Macrophage-specific expression of group IIA $sPLA_2$ results in accelerated atherogenesis by increasing oxidative stress

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Abstract **Group IIA secretory phospholipase A_2 ($sPLA_2$) is an acute-phase protein mediating decreased plasma HDL cholesterol and increased atherosclerosis. This study investigated the impact of macrophage-specific $sPLA_2$ overexpression on lipoprotein metabolism and atherogenesis. Macrophages from $sPLA_2$ transgenic mice have 2.5 times increased rates of LDL oxidation (thiobarbituric acid-reactive substances formation) in vitro (59 ± 5 vs. 24 ± 4 nmol malondialdehyde/mg protein; $P < 0.001$) dependent on functional 12/15-lipoxygenase (12/15-LO). Low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice were transplanted with bone marrow from either $sPLA_2$ transgenic mice ($sPLA_2 \rightarrow LDLR^{-/-}$; n = 19) or wild-type C57BL/6 littermates (C57 BL/6→$LDLR^{-/-}$; n = 19) and maintained for 8 weeks on chow and then for 9 weeks on a Western-type diet. Plasma $sPLA_2$ activity and plasma lipoprotein profiles were not significantly different between $sPLA_2 \rightarrow LDLR^{-/-}$ and C57BL/6→$LDLR^{-/-}$ mice. Aortic root atherosclerosis was increased by 57% in $sPLA_2 \rightarrow LDLR^{-/-}$ mice compared with C57BL/6→$LDLR^{-/-}$ controls ($P < 0.05$). Foam cell formation in vitro and in vivo was increased significantly. Urinary, plasma, and aortic levels of the isoprostane 8,12-*iso*-$iPF_{2\alpha}$-VI and aortic levels of 12/15-LO reaction products were each significantly higher ($P < 0.001$) in $sPLA_2 \rightarrow LDLR^{-/-}$ compared with C57BL/6→$LDLR^{-/-}$ mice, indicating significantly increased in vivo oxidative stress in $sPLA_2 \rightarrow LDLR^{-/-}$. These data demonstrate that macrophage-specific overexpression of human $sPLA_2$ increases atherogenesis by directly modulating foam cell formation and in vivo oxidative stress without any effect on systemic $sPLA_2$ activity and lipoprotein metabolism.**—Tietge, U. J. F., D. Pratico, T. Ding, C. D. Funk, R. B. Hildebrand, T. J. Van Berkel, and M. Van Eck. **Macrophage-specific expression of group IIA $sPLA_2$ results in accelerated atherogenesis by increasing oxidative stress. *J. Lipid Res.* 2005.** 46: **1604–1614.**

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Atherosclerosis is increasingly considered to have an inflammatory component (1). While the atherosclerotic plaque displays the features of a localized inflammation within the vascular wall (2, 3), markers of systemic inflammation such as C-reactive protein have been found to be predictive of future cardiovascular events (4–6).

The type IIA secretory phospholipase A_2 ($sPLA_2$) is an acute-phase protein expressed in response to a variety of proinflammatory cytokines by a number of different tissues and cell types, mainly of mesenchymal origin (7–12). Increased $sPLA_2$ plasma levels have been reported in patients with various acute and chronic inflammatory conditions (8, 10). Notably, patients with atherosclerotic cardiovascular disease have significantly higher circulating $sPLA_2$ levels compared with controls (13, 14), and in one report, $sPLA_2$ levels were even found to have a higher predictive value for future coronary events than C-reactive protein (13).

Data obtained in animal models implied that $sPLA_2$ plays a causative role in the process of atherogenesis and indicated that local as well as systemic expression might be relevant. Transgenic mice overexpressing human $sPLA_2$ (15) have decreased HDL cholesterol plasma levels attributable to increased catabolism of apolipoprotein A-I as well as HDL cholesteryl ester (16–18). These mice develop dramatically increased atherosclerosis when fed an atherogenic diet for 12 weeks but also even on a normal chow diet (19). In addition, increased formation of oxidized

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phospholipids in $sPLA_2$ transgenic mice has been reported (20). In human atherosclerotic plaques, $sPLA_2$ is expressed mainly by vascular smooth muscle cells but also consistently by tissue macrophages within the lesion (21–24).

Therefore, we hypothesized that expression of human $sPLA_2$ exclusively in macrophages might enhance early atherogenesis after bone marrow transplantation (BMT) from $sPLA_2$ transgenic mice into low density lipoprotein receptor-deficient ($LDLR^{-/-}$) recipients. Our results demonstrate that feeding a Western-type diet for 9 weeks after BMT significantly increased atherosclerosis in the group transplanted with the $sPLA_2$ transgenic bone marrow. These data are consistent with the results of a study that was published while our work was in progress (25). However, we provide potential mechanisms for the increased atherogenesis induced by macrophage $sPLA_2$, namely *i*) by increased 12/15-lipoxygenase (12/15-LO)-dependent generation of oxidative stress, as demonstrated by enhanced LDL oxidation by $sPLA_2$ transgenic macrophages in vitro and increased isoprostane formation in vivo, and *ii*) by increased foam cell formation by $sPLA_2$-overexpressing macrophages in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Experimental animals

The human group IIA $sPLA_2$ transgenic mice used in this study have been described previously (15, 16). In these mice, expression of the transgene is driven by the endogenous human group IIA $sPLA_2$ promoter, allowing for regulatability of the transgene by inflammatory stimuli besides high-level baseline $sPLA_2$ expression (26). The $sPLA_2$ transgenic line has been backcrossed extensively to the C57BL/6 genetic background for >14 generations. The animals were caged in animal rooms with ad libitum access to water and mouse chow diet.

Homozygous $LDLR^{-/-}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) as mating pairs and bred at the Gorlaeus Laboratory (Leiden, The Netherlands). Mice were housed in sterilized filter-top cages and given unlimited access to food and water. Mice were maintained on sterilized regular chow, containing 4.3% (w/w) fat without added cholesterol (RM3; Special Diet Services, Witham, UK), or were fed a semisynthetic Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W; Hope Farms, Woerden, The Netherlands). Drinking water was supplied with antibiotics (83 mg/l ciprofloxacin and 67 mg/l polymyxin B sulfate) and 6.5 g/l sucrose. Animal experiments were performed in accordance with the national laws. All experimental protocols were approved by the respective government authorities and the local ethics committees for animal experiments of Leiden University and Humboldt University.

Collection of resident peritoneal macrophages and culture conditions

Resident peritoneal macrophages with and without thioglycollate (Sigma, Deisenhofen, Germany) stimulation for 3 days were harvested by peritoneal lavage with 10 ml of sterile PBS containing 1 mM EDTA, collected by centrifugation (1,400 rpm, 10 min, 4°C), and plated on six-well plates (BD Falcon, Franklin Lakes, NJ) at a cell density of 4×10^6 in RPMI medium (Gibco Invitrogen, Karlsruhe, Germany) supplemented with endotoxin-free 10% fetal calf serum (Specialty Media, Phillipsburg, NJ). After 4 h, plates were washed three times with PBS to remove nonadherent cells and further incubated under various experimental conditions as indicated.

Assessment of $sPLA_2$ expression and $sPLA_2$ activity assay

Resident peritoneal macrophages isolated from $sPLA_2$ transgenic mice and C57BL/6 controls were cultured for 24 h in RPMI supplemented with 10% fetal calf serum. To assess the expression of human type IIA $sPLA_2$, RNA was isolated using the Trizol reagent (Invitrogen), incubated with DNase I (Qiagen, Hilden, Germany), and reverse transcribed using the Omniscript RT Kit (Qiagen). PCR with specific primers to amplify the full-length human type IIA $sPLA_2$ cDNA according to the published sequence (GenBank accession number NM_000300) was performed on aliquots of the RT-PCR product. The presence of the human type IIA $sPLA_2$ protein in cell culture supernatants of the respective macrophage incubations was detected by Western blot as described (18) using a monoclonal antibody (Cayman Chemical, Ann Arbor, MI). The $sPLA_2$ activity assay was performed on cell culture supernatants and mouse plasma essentially as described previously (27). Briefly, using a 1,2-dithio analog of diheptanoyl phosphatidylcholine (Cayman Chemical) as a substrate, free thiols were liberated by $sPLA_2$ action and detected using DTNB.

LDL isolation and measurement of macrophage LDL oxidation and macrophage foam cell formation

Native human LDL was isolated by sequential ultracentrifugation ($1.019 < d < 1.063$), dialyzed extensively against EDTA-free PBS, filter-sterilized, and used within 2 days. The protein content of the preparations was measured using the bicinchoninic acid reagents (Pierce, Rockford, IL). To study macrophage oxidation of LDL (28), unstimulated resident peritoneal macrophages were isolated as described above and incubated in Ham's F-10 medium (Promocell GmbH, Heidelberg, Germany) containing 100 mg LDL protein/ml for the time periods indicated. At the end of the incubations, the media containing LDL were removed and centrifuged for 10 min at 1,400 rpm, and EDTA was added to the supernatants at a final concentration of 0.2 mM to stop further oxidation. The extent of LDL oxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS) formation in 100 μl aliquots as described using malondialdehyde as a standard (28). No-cell controls were included, and the respective TBARS values were subtracted from the experimental values. Cellular protein content was assessed with the bicinchoninic acid assay after adding 0.1 M NaOH and 1% SDS to the wells as described (29). Data for LDL oxidation are expressed in nanomoles of malondialdehyde produced per milligram of cellular protein. To evaluate the role of 12/15-LO in macrophage-mediated LDL oxidation, the specific inhibitor PD146176 (Sigma-Aldrich, Munich, Germany) at a concentration of 20 μM, which is nontoxic to macrophages, was added to mouse peritoneal macrophages from $sPLA_2$ transgenic mice or wild-type C57BL/6 littermates 1 h before the addition of native LDL (30). TBARS formation was assessed after 12 h of incubation. For Western blot analysis of 12/15-LO protein, 30 μg of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal rabbit anti-mouse 12/15-LO antibody at a dilution of 1:1,000 as described previously (31).

To assess macrophage foam cell formation in vitro, oxidized LDL (OxLDL) was prepared by incubating native LDL with 10 μM $CuSO_4$ in Ham's F-10 medium at 37°C for 18 h. Mouse peritoneal macrophages from $sPLA_2$ transgenic mice or wild-type C57BL/6 littermates were incubated for 24 h with OxLDL (25 μg/ml). Then, total cholesterol and free cholesterol were measured using enzymatic colorimetric assays (Wako Chemicals, Neuss,

Germany). Cholesteryl esters were determined by subtracting free cholesterol from total cholesterol values.

Macrophage mRNA expression analyses by real-time quantitative PCR

Total RNA was extracted from thioglycollate-elicited peritoneal macrophages from control C57BL/6 mice and $sPLA_2$ transgenic animals by the Trizol method. cDNA was synthesized from 2 μg of total RNA using RevertAid™ M-MuLV reverse transcriptase. mRNA levels were quantitatively determined on an ABI Prism® 7700 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR-green technology according to the manufacturer's instructions. For detection of scavenger receptor A mRNA, 5′-GGTGGTAGTGGAGCCCATGA-3′ and 5′-CCCGTATATCCCAGCGATCA-3′ were used as forward and reverse primers, respectively. Scavenger receptor A mRNA expression levels were calculated relative to the average of the housekeeping genes HPRT (primers 5′-TTGCTCGAGATGTCATGAAGGA-3′ and 5′-AGCAGGTCAGCAAAGAACTTATAGC-3′) and 36B4 (primers 5′-GGACCCGAGAAGACCTCCTT-3′ and 5′-GCACATCACTCAGAATTTCAATGG-3′).

BMT

To induce bone marrow aplasia, female $LDLR^{-/-}$ mice were exposed to a single dose of 9 Gray (0.19 Gray/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International, Copenhagen, Denmark) with a 6 mm aluminum filter 1 day before transplantation. Bone marrow was isolated by flushing the femurs and tibias from female $sPLA_2$ transgenic mice or female wild-type C57BL/6 littermates with phosphate-buffered saline. Single-cell suspensions were prepared by passing the cells through a 30 μm nylon gauze. Irradiated recipients received 0.5×10^7 bone marrow cells by intravenous injection into the tail vein. The hematologic chimerism of the $LDLR^{-/-}$ mice was determined in genomic DNA from bone marrow by PCR at 17 weeks after transplantation using specific primers located in the promoter region and the first intron of the human type IIA $sPLA_2$ transgene (15), generating a 280 bp product. Notably, two independent BMT experiments were performed, each involving $LDLR^{-/-}$ mice receiving $sPLA_2$ transgenic bone marrow as well as mice receiving control C57BL/6 bone marrow, and the data shown represent the combination of both of these studies.

Serum lipid and lipoprotein analyses

After an overnight fast, ~100 μl of blood was drawn from individual mice by tail bleeding. The concentrations of total and free cholesterol, triglycerides, and phospholipids in serum were determined using enzymatic colorimetric assays (Roche Diagnostics, Mannheim, Germany). The distribution of cholesterol and phospholipids over the different lipoprotein subclasses in serum was determined by fractionation of 30 μl of serum of each mouse using a Superose 6 column (3.2 × 30 mm, Smart-system; Pharmacia, Uppsala, Sweden) as described (32). Total cholesterol and phospholipid contents in the effluent were determined using enzymatic colorimetric assays (Roche Diagnostics).

Histological analysis of the aortic root

To analyze the development of atherosclerosis at the aortic root, transplanted mice were killed at week 17 after transplantation after 9 weeks of feeding the high-cholesterol Western-type diet. The arterial tree was perfused in situ with phosphate-buffered saline (100 mm Hg) for 20 min via a cannula in the left ventricular apex. The aortic arch as well as the thoracic and abdominal aortas were excised and stored in 3.7% formalin (Formal-fixx; Shandon Scientific Ltd.). The total atherosclerotic lesion area in Oil Red O-stained cryostat sections of the aortic root was quantified using the Leica image-analysis system, which consisted of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK). Mean lesion area (in square micrometers) was calculated from 10 Oil Red O-stained sections, starting at the appearance of the triscuspid valves. For the assessment of macrophage area, sections were immunolabeled with MOMA-2 (a generous gift of Dr. G. Kraal, Vrije Universiteit, Amsterdam, The Netherlands; dilution 1:50)

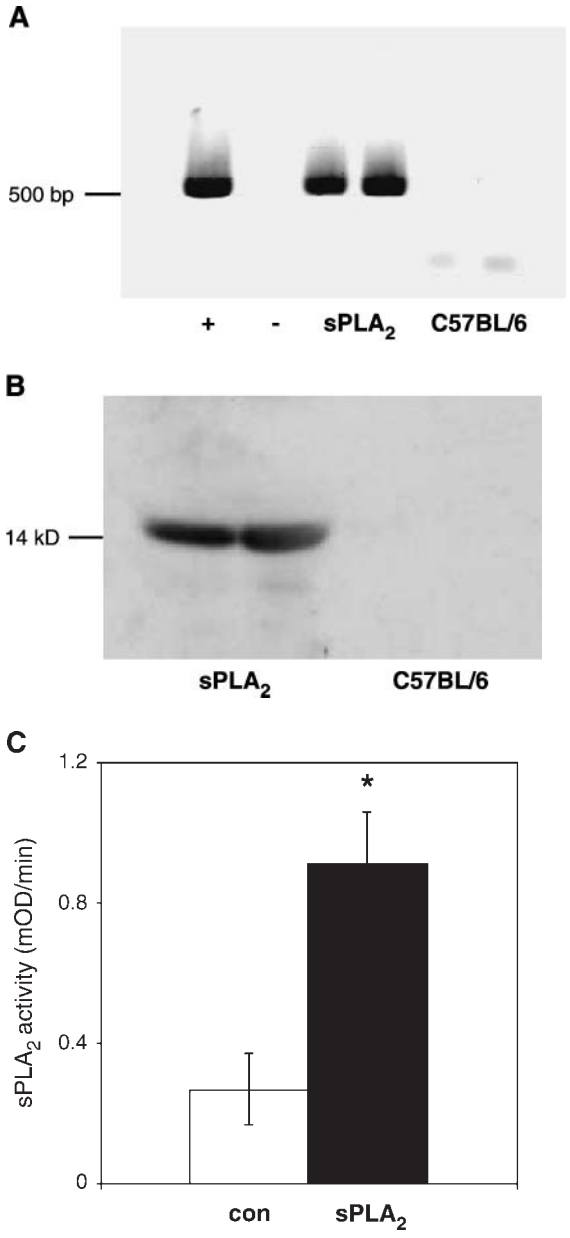

Fig. 1. Expression of functional secretory phospholipase A_2 ($sPLA_2$) enzyme by elicited mouse peritoneal macrophages from human $sPLA_2$ transgenic mice. A: Results of RT-PCR analysis of human $sPLA_2$ mRNA expression in macrophages from $sPLA_2$ transgenic mice and C57BL/6 control littermates, including a water control (−) and as a positive control cloned human $sPLA_2$ cDNA (+). B, C: Western blot analysis for human $sPLA_2$ (B) and $sPLA_2$ activity assay (C), each performed on cell culture supernatants after 24 h of incubation as described in Experimental Procedures. Data in C are given as means ± SEM *Significantly different from control (con) values ($P < 0.001$).

for the specific detection of macrophages. The MOMA-2-positive lesion area was subsequently quantified using the Leica image-analysis system. Cellular density was determined by counting the number of nuclei per macrophage area. The amount of collagen in the lesions was determined using Masson's Trichrome Accustain according to the manufacturer's instructions (Sigma Diagnostics). For detection of the expression of 12/15-LO in atherosclerotic lesions, sections were stained using a rabbit polyclonal antibody for mouse 12/15-LO (dilution 1:100) (31).

Measurement of isoprostanes, 12-hydroxyeicosatetraenoic acid, and 15-hydroxyeicosatetraenoic acid

Urinary, plasma, and aortic levels of the isoprostane 8,12-*iso*-iPF$_{2\alpha}$-VI were measured by gas chromatography-mass spectrometry as described previously (33). Urine was collected for 24 h from groups of animals, blood samples collected from individual mice were immediately centrifuged at 12,000 rpm for 15 min, and plasma was separated and stored at −80°C until analysis. Samples were spiked with a known amount of internal standard, extracted and purified by thin-layer chromatography, and analyzed by negative ion chemical ionization gas chromatography-mass spectrometry (33, 34). Aortas from individual mice were obtained, weighed, minced, and homogenized in PBS containing EDTA (2 mM/l) and butylated hydroxytoluene (2 mM/l), pH 7.4, and total lipid extracted using Folch solution (chloroform-methanol, 2:1, v/v). Next, base hydrolysis was performed using 15% KOH at 45°C for 1 h, and the total levels of 8,12-*iso*-iPF$_{2\alpha}$-VI were processed before analysis as described above. Total 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-HETE levels were assayed by liquid chromatography-tandem mass spectrometry essentially as described (35).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS). Data are expressed as means ± SEM unless indicated otherwise. Results were analyzed using Student's *t*-test, and statistical significance for all comparisons was assigned at $P < 0.05$.

RESULTS

Human sPLA$_2$ transgenic mice express functionally active sPLA$_2$ in macrophages

First, we determined whether human sPLA$_2$ is expressed by peritoneal macrophages from sPLA$_2$ transgenic mice. In unelicited resident peritoneal macrophages, a weak but specific signal was reproducibly detected by RT-PCR, but protein expression could not be visualized by Western blot, indicating low-level constitutive expression of the transgene (data not shown). Consistent with our previous observation that the human sPLA$_2$ transgene is regulatable by inflammatory stimuli in these mice (18, 26), a strong signal was detected for the sPLA$_2$ mRNA by RT-PCR in elicited peritoneal macrophages from sPLA$_2$ transgenic mice, whereas human sPLA$_2$ mRNA was absent in control C57BL/6 mice (**Fig. 1A**). Western blot analysis confirmed significant sPLA$_2$ protein expression in macrophages of sPLA$_2$ transgenic mice (Fig. 1B). In addition, supernatants of macrophages from sPLA$_2$ transgenic mice contained significantly higher sPLA$_2$ activity compared with C57BL/6 controls, indicating the expression of functional sPLA$_2$ protein in macrophages from sPLA$_2$ transgenic mice (0.91 ± 0.15 vs. 0.27 ± 0.10 milli optical density units/min, respectively; $P < 0.001$) (Fig. 1C).

Macrophage expression of sPLA$_2$ increases LDL oxidation in vitro in a 12/15-LO-dependent manner

Next, macrophages from sPLA$_2$ transgenic and C57BL/6 mice were incubated with freshly isolated native LDL to assess LDL oxidation. After 12 and 24 h, TBARS formation was measured in aliquots from the respective incubations. sPLA$_2$ transgenic macrophages exhibited significantly higher TBARS formation after 12 h (54 ± 4 vs. 21 ± 3 nmol malondialdehyde/mg cell protein, respectively; $P < 0.001$) (**Fig. 2**) and 24 h (59 ± 5 vs. 24 ± 4 nmol malondialdehyde/mg cell protein, respectively; $P < 0.001$) (Fig. 2) of incubation than C57BL/6 macrophages.

Because 12/15-LO is critical for macrophage-mediated LDL oxidation and cross-talk between 12/15-LO and sPLA$_2$ has been reported (36, 37), we assessed as a possible mechanism the involvement of 12/15-LO in our model. As determined by Western blot analysis, macrophages from sPLA$_2$ transgenic mice had increased 12/15-LO protein levels compared with wild-type controls (**Fig. 3A**). Using a specific 12/15-LO inhibitor, macrophage-mediated LDL oxidation was inhibited significantly (27%) in the control macrophages ($P < 0.05$; Fig. 3B). However, in sPLA$_2$ transgenic macrophages, LDL oxidation was inhibited by 43% after the addition of the inhibitor ($P < 0.01$; Fig. 3B) and thereby reduced to values that were not significantly different from the levels of LDL oxidation observed in control macrophages either with or without the LO inhibitor.

Macrophage expression of sPLA$_2$ increases foam cell formation in vitro

To determine foam cell formation in vitro, macrophages from sPLA$_2$ transgenic mice and wild-type controls

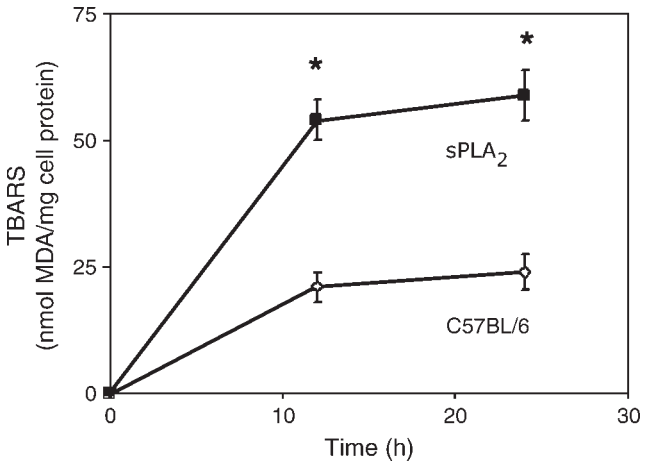

Fig. 2. LDL oxidation by mouse peritoneal macrophages from human sPLA$_2$ transgenic mice and C57BL/6 control littermates. Native LDL was incubated with peritoneal macrophages for 12 and 24 h, and thiobarbituric acid-reactive substances (TBARS) formation was assessed as a parameter of LDL oxidation as described in Experimental Procedures. Experiments were performed in triplicate. Data are given as means ± SEM. * Significantly different from control values. MDA, malondialdehyde.

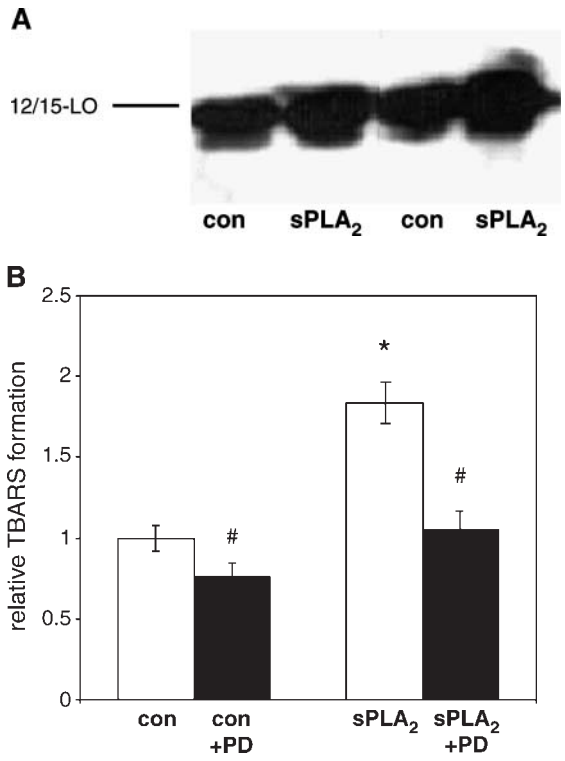

Fig. 3. 12/15-Lipoxygenase (12/15-LO) is involved in LDL oxidation mediated by macrophages from $sPLA_2$ transgenic mice. A: Western blot analysis for mouse 12/15-LO. B: Macrophage-mediated LDL oxidation by peritoneal macrophages from $sPLA_2$ transgenic mice and wild-type littermates in the presence (+PD) or absence of 20 μM of the specific 12/15-LO inhibitor PD146176. Relative data are given as means ± SEM, with the value observed in control (con) macrophages without PD146176 set to 1. * Significantly different from control values ($P < 0.05$). # Significantly different from the values observed in the respective experimental groups without the use of PD146176 ($P < 0.05$). TBARS, thiobarbituric acid-reactive substances.

were incubated with OxLDL for 24 h. $sPLA_2$-overexpressing macrophages displayed a significantly higher cholesteryl ester accumulation compared with controls (9.2 ± 0.9 vs. 6.6 ± 0.8 μg/mg cell protein, respectively; $P < 0.05$) (**Fig. 4**), indicating increased foam cell formation independent of the effects on the oxidation status of LDL. Expression levels of scavenger receptor A as determined by quantitative real-time PCR were not significantly different between both groups of mice (relative expression level, 0.74 ± 0.04 in $sPLA_2$ transgenic macrophages vs. 0.79 ± 0.08 in controls).

Macrophage expression of $sPLA_2$ does not influence plasma $sPLA_2$ activity and lipoprotein levels

To analyze the specific role of macrophage $sPLA_2$ in lipoprotein metabolism and atherosclerotic lesion development, chimeras were created that overexpress $sPLA_2$ in macrophages by transplantation of bone marrow from $sPLA_2$ transgenic mice and control C57BL/6 littermates to $LDLR^{-/-}$ mice, an established model for atherosclerosis.

After BMT, mice were maintained for 8 weeks on chow, then they were fed a Western-type diet for an additional 9

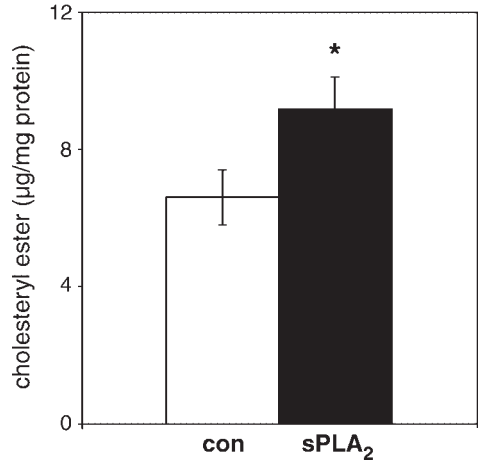

Fig. 4. Foam cell formation in vitro. Peritoneal macrophages from $sPLA_2$ transgenic mice and wild-type littermates were incubated for 24 h in the presence of 25 μg/ml oxidized LDL. Cellular cholesteryl ester content was determined as described in Experimental Procedures. Data are given as means ± SEM. * Significantly different from control (con) values ($P < 0.05$).

weeks. At the end of the study, plasma $sPLA_2$ activity was not significantly different between $LDLR^{-/-}$ mice that received $sPLA_2$ transgenic bone marrow and those transplanted with control bone marrow (1,006 ± 178 vs. 918 ± 171 nmol/h/ml, respectively; NS) (**Fig. 5**).

Plasma levels of total cholesterol, free cholesterol, triglycerides, and phospholipids did not differ significantly between $sPLA_2$ transgenic and control bone marrow transplanted mice at any time point of the study (**Table 1**). In addition, the cholesterol distribution in the different lipoprotein fractions was not different between both groups of mice before initiation of the Western-type diet (**Fig. 6A**) and at the end of the experiment (Fig. 6B). Because $sPLA_2$ activity can influence the phospholipid content

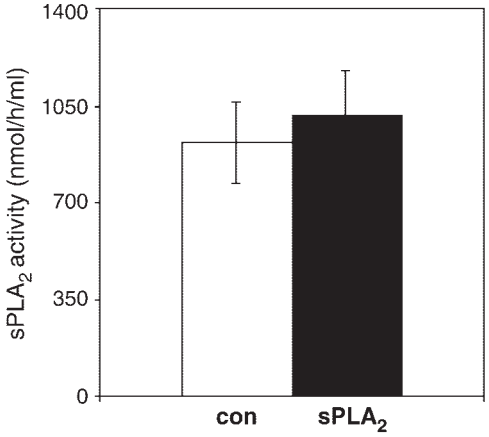

Fig. 5. Plasma $sPLA_2$ activity in low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice transplanted with either $sPLA_2$ transgenic or control (con) C57BL/6 bone marrow after 9 weeks of Western-type diet feeding. The $sPLA_2$ activity assay was carried out as described in Experimental Procedures. Data are given as means ± SEM. n = 19 mice/group.

TABLE 1. Plasma lipid profiles in LDLR$^{-/-}$ mice before BMT and after BMT with either human sPLA$_2$ transgenic bone marrow or wild-type C57BL/6 bone marrow

	C57BL/6→LDLR$^{-/-}$			sPLA$_2$→LDLR$^{-/-}$		
Lipid	0 Weeks	8 Weeks	17 Weeks	0 Weeks	8 Weeks	17 Weeks
Total cholesterol (mg/dl)	335 ± 14	314 ± 9	942 ± 76	339 ± 13	309 ± 13	847 ± 51
Free cholesterol (mg/dl)	92 ± 3	96 ± 5	294 ± 32	98 ± 4	96 ± 6	252 ± 20
Phospholipids (mg/dl)	411 ± 9	479 ± 15	613 ± 30	423 ± 10	481 ± 17	610 ± 26
Triglycerides (mg/dl)	207 ± 11	251 ± 21	200 ± 22	178 ± 9	246 ± 13	167 ± 13

BMT, bone marrow transplantation; LDLR$^{-/-}$, low density lipoprotein receptor-deficient; sPLA$_2$, secretory phospholipase A$_2$. Values are means ± SEM. 0 weeks indicates values before BMT, 8 weeks indicates values before starting the Western-type diet, and 17 weeks indicates the termination of the experiment after 9 weeks of feeding the Western-type diet.

mainly of the HDL lipoproteins (16, 18, 26), we also assessed the phospholipid distribution after fast-protein liquid chromatography fractionation of plasma. Before the start of the Western diet, phospholipid distribution was identical in both groups of mice (Fig. 6C). However, after 9 weeks on the Western-type diet, phospholipids appeared slightly, although not significantly, lower within the HDL fraction of the LDLR$^{-/-}$ mice that had received bone marrow from sPLA$_2$ transgenic mice compared with controls (Fig. 6D).

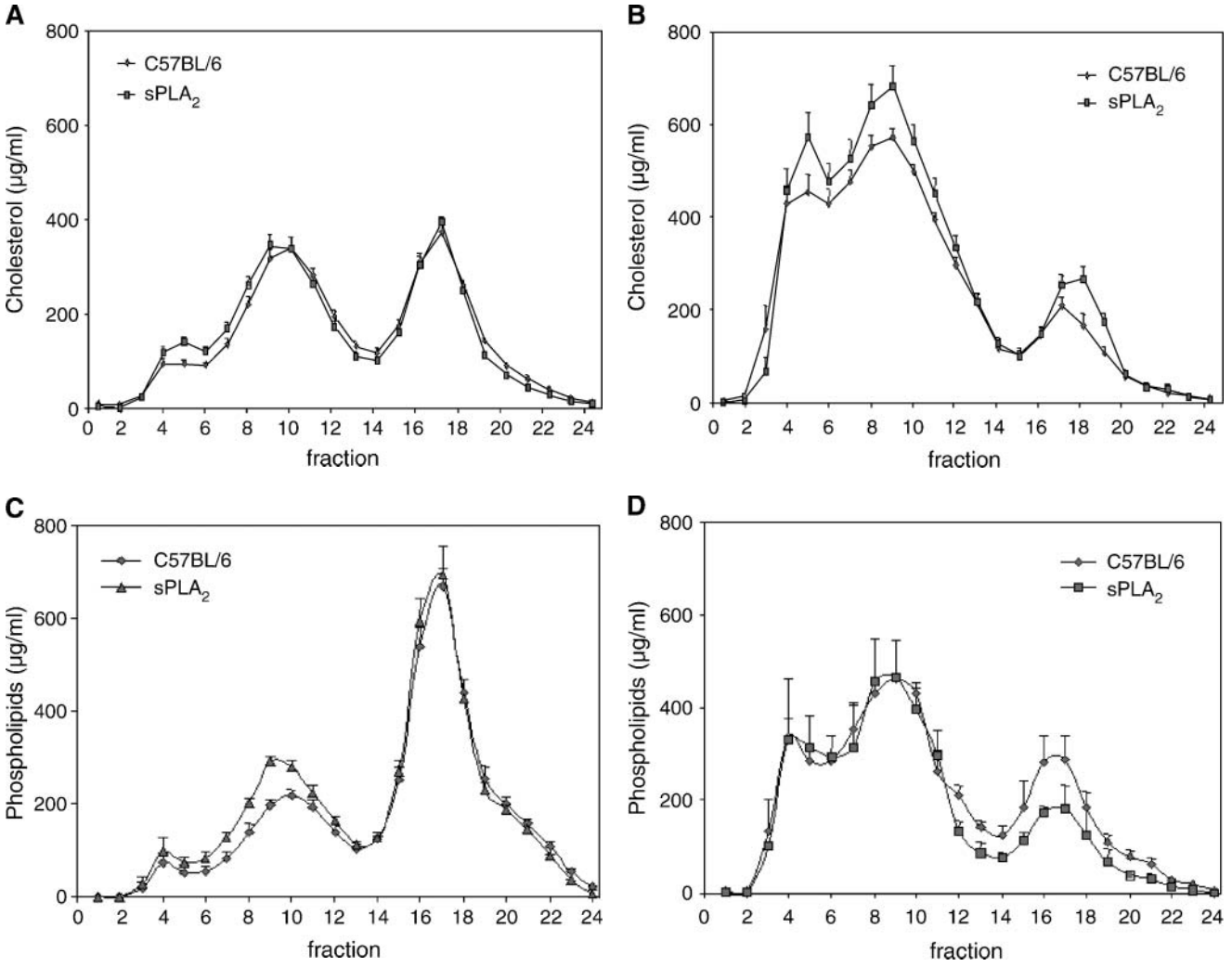

Fig. 6. Fast-protein liquid chromatography cholesterol and phospholipid profiles in LDLR$^{-/-}$ mice transplanted with either sPLA$_2$ transgenic or control C57BL/6 bone marrow. Individual plasma samples were subjected to gel filtration analysis using a Superose 6 column, and cholesterol and phospholipid contents were determined in each fraction with enzymatic assay kits. Relative elution positions are indicated. Depicted are cholesterol profiles before (A) and 9 weeks after (B) feeding a Western-type diet as well as phospholipid profiles before (C) and 9 weeks after (D) feeding a Western-type diet. Data are given as means ± SEM. n = 19 mice/group.

Macrophage expression of $sPLA_2$ increases the formation of aortic atherosclerotic lesions

After 9 weeks on the Western-type diet, mice were killed and the extent of atherosclerosis in the aortic root was determined. $LDLR^{-/-}$ mice that were transplanted with $sPLA_2$ transgenic bone marrow had developed significantly greater (57%) atherosclerotic lesions than $LDLR^{-/-}$ mice transplanted with control C57BL/6 bone marrow (231,415 ± 26,390 vs. 147,211 ± 20,679 μm^2, respectively; $P < 0.05$) (**Fig. 7A–C**).

The collagen content of the atherosclerotic lesions was low in both groups of mice, but a tendency toward slightly higher levels was observed in mice transplanted with bone marrow from $sPLA_2$ transgenic mice compared with controls (4.0 ± 0.6% vs. 1.1 ± 0.3%, respectively; $P = 0.082$) (**Table 2**). In addition, caps covering the atherosclerotic lesion were observed in 12 of the 17 mice transplanted with $sPLA_2$ transgenic bone marrow and in only 5 of the 13 control transplanted mice. No effects on average cap thickness were observed.

Also, the relative macrophage area was not significantly different between mice that had received $sPLA_2$ transgenic bone marrow (34 ± 11%) and those reconstituted with control bone marrow (35 ± 9%) (**Fig. 8**). However, the absolute macrophage area was significantly greater in mice transplanted with the $sPLA_2$ transgenic bone marrow than in controls (124,752 ± 18,696 vs. 67,404 ± 14,076 μm^2, respectively; $P = 0.024$) (Table 2). The number of peritoneal leukocytes was analyzed as a measure of leukocyte infiltration into tissues. No significant difference in the number of peritoneal leukocytes was observed, indicating that leukocyte infiltration is not affected by $sPLA_2$ overexpression in macrophages. Interestingly, despite having an 85% greater total macrophage area, the number of macrophages present within the lesions of mice that had received the $sPLA_2$ transgenic bone marrow was significantly decreased compared with controls (1.680 ± 0.124 vs. 2.330 ± 0.183 nuclei/10^4 μm^2 macrophage area; $P = 0.001$), indicating an increased size of the macrophage foam cells. These findings suggest an increased cholesterol accumulation within the foam cells in vivo, consistent with the in vitro findings.

Macrophage expression of $sPLA_2$ increases in vivo oxidative stress and the aortic formation of 12/15-LO reaction products

Because $sPLA_2$ expression by macrophages significantly increased LDL oxidation in vitro, we next determined whether increased oxidative stress by $sPLA_2$ could represent a potential mechanism to explain the increased atherogenesis in $LDLR^{-/-}$ mice that had received bone marrow from $sPLA_2$ transgenic mice. As a sensitive and robust index of in vivo oxidative stress, we measured the generation of a major isoprostane, 8,12-*iso*-$iPF_{2\alpha}$-VI (34). Compared with mice receiving control bone marrow, those transplanted with $sPLA_2$ transgenic bone marrow had significantly increased levels of 8,12-*iso*-$iPF_{2\alpha}$-VI in plasma (5.77 ± 0.43 vs. 3.15 ± 0.19 ng/ml, respectively; $P < 0.001$) (**Fig. 9A**) as well as in urine (4.1 ± 0.7 vs. 1.8 ± 0.3 ng/mg creatinine, respectively; $P < 0.01$) (Fig. 9B). Moreover, as a parameter of local oxidative stress, we measured aortic 8,12-*iso*-$iPF_{2\alpha}$-VI levels, which were also increased significantly in the aortas of mice that had received the $sPLA_2$ trans-

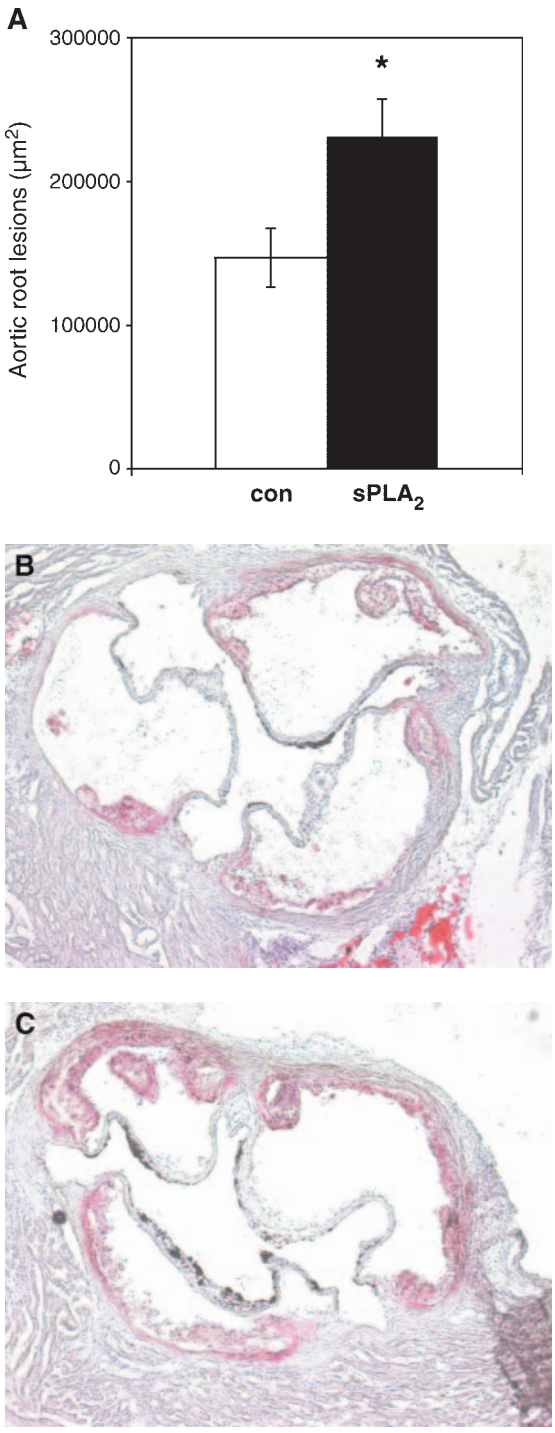

Fig. 7. Aortic atherosclerosis in $LDLR^{-/-}$ mice transplanted with either $sPLA_2$ transgenic or control C57BL/6 bone marrow after 9 weeks of feeding a Western-type diet. A: Summary of aortic root atherosclerosis lesion area assessed as described in Experimental Procedures. Data are given as means ± SEM. * Significantly different from control (con) values ($P < 0.05$). n = 19 mice/group. B, C: Representative histologic images of atherosclerotic lesion formation in a mouse transplanted with C57BL/6 bone marrow (B) and in a mouse transplanted with $sPLA_2$ transgenic bone marrow (C).

TABLE 2. Atherosclerotic lesion composition in LDLR$^{-/-}$ mice transplanted with either human sPLA$_2$ transgenic or wild-type C57BL/6 bone marrow

Variable	C57BL/6→LDLR$^{-/-}$	sPLA$_2$→LDLR$^{-/-}$
Collagen area (%)	1.1 ± 0.3	4.0 ± 0.6
Macrophage area (%)	35 ± 9	34 ± 11
Macrophage area (μm^2)	67,404 ± 14,076	124,752 ± 18,696[a]
Macrophage number (per 10^4 μm^2 macrophage area)	2.330 ± 0.183	1.680 ± 0.124[a]
Cap presence	5 of 13	12 of 17
Cap thickness (μm)	12.0 ± 3.1	12.0 ± 2.9

Values are means ± SEM unless indicated otherwise.
[a] Significantly different from the control group ($P < 0.05$).

genic bone marrow compared with controls (229 ± 13 vs. 146 ± 12 pg/mg tissue, respectively; $P < 0.001$) (Fig. 9C).

We also determined the aortic content of 12/15-LO products. Importantly, in the aortas of mice that received the sPLA$_2$ transgenic bone marrow, levels of 12(*S*)-HETE (6.27 ± 0.53 vs. 3.38 ± 0.42 ng/mg tissue, respectively; $P = 0.001$) (**Fig. 10**) and 15(*S*)-HETE (5.50 ± 0.27 vs. 4.16 ± 0.50 ng/mg tissue, respectively; $P < 0.05$) (Fig. 10) were significantly higher than in aortas of mice transplanted with the wild-type control bone marrow. In addition to our in vitro data, these results demonstrate increased local formation of 12/15-LO reaction products within the vascular wall of LDLR$^{-/-}$ mice reconstituted with the sPLA$_2$ transgenic bone marrow, suggesting an increased enzymatic activity of 12/15-LO in sPLA$_2$-overexpressing macrophages in vivo.

DISCUSSION

The results of this study demonstrate that macrophage-specific overexpression of human sPLA$_2$ accelerates early atherogenesis in LDLR$^{-/-}$ mice by increasing foam cell formation as well as vascular oxidative stress but without affecting plasma cholesterol levels and plasma sPLA$_2$ activity.

Several possible mechanisms for how sPLA$_2$ might promote atherogenesis have been described. *i*) sPLA$_2$ decreases plasma HDL cholesterol levels. Transgenic mice overexpressing human sPLA$_2$ by the control of its endogenous promoter have significantly decreased plasma HDL cholesterol levels (16, 26, 38). Plasma sPLA$_2$ activity (39) as well as protein (18) are found associated mainly with the HDL fraction. However, in this study, plasma cholesterol distribution over the different lipoprotein subclasses was not significantly different between LDLR$^{-/-}$ mice transplanted with sPLA$_2$ transgenic bone marrow and controls. These data further demonstrate that local macrophage-specific sPLA$_2$ expression within the vascular wall is not sufficient to influence systemic HDL levels and lipoprotein metabolism.

ii) sPLA$_2$ induces the formation of aggregated/fused LDL particles (40–42). Aggregated/fused LDL is a prominent feature of atherosclerotic lesions (43). This type of LDL modification increases the affinity of LDL particles for proteoglycans and subsequently enhances the forma-

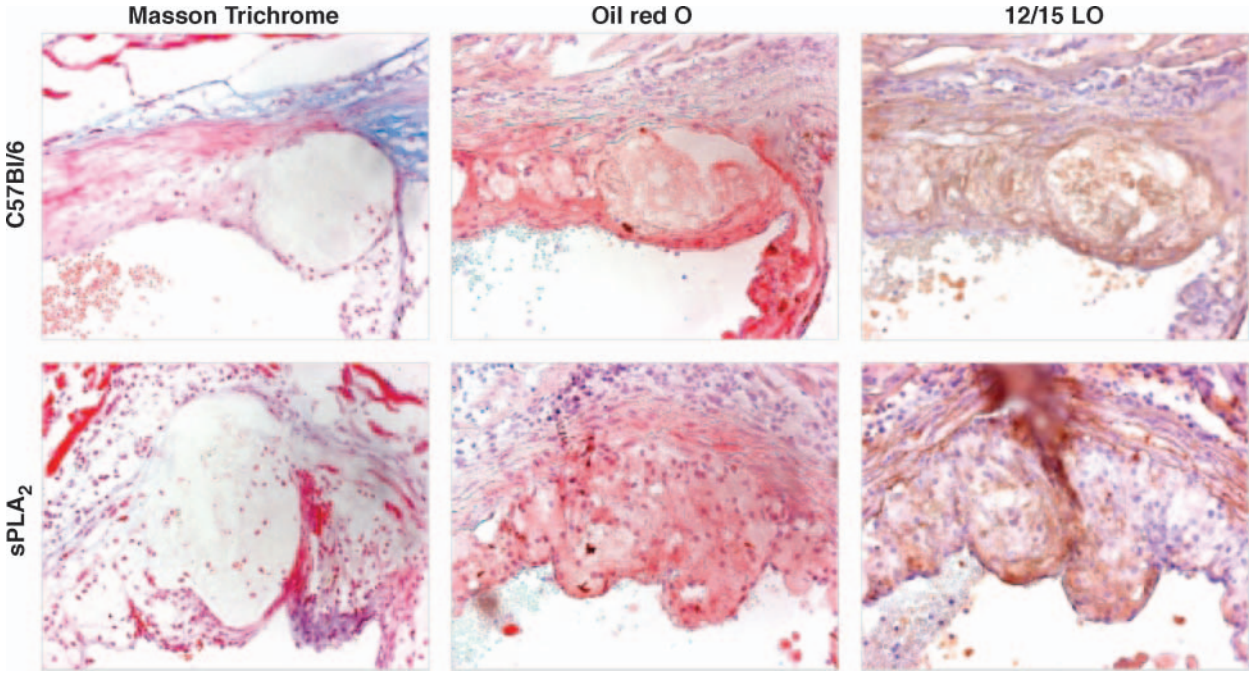

Fig. 8. Representative photomicrographs of aortic atherosclerotic lesion areas from LDLR$^{-/-}$ mice transplanted with bone marrow from either sPLA$_2$ transgenic or C57BL/6 control bone marrow stained for collagen (Masson trichrome), lipids (Oil Red O), and mouse 12/15-LO. Stainings were performed as described in Experimental Procedures.

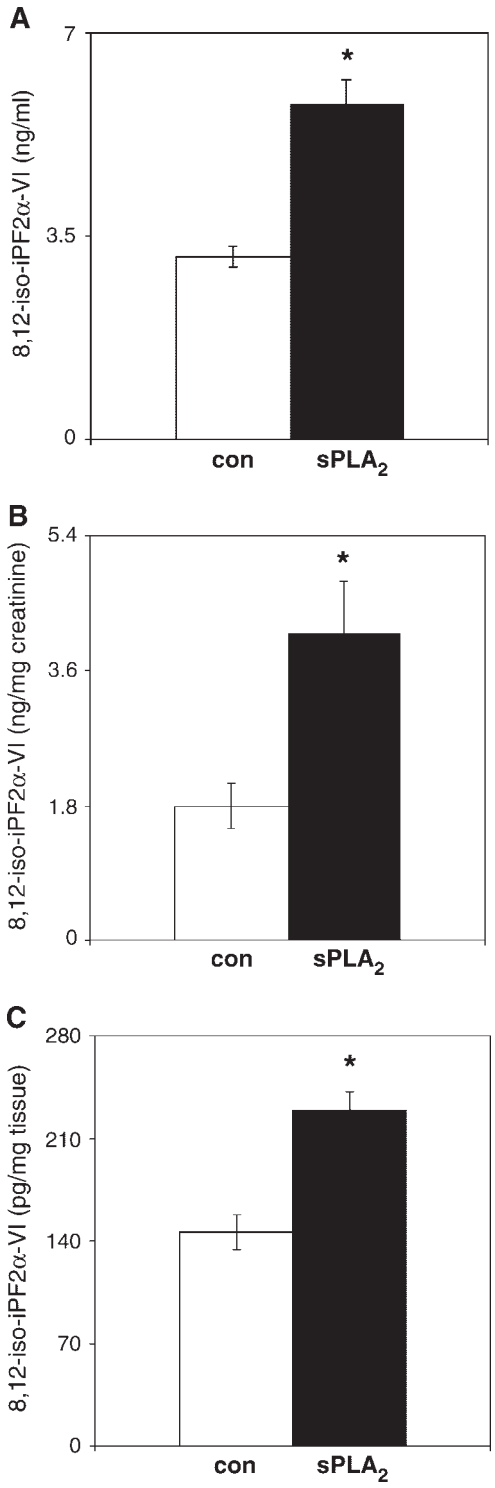

Fig. 9. Formation of the isoprostane 8,12-*iso*-iPF$_{2\alpha}$-VI as a sensitive marker of in vivo oxidative stress. At the end of the experiment, after 9 weeks of Western-type diet feeding to LDLR$^{-/-}$ mice transplanted with either sPLA$_2$ transgenic or control (con) C57BL/6 bone marrow 8,12-*iso*-iPF$_{2\alpha}$-VI concentrations were determined as described in Experimental Procedures for plasma (A), urine (B), and aorta (C). Data are given as means ± SEM. * Significantly different from control values. n = 19 mice/group.

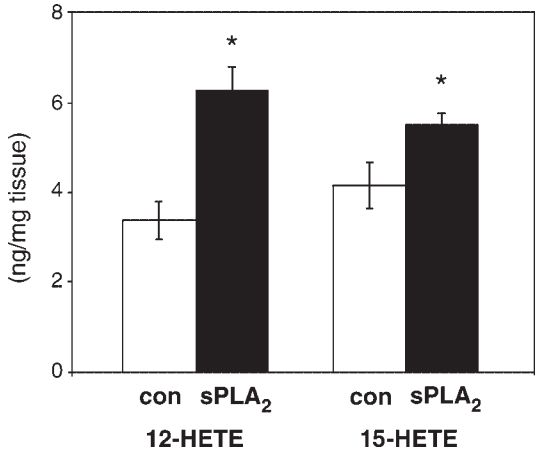

Fig. 10. Aortic contents of the 12/15-LO reaction products 12(*S*)-hydroxyeicosatetraenoic acid (12-HETE) and 15-HETE. At the end of the experiment, after 9 weeks of Western-type diet feeding to LDLR$^{-/-}$ mice transplanted with either sPLA$_2$ transgenic or control (con) C57BL/6 bone marrow, concentrations of the 12/15-LO metabolites were determined as described in Experimental Procedures. Data are given as means ± SEM. * Significantly different from control values ($P < 0.05$).

tion of foam cells and lipid accumulation within the vascular wall (43, 44). This mechanism might have contributed to the increase in foam cell formation that we observed in vivo. Although the relative percentage of foam cell area within the plaque was not different between the experimental groups, absolute foam cell area was increased in vivo in mice transplanted with sPLA$_2$ transgenic bone marrow. Importantly, the number of macrophages per square micrometer of macrophage area was decreased in this group, indicating an increased size of the accumulated macrophage foam cells. In agreement, sPLA$_2$ transgenic macrophages also displayed increased uptake of OxLDL and cholesteryl ester accumulation in vitro.

iii) sPLA$_2$-modified HDL loses its protective properties against LDL oxidation. In vitro studies using endothelial cell and vascular smooth muscle cell cocultures indicated that sPLA$_2$-treated HDL does not protect against LDL oxidation (20). This finding has been attributed to a loss of paraoxonase activity from sPLA$_2$-modified HDL (20). Paraoxonase is a major enzyme able to degrade biologically active oxidized phospholipids (45). Livers from sPLA$_2$ transgenic mice maintained on an atherogenic diet with added cholate for 12 weeks contained increased levels of biologically active oxidized phospholipids (20). However, macrophage-specific overexpression of sPLA$_2$ in transgenic mice did not affect HDL paraoxonase activity (46). In addition, our in vitro data demonstrate that sPLA$_2$ expression by peritoneal macrophages significantly enhances LDL oxidation, even in the absence of HDL particles, suggesting a novel causative role for sPLA$_2$ in LDL oxidation.

To our knowledge, our data provide the first direct in vivo evidence that macrophage sPLA$_2$ induces increased oxidative stress, an established major contributing factor to atherogenesis (2, 47–49). To assess oxidative stress, we chose to measure the formation of the isoprostane 8,12-

iso-iPF$_{2\alpha}$-VI, a specific and highly sensitive marker of lipid peroxidation and oxidative stress (50). We demonstrate that its levels were increased in urine and plasma as systemic parameters as well as directly within the aortas of LDLR$^{-/-}$ mice transplanted with sPLA$_2$ transgenic bone marrow compared with controls.

With relevance to our findings, in vitro studies reported a possible cooperative action of sPLA$_2$ and 12/15-LO in the generation of OxLDL (36, 37). Even in the absence of cells, purified 12/15-LO and sPLA$_2$ enzymes were able to mimic the cell-mediated oxidation of LDL. Notably, one study used bee venom sPLA$_2$ as well as *Naja naja* venom sPLA$_2$ (36), whereas the other study was performed with recombinant human type IIA sPLA$_2$ enzyme (37). Interestingly, the sole addition of sPLA$_2$ to LDL caused a significant shift in the electrophoretic mobility of the particle, consistent with oxidative modification (36). 12/15-LO is expressed by mouse peritoneal macrophages, and the respective cells from 12/15-LO knockout mice exhibited significantly reduced LDL oxidation (51). Importantly, these studies were performed in mice on a C57BL/6 genetic background that lack the endogenous mouse sPLA$_2$ enzyme as a result of a frameshift mutation (52). Therefore, it is highly conceivable that the additional expression of human sPLA$_2$, as in the transgenic mouse macrophages used in our study, might result in a further significant enhancement of LDL oxidation and the generation of oxidative stress. This cooperative action of sPLA$_2$ and 12/15-LO is supported by the increased 12/15-LO protein levels that we observed in the sPLA$_2$ transgenic macrophages as well as by the finding that blocking 12/15-LO in sPLA$_2$ transgenic macrophages with a specific inhibitor reduced the levels of in vitro LDL oxidation to values observed in control mice. Based on our isoprostane data as well as on the increased levels of 12/15-LO products detected within the aortas of LDLR$^{-/-}$ mice transplanted with the sPLA$_2$ transgenic bone marrow, we suggest that this mechanism is also active in vivo. Further studies are required, however, to delineate the pathophysiological basis of the cooperation between 12/15-LO and sPLA$_2$ in the generation of increased oxidative stress at the molecular level.

Notably, while this work was in progress, Webb et al. (25) reported that macrophage-specific expression of sPLA$_2$ resulted in increased atherosclerosis after 12 weeks of feeding a Western-type diet after BMT. In agreement with our data, they found that macrophage expression of sPLA$_2$ in LDLR$^{-/-}$ mice changed neither plasma cholesterol distribution nor plasma sPLA$_2$ activity. In addition, and with relevance to the previously reported action of sPLA$_2$ (16–18, 26, 38), our study also demonstrates that the phospholipid distribution among lipoprotein subclasses remained unchanged in LDLR$^{-/-}$ mice transplanted with bone marrow from sPLA$_2$ transgenic mice compared with controls. However, we extend their results by providing novel potential mechanisms for how sPLA$_2$ expression by macrophages might accelerate atherogenesis in vivo, namely, enhanced foam cell formation and increased LDL oxidation in vitro as well as increased generation of oxidative stress in vivo.

In summary, this study demonstrates that macrophage-specific overexpression of human sPLA$_2$ increases atherogenesis in LDLR$^{-/-}$ mice without affecting systemic lipoprotein metabolism. In addition, our data provide new insights on the proatherogenic role of sPLA$_2$ by showing novel mechanisms of sPLA$_2$ action in vivo: increased foam cell formation and enhancement of oxidative stress. Finally, our study suggests that macrophage sPLA$_2$ might be a suitable target for pharmacological inhibition as a treatment strategy against atherosclerotic cardiovascular disease, a major health burden of modern societies.

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