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Adenovirus-mediated gene transfer of Lp-PLA_2 reduces LDL degradation and foam cell formation in vitro

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Abstract Oxidation of LDL generates biologically active platelet-activating factor (PAF)-like phospholipid derivatives, which have potent proinflammatory activity. These products are inactivated by lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme capable of hydrolyzing PAF-like phospholipids. In this study, we generated an adenovirus (Ad) encoding human Lp-PLA_2 and injected 10^8, 10^9, and 10^{10} plaque-forming unit doses of Adlp-PLA_2 and control AdlacZ intra-arterially into rabbits to achieve overexpression of Lp-PLA_2 in liver and in vivo production of Lp-PLA_2-enriched LDL. As a result, LDL particles with 3-fold increased Lp-PLA_2 activity were produced with the highest virus dose. Increased Lp-PLA_2 activity in LDL particles decreased the degradation rate in RAW 264 macrophages after standard in vitro oxidation to 60–80% compared with LDL isolated from LacZ-transduced control rabbits. The decrease was proportional to the virus dose and Lp-PLA_2 activity. Lipid accumulation and foam cell formation in RAW 264 macrophages were also decreased when incubated with oxidized LDL containing the highest Lp-PLA_2 activity. Inhibition of the Lp-PLA_2 activity in the LDL particles led to an increase in lipid accumulation and foam cell formation. It is concluded that increased Lp-PLA_2 activity in LDL attenuates foam cell formation and decreases LDL oxidation and subsequent degradation in macrophages.—Turunen, P., J. Jalkanen, T. Heikura, H. Puhakka, J. Karppi, K. Nyyssönen, and S. Ylä-Herttuala. **Adenovirus-mediated gene transfer of Lp-PLA_2 reduces LDL degradation and foam cell formation in vitro.** ***J. Lipid Res.*** **2004.** 45: **1633–1639.**

Supplementary key words lipoprotein-associated phospholipase A_2 • low density lipoprotein • macrophage

Oxidized low density lipoprotein (oxLDL) plays an important role in atherosclerosis. Uptake of oxLDL contributes to the formation of foam cells by arterial macrophages. OxLDL also plays other roles in atherogenesis, such as being cytotoxic and stimulating the migration of monocytes into the arterial wall (1). Oxidation of LDL is a free radical-mediated, autocatalytic process. Polyunsaturated fatty acids, which are present in LDL lipids, are main targets of the reactive forms of oxygen. Initial products of phospholipid oxidation are usually hydroperoxy derivatives, which give rise to a variety of aldehyde products (2). This fragmentation also leads to the formation of polar phospholipids containing short-chain acyl groups at the *sn*-2 position (3). These molecules serve as substrates for lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as platelet-activating factor-acetylhydrolase (PAF-AH), which hydrolyzes them to lysophospholipids (4). Some of the biologic effects of oxLDL can be mimicked by phospholipids that are subjected to oxidation in vitro. The ability of oxidized phospholipids to induce monocyte binding can be completely abolished by Lp-PLA_2 (5).

Atherosclerosis has features of chronic inflammation, and oxLDL has been implicated as a factor involved in arterial wall inflammation (6). During LDL oxidation, biologically active PAF-like phospholipid derivatives are produced that may contribute to the proinflammatory properties of oxLDL (7). PAF levels are found to be higher in coronary arteries from patients with severe atherosclerosis, and antibodies to PAF are associated with borderline hypertension, early atherosclerosis, and metabolic syndrome (8–10). Because it is difficult to increase Lp-PLA_2 activity in LDL particles in vitro without the risk of causing alterations in lipoprotein structure and physiological properties, we generated adenoviruses encoding Lp-PLA_2 cDNA and injected these viruses intra-arterially into rabbits to achieve overexpression of Lp-PLA_2 in liver and in vivo production of LDL particles with increased Lp-PLA_2 activity to study the effects of Lp-PLA_2 on LDL

Abbreviations: Ad, adenovirus; ALAT, alanine aminotransferase; CRP, C-reactive protein; IL-6, interleukin-6; LPDS, lipoprotein-deficient serum; Lp-PLA_2, lipoprotein-associated phospholipase A_2; lyso-PC, lysophosphatidylcholine; oxLDL, oxidized low density lipoprotein; PAF-AH, platelet-activating factor-acetylhydrolase; pfu, plaque-forming units; SMC, smooth muscle cell; TBARS, thiobarbituric acid-reactive substance.

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degradation and foam cell formation in vitro. It was found that Lp-PLA_2 gene transfer led to an increased enzyme activity in isolated LDL particles, with potentially antiatherogenic effects on LDL oxidation, subsequent degradation, and decreased foam cell formation in RAW 264 macrophages in vitro.

METHODS

Materials

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO). Cell culture reagents were from Gibco BRL (Paisley, UK) unless otherwise stated. Cell lines were from ATCC (Manassas, VA), and chamber slides were from LabTek Brand, Nunc International (Roskilde, Denmark). ^{125}I was from Wallac Finland Oy (Helsinki, Finland). The PAF-AH assay kit was from Cayman Chemical (Ann Arbor, MI), and the Lp-PLA_2 inhibitor Pefabloc (4-[2-aminoethyl]benzosulfonylfluoride) was purchased from Roche Diagnostics (Mannheim, Germany).

Generation of adenoviruses

The Lp-PLA_2 adenoviruses were constructed and produced with the Adeno-X™ Expression System (Clontech, Palo Alto, CA), which is based on serotype 5 adenovirus. Human Lp-PLA_2 cDNA (11) was cloned into Adeno-X™ Viral DNA with cytomegalovirus immediate early promoter and bovine growth hormone poly(A). The recombinant Adeno-X™ DNA was packaged into adenoviruses by transfecting HEK 293 cells using Fugene 6 reagent (Boehringer Mannheim, Mannheim, Germany). Replication-deficient adenoviruses were produced in HEK 293 cells and purified by CsCl gradient centrifugation. The identity of the viruses was confirmed from viral DNA by PCR using human Lp-PLA_2-specific primers. The production of LacZ control adenoviruses has been previously described (12). Purified virus preparations were analyzed for the absence of toxicity, wild-type viruses, microbiological contaminants, and lipopolysaccharide as described (12).

Gene transfer in vitro

RAW 264 cells (ATCC) and rabbit aortic smooth muscle cells (SMCs) (13) were transduced with adenoviruses at multiplicities of infection of 100, 1,000, and 5,000. The medium was changed to Optimem (Gibco BRL) containing 0.5% lipoprotein-deficient serum (LPDS) and 1% penicillin streptomycin. The cells were grown for 48 h, and medium was collected and lyophilized for enzyme activity and immunoblot analyses. Total RNA was isolated for RT-PCR analysis using Trizol reagent (Gibco BRL).

Immunoblot analysis

Lyophilized medium of the adenovirus Lp-PLA_2 (Adlp-PLA_2)-transduced RAW 264 cells and SMCs was subjected to 12% SDS-PAGE. Samples were mixed with loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) and incubated for 4 min at 95°C before application to the gel. The resolved proteins were blotted on Immobilon polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Lp-PLA_2 was detected with human PAF-AH polyclonal antiserum (dilution, 1:1,000) according to the manufacturer's instructions (Cayman Chemical). Human plasma PAF-AH was used as a positive control (Cayman Chemical).

Animal work

LDL was isolated for in vitro studies from 12 New Zealand White rabbits after the gene transfer. Fentanyl-fluanisone (0.3 ml/kg sc; Janssen Pharmaceutica, Beerse, Belgium) and midazolam (1.5 mg/kg im; Roche, Basel, Switzerland) were used for anesthesia. The gene transfer was done via common carotid artery with 5F introducer (Cordis Corp., Miami Lakes, FL) after 2 weeks of a 0.5% cholesterol diet. The doses of adenovirus (Adlp-PLA_2 and AdlacZ) were 10^8, 10^9, and 10^{10} plaque-forming units (pfu). As an additional control, a subgroup of rabbits ($n = 3$) were injected with physiological saline only. Serum (40–60 ml/rabbit) was collected for LDL isolation and enzyme activity measurements. Livers were removed for histological analyses. The animal protocol was approved by the Experimental Animal Committee of the University of Kuopio, Finland.

Isolation and modification of LDL

LDL was isolated from fasting serum of the transduced rabbits by ultracentrifugation (14) and radioiodinated with ^{125}I using Iodogen (Pierce Chemical Co., Rockford, IL) as an oxidizing agent (15) before standardized 18 h incubation with Cu^{2+} (20 μM) (14). Specific activities of the labeled LDLs were 250–500 cpm/ng protein. In one group, Lp-PLA_2 activity was irreversibly inhibited by 0.1 mM Pefabloc (16) at 37°C for 30 min before LDL iodination and oxidation. Dialysis steps were performed overnight in 0.9% NaCl and 0.01% EDTA, pH 7.4, at 4°C. After incubation, the medium was analyzed using agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis Kit; Beckman, Namur, Belgium).

Analysis of blood samples

Serum and LDL Lp-PLA_2 activities were determined by using a commercially available assay kit according to the manufacturer's instructions (Cayman Chemical). The assay uses 2-thio-PAF, which serves as a substrate for Lp-PLA_2. Upon hydrolysis of the acetyl thioester bond by Lp-PLA_2, free thiols are detected using 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent). The absorbance is read at 414 nm over a period of time using an ELISA plate reader. Absorbance values were plotted as a function of time, and Lp-PLA_2 activity was calculated from the linear portion of the curve and expressed as nanomoles per milliliter per minute. The lipid peroxide content of LDL (150 μg) was estimated by measuring the thiobarbituric acid-reactive substance (TBARS) produced in terms of malondialdehyde (14). Conjugated diene formation was measured from plasma samples as described previously (17) and expressed as millimoles per mole of cholesterol. Blood plasma values of total cholesterol, alanine aminotransferase (ALAT), and C-reactive protein (CRP) were measured on day 7 using routine clinical chemistry assays at the Kuopio University Hospital Laboratory.

RT-PCR

For assessment of Lp-PLA_2 mRNA expression, RT-PCR was performed from liver tissue 7 days after the gene transfer and also from RAW 264 cells and SMCs at 48 h after virus transduction. Total RNA was isolated from the liver samples and from cell cultures after homogenization in Trizol reagent and treated with RQ1 RNase-free DNase (Promega, Madison, WI). Four micrograms of total RNA was reverse-transcribed using random hexamer primers (Promega) and M-MULV Reverse Transcriptase (New England Biolabs, Beverly, MA). cDNA was amplified by PCR using DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, Finland) and primers specific for human Lp-PLA_2 sequence as follows: forward, 5′-TGGAGCAACGGTTATTCAG-3′; reverse, 5′-TGGTTGTGTTAATGTTGGTCC-3′. The reaction was subjected

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to 45 cycles of denaturing at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. Extension in the final cycle was 7 min.

Histological analysis

Immunohistochemical staining for paraffin-embedded liver sections was performed for detection of Lp-PLA_2 protein expression after gene transfer. As a primary antibody, anti-human PAF-AH polyclonal antiserum was used (dilution, 1:500). Control immunostainings were conducted with sections from AdlacZ-transduced rabbits and also with Lp-PLA_2 sections without the primary antibody. Hematoxylin was used as a counter stain. The horseradish peroxidase system 3,3′-diaminobenzidine tetrahydrochloride-plus kit (DAB-Plus Substrate Kit; Zymed Laboratories, South San Francisco, CA) was used for signal detection according to the manufacturer's instructions.

Degradation assays and interleukin-6 measurements

Degradation assays were performed in RAW 264 cells in Optimem containing 10% LPDS with 10 μg/ml of the isolated, labeled, and oxLDL fractions (14). The medium was collected at different time points (3, 6, 12, and 24 h), and the amount of ^{125}I-labeled acid-soluble material in the medium (degradation) was determined. Values obtained from empty wells were subtracted before calculating the results. Protein concentrations from the RAW 264 cells and the LDL fractions were determined by the method of Lowry et al. (18). Interleukin-6 (IL-6) concentrations in the culture supernatants were determined with an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Foam cell formation

RAW 264 cells were plated on chamber slides and incubated for 18 h with Optimem and 10% LPDS containing 100 μg/ml of the LDL fractions isolated from rabbits given different adenovirus doses (19). One Lp-PLA_2 group was treated with the Lp-PLA_2 inhibitor Pefabloc. After incubation, the cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with Oil Red O in 60% isopropanol, 0.4% dextrin, and hematoxylin. Foam cell formation was evaluated by light microscopy.

Statistics

All data are expressed as means ± SD. ANOVA followed by unpaired Student's *t*-test was used to evaluate statistical significances. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Transgene expression and the functionality of the Adlp-PLA_2 were verified at mRNA, protein, and enzyme activity levels in vitro and in vivo. Rabbit aortic SMCs and RAW 264 cells were transduced with Adlp-PLA_2. The total RNA was collected after 48 h for RT-PCR. The presence of Lp-PLA_2 transcript was detected in Adlp-PLA_2-transduced cells by using primers specific for human Lp-PLA_2 (**Fig. 1A**). Western blot analysis of lyophilized medium of the same cells showed in the Adlp-PLA_2-transduced cell supernatants but not in the untransduced control cell supernatants the presence of an ~65 kDa protein (Fig. 1B), which corresponds to the molecular mass identified for the glycosylated form of serum Lp-PLA_2 (11). The maximal Lp-PLA_2 enzyme activity in the lyophilized medium showed over 10-fold increased activity in SMCs and RAW 264 cells compared with the activity in the untransduced controls (Fig. 1C). In vivo, Adlp-PLA_2-transduced rabbit livers showed human Lp-PLA_2 mRNA expression in RT-PCR analysis 7 days after the gene transfer, but no signal was present in AdlacZ-transduced control livers (**Fig. 2A**). Immunostaining with anti-human PAF-AH antiserum showed protein expression in liver sections from Adlp-PLA_2-trans-

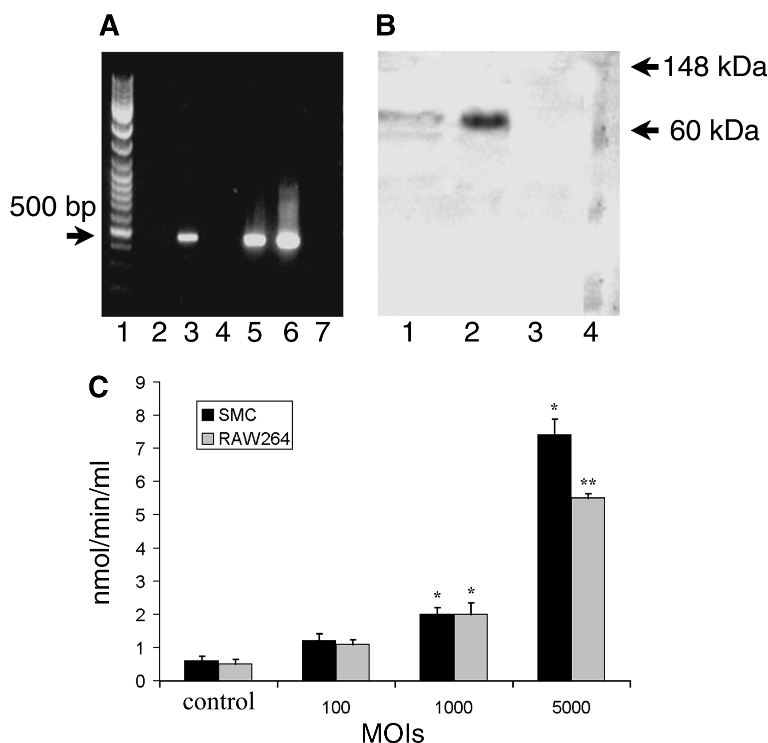

Fig. 1. Expression of human lipoprotein-associated phospholipase A_2 (Lp-PLA_2) after adenoviral gene transfer. A: Lp-PLA_2 mRNA was detected by RT-PCR from smooth muscle cell (SMC) and RAW 264 cell cultures 48 h after transduction with adenovirus Lp-PLA_2 (Adlp-PLA_2). Lane 1, ladder; lane 2, untransduced RAW 264 cells; lane 3, transduced RAW 264 cells; lane 4, untransduced SMCs; lane 5, transduced SMCs; lane 6, positive control (Lp-PLA_2 Adeno-X™ plasmid); lane 7, negative control. B: Western blot analysis of SMCs transduced with Adlp-PLA_2. Media from cells were collected after 48 h of incubation and lyophilized, and 20 ng of protein was electrophoresed on SDS-PAGE. Lane 1, medium from transduced SMCs; lane 2, positive control [purified human platelet-activating factor-acetylhydrolase (PAF-AH)]; lane 3, medium from untransduced SMCs; lane 4, molecular mass marker. Lp-PLA_2 was detected with human polyclonal PAF-AH antiserum as described in Methods. C: Lp-PLA_2 enzyme activities measured from media collected from SMCs and RAW 264 cells transduced with different multiplicities of infection (MOIs) of Lp-PLA_2. Media from untransduced cells were used as a control. All values are means ± SD of three determinations. ANOVA was followed by Student's *t*-test (* $P < 0.05$, ** $P < 0.002$).

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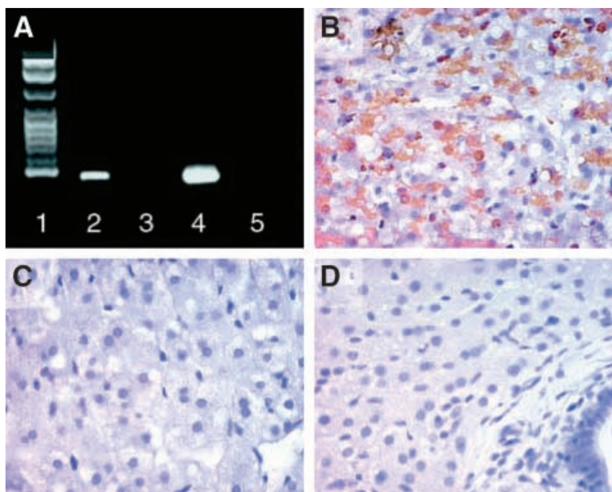

Fig. 2. Expression of human Lp-PLA$_2$ alter adenoviral gene transfer in vivo. A: Lp-PLA$_2$ mRNA was detected by RT-PCR from liver tissue 7 days after Lp-PLA$_2$ gene transfer. Lane 1, ladder; lane 2, liver from Adlp-PLA$_2$-transduced rabbits; lane 3, liver from AdlacZ-transduced rabbits; lane 4, positive control (Lp-PLA$_2$ Adeno-X™ plasmid); lane 5, negative control. B: Immunostaining with human polyclonal PAF-AH antiserum (dilution, 1:500) shows positive staining 7 days after gene transfer in liver sections from Adlp-PLA$_2$-transduced [10^{10} plaque-forming units (pfu)] rabbits. C: No immunostaining is seen in liver in LacZ-transduced rabbits. D: Nonimmune control for the immunostainings. Magnification, ×40.

duced rabbits but not in the control LacZ rabbits (Fig. 2, B–D). The gene transfer also led to increased Lp-PLA$_2$ activity in serum: at an adenovirus dose of 10^8 pfu (n = 4), the activities were 49 ± 1.4 nmol/min/ml in the Lp-PLA$_2$ group and 42 ± 2.8 nmol/min/ml in the LacZ group; at a dose of 10^9 pfu (n = 4), the activities were 51 ± 4.9 nmol/min/ml in the Lp-PLA$_2$ group and 38 ± 4.2 nmol/min/ml in the LacZ group; and at a dose 10^{10} pfu (n = 4), the activities were 63 ± 1.4 nmol/min/ml in the Lp-PLA$_2$ group and 38 ± 2.8 nmol/min/ml in the LacZ group. Lp-PLA$_2$ activity was also measured from isolated rabbit LDL, and an aliquot of the LDL from each dose group was treated with Pefabloc. Lp-PLA$_2$ inhibitor treatment irreversibly inhibited the Lp-PLA$_2$ activity in rabbit LDL (**Fig. 3A**).

Blood plasma values (means ± SEM) of total cholesterol, CRP, and ALAT at 7 days after the gene transfer are shown in Fig. 3, B–D. There were no statistically significant differences between the study groups. In addition, TBARS and diene measurements from plasma and isolated LDLs did not show any statistically significant differences between the groups (data not shown). Incubation of native LDL or oxLDL with RAW 264 cells for 18 and 48 h did not lead to any increased IL-6 production by the cells, indicating no major inflammatory activation of RAW 264 macrophages during the experiments (data not shown).

The effect of the increased Lp-PLA$_2$ activity on LDL oxidation and subsequent degradation in macrophages was analyzed using LDLs isolated from rabbits given different doses of Adlp-PLA$_2$. Agarose gel electrophoresis showed a slight decrease in the migration of ^{125}I-LDL isolated from the Adlp-PLA$_2$ group (10^{10} pfu) compared with the migration of control LacZ ^{125}I-LDL and ^{125}I-LDL treated with the Lp-PLA$_2$ inhibitor Pefabloc (**Fig. 4**). Degradation in RAW 264 macrophages of the isolated LDL fractions subjected to standardized oxidation was followed at different time points (3, 6, 12, and 24 h). Increased Lp-PLA$_2$ content in LDL particles decreased the degradation of LDL after oxidation to 60–87% of the control LacZ LDL degradation. The values at different time points were 87% for 3 h, 60% for 6 h, 77% for 12 h, and 74% for 24 h (data not shown). Next, we studied the degradation of LDL isolated from rabbits given different adenovirus doses. Enzyme activities of the isolated LDLs are shown in Fig. 3A. The cells were incubated with medium containing 10 μg/ml LDL for 6 h. As a result, the increased Lp-PLA$_2$ activity in the rabbit LDLs decreased the degradation of LDL after oxidation to 63–87% of the LacZ control LDL values (**Fig. 5**). Inhibition of the Lp-PLA$_2$ activity by Pefabloc led to a 2-fold increase in the degradation compared with the LacZ control LDL. Inhibition of the degradation was most effective with LDL containing the highest level of Lp-PLA$_2$ activity. To test whether Lp-PLA$_2$ could inhibit the uptake of larger quantities of oxLDL, we tested the effect of increased Lp-PLA$_2$ activity on foam cell formation in RAW 264 macrophages, incubating the cells for 18 h with 100 μg/ml LDLs isolated from the transduced rabbits and subjected to standardized oxidation. The lipid accumulation in RAW 264 macrophages and foam cell formation were decreased when incubated with oxLDL containing the highest Lp-PLA$_2$ activity (**Fig. 6**). When Lp-PLA$_2$ was irreversibly inhibited in the LDL particle, it led to an increase in lipid accumulation and foam cell formation compared with that in the Lp-PLA$_2$ and LacZ control LDL groups (Fig. 6).

DISCUSSION

Lp-PLA$_2$ is a member of the phospholipase A$_2$ superfamily, which consists of a large number of enzymes defined by their ability to catalyze the hydrolysis of the *sn*-2 ester bond in phospholipids (20). The biological role of Lp-PLA$_2$ is to hydrolyze PAF and other polar phospholipids with a short, oxidized acyl chain in the *sn*-2 position of glycerol (21). The specificity of the enzyme for this type of acyl group ensures that phospholipid components of cellular membranes and lipoproteins remain intact while products of oxidation and fragmentation are hydrolyzed. Lp-PLA$_2$ is a hydrophobic protein, and in plasma two-thirds of the enzyme activity is associated with LDL, the rest of the activity being associated mainly with HDL (22). On the other hand, less than 1% of the LDL particles contain Lp-PLA$_2$ (23). Thus, even a minor increase in Lp-PLA$_2$ content could have important effects on the properties of LDL. Because it is difficult to get hydrophobic proteins in LDL in a test tube, we wanted to direct overexpression of Lp-PLA$_2$ into the liver, where lipoprotein particles are produced. This was achieved with adenovirus-mediated gene transfer, because adenovirus given via a systemic route is known to lead to strong transgene expression in the liver

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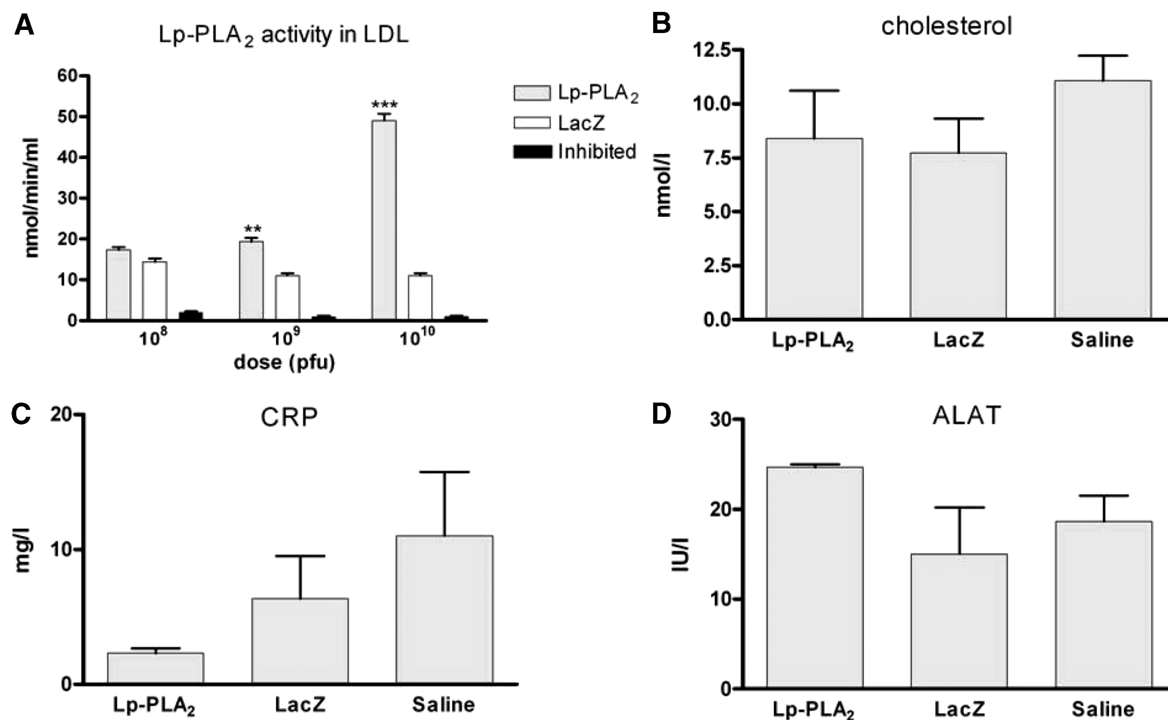

Fig. 3. Lp-PLA$_2$ activity in isolated LDL and analysis of plasma samples of the donor rabbits. A: Lp-PLA$_2$ activity in LDL particles isolated from rabbits treated with 10^8, 10^9, and 10^{10} pfu adenoviruses. An aliquot of LDL at each dose level was treated with Lp-PLA$_2$ inhibitor (0.1 mM Pefabloc for 30 min at 37°C), which irreversibly destroys Lp-PLA$_2$ enzyme activity in the particle. B–D: Total cholesterol (B), CRP (C), and ALAT (D) 7 days after the gene transfer (10^{10} pfu). All values are means ± SD of three determinations. ANOVA was followed by Student's *t*-test (Lp-PLA$_2$ vs. LacZ, ** $P < 0.002$, *** $P < 0.0001$).

(24). The purpose of the study was not to evaluate the effect of adenovirus-mediated Lp-PLA$_2$ gene transfer on atherogenesis in rabbits per se, because gene expression achieved in large animals by adenovirus only lasts for 1–2 weeks (24). Rather, the main goal was to achieve a short-term, effective Lp-PLA$_2$ overexpression in the liver for the production of LDLs with increased Lp-PLA$_2$ activity for in vitro studies. We considered these in vitro studies essential before trying any antiatherosclerotic gene transfer protocols in rabbits that would require the construction of alternative gene transfer vectors (25).

LDL oxidation plays an important role in atherogenesis (1). Oxidation of LDL is a multistep process in which the peroxidation of polyunsaturated fatty acids leads to the accumulation of lipid peroxidation products in the lipoprotein particle (so-called minimally modified LDL), followed by propagation of the lipid peroxidation, modification of apolipoprotein B, and accumulation of lysophosphatidylcholine (lyso-PC) in the LDL (26). It has been demonstrated that PAF-like lipids are also generated during LDL oxidation (3, 5). These lipids mimic PAF and can activate many types of cells via the PAF receptor and cause the stimulation of platelet aggregation, leukocyte activation and adhesion to endothelium, increased vascular permeability, monocyte activation, and production of superoxide anion by macrophages (27). The formation of PAF-like lipids is an important part of the biological activity of minimally modified LDL, whereas their role in fully ox-LDL remains unknown. Extensive oxidation of LDL creates particles that are metabolized in macrophages by scavenger receptors (28), which leads to the accumulation of intracellular cholesterol (1). In this study, LDL degradation and foam cell formation were used as biological indicators of the effects of increased Lp-PLA$_2$ activity. However, it should be kept in mind that both of these methods measure late stages in the sequence of LDL oxidation and require extensive modification of both apolipoprotein B-100 and lipid components of the studied LDL fractions. Nevertheless, we consider these analyzes important surrogate markers of LDL atherogenicity in vivo, because in-

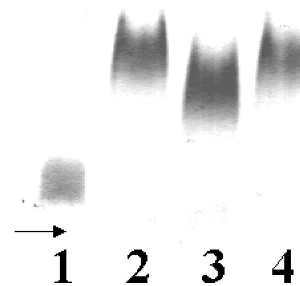

Fig. 4. ^{125}I-LDL isolated from rabbits given 10^{10} pfu of Lp-PLA$_2$ or LacZ adenovirus was incubated overnight with 20 μM Cu^{2+} and agarose gel electrophoresis was performed. Lane 1, native ^{125}I-LDL; lane 2, ^{125}I-oxidized low density lipoprotein (^{125}I-oxLDL; Pefabloc inhibition); lane 3, ^{125}I-oxLDL (Lp-PLA$_2$); lane 4, ^{125}I-oxLDL (LacZ). The arrow indicates the point of application.

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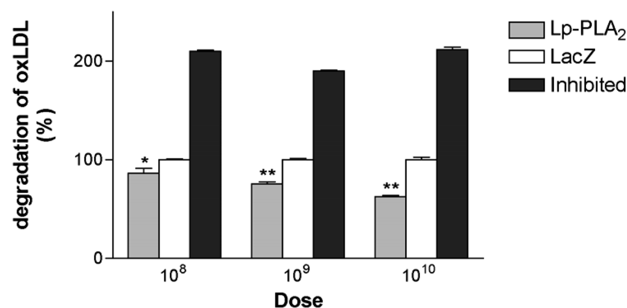

Fig. 5. Effect of Lp-PLA_2 on the degradation of oxLDL. RAW 264 cells were incubated for 6 h with 10 μg/ml ^{125}I oxLDL isolated from rabbits given different doses of adenovirus. An aliquot of LDL at each dose level was treated with 0.1 mM Pefabloc. Inhibition of the Lp-PLA_2 activity led to a 2-fold increase in degradation compared with that in the LacZ control LDL (marked as 100%). Inhibition of the degradation was most effective (63%) with LDL containing the highest level of Lp-PLA_2 activity. Absolute values of degraded oxLDL (ng/mg cell protein) in Lp-PLA_2 groups were as follows: 729 ± 66 (10^8 pfu), 632 ± 30 (10^9 pfu), and 429 ± 17.7 (10^{10} pfu); values in LacZ groups were 843 ± 13.6 (10^8 pfu), 837 ± 18.1 (10^9 pfu), and 683 ± 30.1 (10^{10} pfu); values in Pefabloc groups were 1,771 ± 18.6 (10^8 pfu), 1,592 ± 15.2 (10^9 pfu), and 1,444 ± 33.2 (10^{10} pfu). ANOVA was followed by Student's *t*-test (* $P < 0.05$, ** $P < 0.002$ for Lp-PLA_2 vs. LacZ). Degradation in LacZ medium is presented as 100% of degradation.

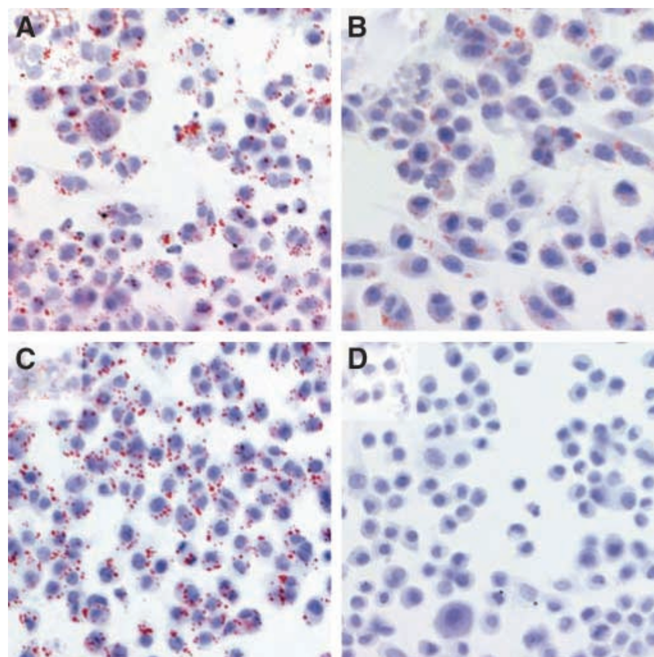

Fig. 6. Prevention of foam cell formation by Lp-PLA_2 in LDL particles. RAW 264 cells were incubated for 18 h with 100 μg/ml oxLDL. A: Foam cell formation with rabbit oxLDL from the LacZ control group. B: Lipid accumulation was decreased when cells were incubated with rabbit oxLDL containing the highest Lp-PLA_2 activity. C: When Lp-PLA_2 activity was inhibited with 0.1 mM Pefabloc, foam cell formation was markedly increased. D: Control cells incubated without LDL. Oil Red O staining is shown.

creased intracellular cholesterol accumulation is the hallmark of early human atherosclerotic lesions (1, 6).

Protective effects of Lp-PLA_2 on the atherogenic properties of LDL are most likely related to its ability to destroy oxidatively fragmented phospholipids, which are one class of ligands on oxLDL for macrophage scavenger receptor recognition (29). Reduction of these substances can also prevent the subsequent formation of fully oxLDL and uptake by macrophages. On the other hand, lyso-PC accumulating in fully oxLDL has several proatherogenic effects (30, 31). Thus, Lp-PLA_2 can potentially affect the LDL oxidation process in both the early and late phases by reducing bioactive PAF-like lipids in minimally modified LDL and causing lyso-PC accumulation in the lipoprotein particle. However, it is important to note that lyso-PC is water soluble and can diffuse out from LDL. In addition, some antiatherogenic actions of lyso-PC have also been reported, for example, the promotion of cholesterol efflux from macrophage foam cells (32). Therefore, it is possible that while protecting LDL particles from becoming oxidized, Lp-PLA_2 activity may lead to enhanced production of lyso-PC, which has multiple effects on atherogenesis. Recently, it was shown that inhibition of Lp-PLA_2 diminished the toxicity and apoptosis induced by fully oxLDL and that this protection was apparent only after a prolonged incubation (33). It has also been shown that HDL-associated Lp-PLA_2 activity prevents the oxidation of atherogenic lipoproteins and macrophage homing into apolipoprotein $E^{-/-}$ mouse lesions (34).

When interpreting these results, it should be remembered that we have expressed human Lp-PLA_2 in rabbits and that binding of the human enzyme to rabbit LDL may not fully resemble the human situation. Also, while preparing radiolabeled oxLDL for degradation studies, lipoprotein preparations have been dialyzed, and this could have reduced the lyso-PC content in the lipoprotein particles, modifying their biological properties. Thus, these results may not fully reflect the in vivo situation regarding the effects of Lp-PLA_2 on atherogenesis. Lastly, the inhibitor used for the study is not absolutely specific for Lp-PLA_2 and may have additional unknown effects on LDL oxidation and lipid uptake by macrophages.

In the context of atherosclerosis, the role of Lp-PLA_2 is still somewhat unclear; indeed, Lp-PLA_2 is considered to have a dual role: one that is anti-inflammatory (35) and one that is proinflammatory as a result of the generation of lyso-PC, which is an abundant component of oxLDL (30). Increased Lp-PLA_2 expression and activity have been demonstrated in human and rabbit atherosclerotic lesions (36). However, it remains unclear whether Lp-PLA_2 contributes to the progression of human lesions, and definitive conclusions about the proatherogenic and antiatherogenic roles of Lp-PLA_2 activity and its inhibition can only be obtained from prospective human intervention studies. Our results show that when subjected to in vitro oxidation, increased levels of Lp-PLA_2 activity in LDL reduce subsequent lipoprotein degradation and foam cell formation in macrophages, which suggest that in the early fatty streaks Lp-PLA_2 may have antiatherogenic effects by reducing proinflammatory changes and lipid uptake in lesion macrophages.

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