL)–treated total RNA by use of Superscript II and random hexamer primers (GIBCO BRL) according to the manufacturer’s instructions. A series of standards were also prepared by performing a 4-fold serial dilution of total RNA from a 6-day primary culture of human monocyte–derived macrophages in the range 2 to 0.12 ng RNA per reverse transcription (RT) reaction.

cDNA samples (5 μL of each) were analyzed for expression of Lp-PLA₂, and the housekeeping gene GAPDH by a real-time quantitative reverse transcriptase—polymerase chain reaction (RT-PCR) by use of the fluorescent TaqMan 5′ nuclease assay. TaqMan assay oligonucleotide primers and probes were designed using Primer Express software, version 1.0 (PE Biosystems). Each TaqMan hydrolysis probe consisted of the fluorescent reporter dye 6-carboxyfluorescein (FAM), covalently linked to the 5′ end of the oligonucleotide, and the quencher dye 6-carboxytetramethylrhodamine (Tamra), attached to the 3′ end via a linker group (PE Biosystems).

PCRs (5′>3′ nucleic acid) were performed in MicroAmp Optical 96-Well Reaction Plates with Optical Caps (PE Biosystems) by use of the ABI PRISM 7700 Sequence Detection System for thermal cycling and real-time fluorescence measurements (PE Biosystems). Each 25-μL reaction consisted of 1× TaqMan Universal PCR Master Mix (10 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl, 10 mmol/L EDTA, 60 mmol/L passive reference dye 1 [6-carboxy-X-rhodamine], 0.2 mmol/L dATP, 0.2 mmol/L dCTP, 0.2 mmol/L dGTP, 0.4 mmol/L dUTP, 5.5 mmol/L MgCl₂, 8% glycerol, 0.625 U AmpliTaq Gold DNA polymerase, and 0.25 U AmplEase uracil N-glycosylase), 300 mmol/L forward primer, 300 mmol/L reverse primer, 100 mmol/L TaqMan quantification probe, and 5 μL template and was set to 1) to give a fold increase value for each sample. As additional controls, monocytes and lymphocytes were included in the RT-PCR analysis. Monocytes and lymphocytes were isolated from human blood by countercurrent centrifugal elution with a minor modification. Monocytes were obtained at 95% purity; lymphocytes, at 100% purity. The macrophage sample was generated by culturing monocytes for 4 days in RPMI supplemented with 2% fetal bovine serum, 2% human serum, and 2 mM glutamine. The macrophage, macrophage, and lymphocyte samples used in the analysis were from the same donor. Aortic smooth muscle cells were also included in the assay. The cells were primary smooth muscle cells from a human donor that were made quiescent in SmGM-2 medium (Clonetics) over a 2-day period.

**Figure 4.** RT-PCR analysis of Lp-PLA₂ expression in human type II, type IV, and type V lesions in cultured human plasma leukocytes and human arterial smooth muscle cells (SMCs). mRNA was isolated from all samples, transcribed to first-strand cDNA, and used for real-time fluorescence RT-PCR analysis as described in Methods. Results from duplicate determinations are expressed in relation to the expression in primary aortic SMCs, whose value was set to 1.

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averaged for each rabbit, and protein content was determined by a modified Lowry method.36

Results

In situ hybridization analysis with human Lp-PLA2 antisense riboprobe showed that Lp-PLA2 mRNA expression was localized over the macrophage-rich regions in all types of human atherosclerotic lesions (type I to V lesions according to the classification of Stary et al28). Two representative examples of Lp-PLA2 mRNA expression in human type I and II lesions (diffuse intimal thickening and fatty streak, respectively) are seen in Figures 1C and 2A. In the type I lesion, the thickened intima consists mostly of smooth muscle cells (Figure 1B) and connective tissue, among which are scattered solitary macrophages showing a strong signal (Figure 1C). In type II lesion intima there are, among smooth muscle cells (Figure 2D), a streak of macrophage foam cells (Figure 2C), which show a positive hybridization signal (Figure 2A). An example of Lp-PLA2 mRNA expression in an advanced type IV lesion (atheroma) is seen in Figure 3A. This lesion has a macrophage foam cell–rich cap. The micrograph shows the shoulder area of the lesion with scattered macrophage foam cells expressing Lp-PLA2. No hybridization signal was seen with the corresponding sense probe (Figure 2B and 3B).

Immunostaining of serial sections with monoclonal antibody 2C10 against Lp-PLA2 protein showed positive staining in the same areas where Lp-PLA2 mRNA was detected (Figures 1D, 2F, and 3E and the Table). By use of a combined in situ hybridization and immunocytochemical analysis, macrophages (CD68 mAbs) were clearly identified as the source of Lp-PLA2 mRNA (Figure 1C). Immunostainings with an antibody for oxidized LDL (MAL-2) showed a positive signal in the same areas that were positive for the antibody 2C10 (results not shown). Medial smooth muscle cells showed no detectable hybridization signal or immunostaining for Lp-PLA2.

RT-PCR analysis from human type II, type IV, and type V lesions confirmed the induced expression of Lp-PLA2 mRNA in atherosclerotic lesions (Figure 4). It is also clear from the RT-PCR analysis that macrophages and lymphocytes are the major source of Lp-PLA2 expression, in view of the fact that only a very low level of expression was found in human aortic smooth muscle cells (Figure 4).

Positive in situ hybridization (data not shown) and immunocytochemistry for Lp-PLA2 were detected in WHHL rabbit and NZW rabbit atherosclerotic lesions. Examples of advanced macrophage-rich atherosclerotic plaques from rabbit aorta are seen in Figure 5. Immunostainings for Lp-PLA2 protein with monoclonal antibody 3H2 (Figure 5, panels B and F) colocalized with macrophages (Figure 5, panels A and E). As with the human lesions, no positive Lp-PLA2 signal

Figure 5. Lp-PLA2 protein in rabbit atherosclerotic lesion. Serial sections of a WHHL (panels A to D) and NZW (panels E to H) rabbit atherosclerotic lesion. A and E, Antibody specific for rabbit macrophages (RAM11, dilution 1:50). B and F, Antibody specific for Lp-PLA2 protein (3H2, dilution 1:50). Positive cells are shown in red. C and G, Antibody specific for muscle actin (HHF35, dilution 1:50). D and H, Nonimmune controls for the immunostainings. An asterisk indicates the same intimal smooth muscle cell area in panels E to H. Original magnification ×19.9.
was detected in medial smooth muscle areas (Figure 5, panels C and G).

Compared with aortas from age- and sex-matched control rabbits, extracts from diseased aortas of the WHHL rabbits were shown to contain increased PLA2 activity (Figure 6). The identity of the increased PLA2 activity was confirmed as Lp-PLA2, in view of the fact that all of the elevated PLA2 activity could be inhibited by preincubation of the extract with Lp-PLA2-specific inhibitor SB-222657. From these findings, it was also demonstrated that whereas in control rabbits ≈60% of the aortic PAF-hydrolyzing activity could be attributed to Lp-PLA2, this proportion was increased to 90% in aortas from diseased rabbits (Figure 6). This actually represents a 6-fold increase in Lp-PLA2 activity in atherosclerotic lesions from WHHL rabbits compared with aortas from the control rabbits.

Discussion

Lp-PLA2 is found to be predominantly associated with LDL in human plasma,25,37 and it is also known that macrophages secrete Lp-PLA2 activity in cell culture.20,26 How much macrophages contribute to blood levels of Lp-PLA2 is presently unknown. More recently, it has been speculated that the level of Lp-PLA2 in the blood is completely dependent on the rate of lipoprotein clearance.38 In this model, blood Lp-PLA2 levels will be lower when the rate of lipoprotein, particularly LDL, removal is high, and the opposite is true when the clearance rate of lipoproteins is low. Consistent with this notion is the observation that small dense LDL, a lipoprotein pool that is slowly metabolized and very atherogenic,39 is actually enriched with Lp-PLA2.40

Lp-PLA2 would appear to play an important role in inflammatory reactions. On one hand, this enzyme is capable of hydrolyzing and inactivating PAF and related oxidized or polar phospholipids, whereas on the other hand, it has the capacity for generating large quantities of 2 proinflammatory lipid mediators, lyso-PC and free oxidized fatty acids, after the hydrolysis of oxidized phosphatidylycerolines. Which of these activities predominate in atherogenesis remains unknown.21,23 In the present study, we show that Lp-PLA2 is expressed in lesion macrophages and that Lp-PLA2 enzyme activity is 6-fold higher in WHHL rabbit atherosclerotic arteries than in control rabbit arteries. Thus, the expression and enzyme activity of Lp-PLA2 are increased in atherogenesis, which is characterized by a microenvironment of high oxidative stress and the presence of oxidized LDL.41 In situ hybridization and RT-PCR were used to confirm arterial expression of Lp-PLA2, which cannot be distinguished from LDL or plasma-derived Lp-PLA2 on the basis of immunocytochemistry or enzyme activity analyses. Also, simultaneous in situ hybridization and cell typing by immunocytochemistry were used to confirm that macrophages are the source of the enzyme in atherosclerotic lesions.

Oxidized LDL plays an important role in the pathogenesis of atherosclerosis.2 Oxidized LDL is present in atherosclerotic lesions in vivo,41 and at least part of the proinflammatory effects of oxidized LDL are mediated by lyso-PC.3–7 Indeed, several studies have indicated that elevated levels of lyso-PC are found in atherosclerotic lesions.42,43 Lp-PLA2 may be a key enzyme responsible for the increased formation

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Lp-PLA2 activity is elevated in rabbit atherosclerotic lesions. Upper aortic segments from sex- and age-matched WHHL (n=6), normal H/LOP (n=4), and normal NZW (n=4) rabbits were homogenized and assayed for PLA2 activity, in the absence or presence of 300 nmol/L SB-222657, which is a selective Lp-PLA2 inhibitor as outlined in Methods. PAF-AH indicates PAF acetylhydrolase. Data represent the mean±SD.

### Human and Rabbit Atherosclerotic Samples Used for In Situ Hybridization (ISH) and Immunocytochemistry (ICC) Studies

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NA indicates not analyzed; −, absent; +, moderate; ++, strong Lp-PLA2 mRNA expression and positive immunocytochemistry signal with antibodies 2C10 (human samples) or 3H2 (rabbit samples). Lesions were classified according to Stary et al.28
of lyso-PC in atherosclerotic lesions, in view of the fact that oxidative modification of LDL generates substrates for the enzyme. Thus, expression of Lp-PLA2 in activated macrophages will probably lead to the release in atherosclerotic lesions of lyso-PC and free oxidatively modified fatty acids in potentially large quantities. Several biological activities have been assigned to increased lyso-PC content, such as chemotactic activity for human monocytes,3 endothelial dysfunction,18,34 induction of the expression of endothelial leukocyte adhesion molecules,7 and increased expression of platelet-derived growth factor and heparin-binding epidermal growth factor–like proteins.17 Thus, although preventing the proposed biological activities of PAF-like substances, Lp-PLA2 could augment the atherosclerotic process by releasing into the microenvironment increased concentrations of lyso-PC and oxidatively modified free fatty acids from oxidized LDL. It has been shown previously that Lp-PLA2 is able to inhibit LDL oxidation in vitro.8 On the other hand, others have not been able to confirm these observations.6,9,32,44 Whether Lp-PLA2 activity is primarily proatherogenic or antiatherogenic remains to be elucidated. The final test will come from evaluating potent and selective inhibitors of the enzyme in animal models of atherosclerosis. It appears that Lp-PLA2 expression is clearly derived from monocyte/macrophages and lymphocytes, whereas group II secretory phospholipase A2 is highly expressed in smooth muscle cells in both normal and atherosclerotic arteries.13 Also, group II phospholipase A2 cleaves normal unmodified LDL phospholipids, whereas Lp-PLA2 requires oxidation to generate a substrate.32 Thus, Lp-PLA2 is closely associated to the inflammatory aspects of atherogenesis and oxidation of LDL. Even though Lp-PLA2 can be anti-inflammatory under certain conditions, such as in a rat foot pad model after exogenous PAF application,20 lyso-PC and free oxidized fatty acids in atherosclerotic lesions can substantially amplify the pathological process and cause chronic monocyte/macrophage-dominated inflammation, which is typical of atherosclerosis, in view of the fact that the arterial wall contains much higher concentrations of LDL than most other physiological compartments.45,46 Increased expression of Lp-PLA2 in lesion macrophages suggests that modulation of the enzyme activity could become a potential target for the development of antiatherogenic therapy in the vessel wall.

Acknowledgments

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Lipoprotein-Associated Phospholipase A₂, Platelet-Activating Factor Acetylhydrolase, Is Expressed by Macrophages in Human and Rabbit Atherosclerotic Lesions
Tomi Häkkinen, Jukka S. Luoma, Mikko O. Hiltunen, Colin H. Macphee, Kevin J. Milliner, Lisa Patel, Simon Q. Rice, David G. Tew, Kari Karkola and Seppo Ylä-Herttuala

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