Analyses of Group III Secreted Phospholipase A$_2$ Transgenic Mice Reveal Potential Participation of This Enzyme in Plasma Lipoprotein Modification, Macrophage Foam Cell Formation, and Atherosclerosis*§

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Among the many mammalian secreted phospholipase A$_2$ (sPLA$_2$) enzymes, PLA2G3 (group III secreted phospholipase A$_2$) is unique in that it possesses unusual N- and C-terminal domains and in that its central sPLA$_2$ domain is homologous to bee venom PLA$_2$ rather than to other mammalian sPLA$_2$S. To elucidate the in vivo actions of this atypical sPLA$_2$, we generated transgenic (Tg) mice overexpressing human PLA2G3. Despite marked increases in PLA$_2$ activity and mature 18-kDa PLA2G3 protein in the circulation and tissues, PLA2G3 Tg mice displayed no apparent abnormality up to 9 months of age. However, alterations in plasma lipoproteins were observed in PLA2G3 Tg mice compared with control mice. In vitro incubation of low density (LDL) and high density (HDL) lipoproteins with several sPLA$_2$S showed that phosphatidylcholine was efficiently converted to lysophosphatidylcholine by PLA2G3 as well as by PLA2G5 and PLA2G10, to a lesser extent by PLA2G2F, and only minimally by PLA2G2A and PLA2G2E. PLA2G3-modified LDL, like PLA2G5- or PLA2G10-treated LDL, facilitated the formation of foam cells from macrophages ex vivo. Accumulation of PLA2G3 was detected in the atherosclerotic lesions of humans and apoE-deficient mice. Furthermore, following an atherogenic diet, aortic atherosclerotic lesions were more severe in PLA2G3 Tg mice than in control mice on the apoE-null background, in combination with elevated plasma lysophosphatidylcholine and thromboxane A$_2$ levels. These results collectively suggest a potential functional link between PLA2G3 and atherosclerosis, as has recently been proposed for PLA2G5 and PLA2G10.

Secreted phospholipase A$_2$ (sPLA$_2$)$^3$ enzymes represent a group of structurally related, disulfide-rich, low molecular mass (typically 14–18 kDa) enzymes with strict Ca$^{2+}$ dependence and a His-Asp catalytic dyad (1, 2). To date, 10 catalytically active sPLA$_2$ enzymes have been identified in mammals (IB, IIA, IIC, IID, IIE, III, V, X, and XIIA). These enzymes are further subdivided into three branches, namely groups I/II/V, group III, and group XII (1, 2). Recent studies employing transgenic (Tg) (gain-of-function) and knock-out (loss-of-function) mice for several sPLA$_2$S have revealed that individual enzymes exert distinct functions in vivo. Thus, PLA2G1B (group IB sPLA$_2$) plays a role in digestion of dietary phospholipids (3, 4), PLA2G2A (group IIA sPLA$_2$) plays a role in antibacterial defense and possibly tumorigenesis (5–8), PLA2G5 (group V sPLA$_2$) plays a role in zymosan-induced eicosanoid synthesis and phagocytosis by macrophages (9, 10) and airway hypersensitivity (11), and PLA2G10 (group X sPLA$_2$) plays a role in allergen-induced asthma (12) and myocardial ischemia/

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§ The abbreviations used are: sPLA$_2$, secretory PLA$_2$; PLA$_2$, phospholipase A$_2$; ESI-MS, electrospray ionization-mass spectrometry; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; Ac-LDL, acetyl-LDL; LPC, lysophosphatidylcholine; NEFA, nonesterified fatty acids(s); PEP, phosphate-buffered saline; PC, phosphatidylcholine; PGE$_2$, prostaglandin E$_2$; TXA$_2$, thromboxane A$_2$; VLDL, very low density lipoprotein; WT, wild-type; SMA, smooth muscle actin.
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reperfusion injury (13). Thus far, beyond the well accepted regulatory role of group IVA cytosolic PLA<sub>2</sub> in arachidonate metabolism (14), PLA2G5 and PLA2G10 are the only two sPLA<sub>2</sub>s that have been proven to have the capacity to modulate arachidonic acid metabolism in vivo (15, 16).

PLA2G3 (group III sPLA<sub>2</sub>) is distinctive among mammalian sPLA<sub>2</sub>s. It consists of a central sPLA<sub>2</sub> (S) domain flanked by unique N-terminal and C-terminal domains, the molecular mass of its full-length protein (55 kDa) is larger than that of other sPLA<sub>2</sub>s (14–18 kDa), and its S domain is homologous to bee venom group III sPLA<sub>2</sub> rather than to other mammalian sPLA<sub>2</sub>s (17). The central S domain alone is sufficient for its enzymatic function (17–19). PLA2G3 undergoes proteolytic processing to produce the S domain-only form in cultured cells (19), yet it remains uncertain whether the same processing occurs in vivo. Forced expression of PLA2G3 in several cell types results in increased arachidonic acid metabolism, for which its potency is superior to that of PLA2G2A and next to that of PLA2G10 and PLA2G5 (18, 19). PLA2G3 is immunohistochemically detected in the vascular endothelium of various tissues, alveolar epithelium and macrophages, peripheral and central nervous systems, and several types of cancer (19–21). However, the pathophysiological roles and relevant substrates for PLA2G3 in vivo remain to be elucidated.

In an effort to gain new insight into the in vivo actions of this unique sPLA<sub>2</sub>, we generated Tg mice overexpressing human PLA2G3. Despite marked increases in PLA<sub>2</sub> activity in sera and tissues, PLA2G3 Tg mice exhibit no signs of lung surfactant tissue, alveolar epithelium and macrophages, peripheral and central nervous systems, and several types of cancer (19–21). PLA2G3 Tg mice, in which the active PLA2G3 transgene, but not in LNL-PLA2G3 Tg mice, in which the PLA2G3 transgene remains silent, were regarded as events caused by the overexpressed PLA2G3. Generation of PLA2G10 Tg mice was described previously (36).

**PCR Genotyping**—Approximately 0.1 μg of genomic DNA obtained from the mouse tails was subjected to PCR amplification with ExTaq polymerase (Takara Biomedicals) and a set of primers (CAG-F1 (5′-ctgctaaccatgttcagcc-3′) and CAG-III-R1 (5′-gggctaagcagttagcaatc-3′) for PLA2G3; CAG-F1 and CAGX-R1 (5′-gggctaagcagttagcaatc-3′) for PLA2G10) obtained from Fasmac. The PCR conditions were 95 °C for 5 min and then 35 cycles of 95 °C for 30 s and 68 °C for 3 min on a thermal cycler (Applied Biosystems). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide.

**Northern Blotting**—Equal amounts (−10 μg) of total RNA obtained from tissues by use of TRIzol reagent (Invitrogen) were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with appropriate cDNA probes that had been labeled with [32P]dCTP (PerkinElmer Life Sciences) by random priming (Takara Biomedicals). Hybridization and subsequent membrane washing were carried out as described previously (15).

**Purification of Recombinant Human PLA2G3**—The cDNA encoding the S domain of human PLA2G3 (III-S; amino acid residues 136–289) was inserted after a coding sequence for the His<sub>6</sub> tag of the pMSNHT expression vector (Katakura Industries). Baculovirus carrying the cDNA for the N-terminally His<sub>6</sub>-tagged III-S was injected into silkworms (Katakura Industries). After 6 days, 2 ml of the silkworm body fluid was diluted in 40 ml of 100 mM phosphate buffer (pH 7.4) containing 1.5 M NaCl, 0.01% Tween 20, and protease inhibitors (catalogue number 1 873 580; Roche Applied Science). The soluble fraction of the homogenate was applied to HisTrap HP (1-ml column volume; GE Healthcare) on an AKTA system (Amersham Biosciences). The column was sequentially washed with phos-
phate buffer containing 1.5 M NaCl and 0.01% Tween 20 and then with the same buffer containing 0.5 M NaCl, followed by elution of the bound proteins with phosphate buffer (pH 7.4) with a 0–100% gradient of NaCl and imidazole (both up to 0.5 M) for 90 min at a flow rate of 1 ml/min. Fractions containing III-S protein (~20% purity as assessed by SDS-PAGE followed by staining with Coomassie Brilliant Blue; not shown) were collected and dialyzed against 20 mM Tris-HCl (pH 8.0).

Measurement of \( \text{PLA}_2 \) Activity—Tissues (100 \( \mu \)g) were soaked in 500 \( \mu \)l of SET buffer comprising 20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Sigma) and then homogenized with a Polytron homogenizer and sonicator. \( \text{PLA}_2 \) activities in the tissue homogenates and sera were assayed by measuring the amounts of radiolabeled linoleic acid released from the substrate 1-palmitoyl-2-\[^{14}\text{C} \]linoleoyl-phosphatidylethanolamine (PerkinElmer Life Science). The substrate in ethanol was dried under a stream of \( \text{N}_2 \) and dispersed in water by sonication. Each reaction mixture (total volume 250 \( \mu \)l) consisted of appropriate amounts of the required samples, 100 mM Tris-HCl (pH 7.4), 4 mM CaCl\(_2\) and 2 \( \mu \)mol of substrate. After incubation for 30 min at 37 °C, \[^{14}\text{C} \]linoleic acid was extracted, and the radioactivity was quantified with a liquid scintillation counter, as described previously (15).

Preparation of Anti-\( \text{PLA}_2 \)G3 Antibodies—The cDNA encoding the S domain of human \( \text{PLA}_2 \)G3 (amino acid residues 150–290) was inserted after a coding sequence for His\(_6\) tag of the pPROExHtB expression vector (Invitrogen). The expressed protein was purified by nickel-Sepharose and further purified by the Prep Cell system (Bio-Rad), followed by removal of the His\(_6\) tag from the S domain. Two rabbit antisera against the purified S domain were prepared by Kitayama Laboratories (designated Nov1 and Nov2). Alternatively, rabbit antiserum against the synthetic peptide CPQNISPLQYNYGIRN (corresponding to amino acid residues 187–202, a portion that shows high homology between human and mouse \( \text{PLA}_2 \)G3 proteins) was prepared by BioLogica (designated S3). The specificity of the antibodies thus obtained was evaluated by immunoblotting (Fig. S1A) using SP9 cells (cell homogenates and culture supernatants) infected with baculovirus expressing III-S of human \( \text{PLA}_2 \)G3. The procedure for immunoblotting has been described previously (15).

Mouse monoclonal antibodies for human \( \text{PLA}_2 \)G3 were prepared by a standard protocol using recombinant human III-S protein as an antigen (see above). Anti-\( \text{PLA}_2 \)G3 mouse monoclonal antibody was produced by culturing the hybridoma cell line 7B5B7, 4A10C5, 1F4E2, or 5D2F1 in BD Cell mAb Serum-free Medium with CELLine CL-1000 flasks (BD Biosciences) at 37 °C under a 5% \( \text{CO}_2 \) atmosphere for 7 days. Culture supernatant was centrifuged at 1000 rpm for 10 min to remove cells and then filtered through a 0.45-\( \mu \)m filter. Phosphate buffer (pH 7.4) was added to the filtered culture supernatant at a final concentration of 20 mM before loading the sample onto a Protein G-Sepharose column (GE Healthcare). After washing the column with 10 times the column volume of 20 mM phosphate buffer, the anti-\( \text{PLA}_2 \)G3 antibody was eluted with 100 mM citric acid buffer (pH 2.7) and dialyzed against 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS). The specificity of each monoclonal antibody was evaluated by Western blotting (Fig. S1B).

Histochemistry—Immunohistochemistry of mouse and human tissue sections was performed as described previously (19). Use of human tissues was approved by the ethics committee of our faculty. In brief, formalin-fixed tissues were embedded in paraffin, sectioned, mounted on glass slides, deparaffinized in xylene, and rehydrated in ethanol with increasing concentrations of water. The tissue sections (4 \( \mu \)m thick) were incubated with Target Retrieval Solution (Dako Cytomation) as required, incubated for 10 min with 3% (v/v) \( \text{H}_2\text{O}_2\), washed three times with TBS for 5 min each, incubated with 5% (v/v) skim milk in TBS for 30 min, washed three times with TBS for 5 min each, and incubated with anti-\( \text{sPLA}_2 \)G3 antibodies or control serum at a 1:200–500 dilution in TBS overnight at 4 °C. The sections were then treated with a CSA (catalyzed signal-amplified) system staining kit (Dako Cytomation) with diaminobenzidine substrate, followed by counterstaining with hematoxylin and eosin. The cell type was identified by conventional hematoxylin and eosin staining of serial sections adjacent to the specimen used for immunohistochemistry. Rabbit antisera for human and mouse \( \text{PLA}_2 \)G2A, \( \text{PLA}_2 \)G5, and \( \text{PLA}_2 \)G10 have been described previously (15, 19). Immunostaining for human \( \alpha \)-smooth muscle actin (SMA) (clone 1A4; Dako Cytomation) and human macrophages (clone HAM56; Dako Cytomation) was also performed using the EnVision + kit (Dako Cytomation) for identification of smooth muscle cells and monocytes/macrophages, respectively (40). Double immunostaining with antibodies against \( \alpha \)-SMA or HAM56 and \( \text{PLA}_2 \)G3 was performed on the sections to determine the localization of \( \text{PLA}_2 \)G3 in intimal cells. In brief, the sections were first immunostained for \( \alpha \)-SMA or HAM56 and visualized by diaminobenzidine. They were then reimmunostained with antibody against \( \text{PLA}_2 \)G3 using EnVision System-alkaline phosphatase (Dako Cytomation) and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate system (Dako Cytomation). When colocalized, the color turned into dark blue.

Immunoprecipitation and Western Blotting—A monoclonal antibody for \( \text{PLA}_2 \)G3 (5D2F1) or control mouse IgG\(_1\) (2 mg) was conjugated with 0.5 ml of formyl Cellulofine (Seikagaku Kogyo). The beads (20 \( \mu \)l) were incubated with 250 \( \mu \)l of the plasma obtained from \( \text{PLA}_2 \)G3 Tg mice as well as control mice at 4 °C overnight. After centrifugation, the beads were washed five times with 1 ml of phosphate-buffered saline (PBS) and boiled for 5 min in 20 \( \mu \)l of SDS-PAGE sample buffer. Then the resulting supernatants were applied to 12.5% SDS-polyacrylamide gels, and separated proteins were transferred to nitrocellulose membranes. The membranes were subjected to immunoblotting with rabbit anti-\( \text{PLA}_2 \)G3 polyclonal antibody, as described previously (15, 19).

Measurement of Serum Biochemical Markers—With or without an overnight fast, 10-week-old mice were anesthetized, and blood samples were immediately collected by cardiac puncture. Sera were applied to a clinical chemistry analyzer VetScan with V-DPP rotors (Abaxis).

Foam Cell Formation from Macrophages—The mouse macrophage cell line J774 (American Type Culture Collection) was
maintained in DMEM (Wako) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. LDL (d = 1.019 – 1.063 g/ml) was prepared from human plasma by ultracentrifugation method modified by Hatch (38). LDL (2 mg/ml) was treated with partially purified human PLA2G3 (200 μg/ml) or human PLA2G5 (5 μg/ml) (Cayman Chemicals) in a buffer comprising 6 mM HEPES (pH 7.4), 6 mM CaCl2, 84 mM NaCl, 2.4 mM MgCl2, and 20 mg/ml bovine serum albumin). After incubation for 24 h at 37 °C, the reaction was terminated by the addition of 10 mM EDTA. Subsequently, the concentrations of nonesterified fatty acids (NEFA) released were determined by colorimetric assay with an NEFA C Kit (Wako).

J774 cells were seeded on 24-well plates at a cell density of 10^5 cells/well. After incubation for 2 h, the medium was replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 200 μg/ml LDL with or without pretreatment with human PLA2G3 or PLA2G5. Alternatively, 50 μg/ml acetylated LDL (Ac-LDL) (Biomedical Technology Inc.) was used as a positive control for foam cell formation. After 24 h, the cells were fixed in 10% formalin in PBS for 10 min and washed with PBS. Saturated oil red O solution was prepared by dilution of 3 mg/ml oil red O in 60% isopropyl alcohol, and washed with PBS. After 24 h, the cells were stained with oil red O. Incubation, the cells were stained with oil red O. After a brief wash with 60% isopropyl alcohol, the cells were equilibrated in PBS.

For quantification of cholesterol, the cells were washed with 0.3% bovine serum albumin in PBS and then twice with PBS. Cellular cholesterol was extracted with hexane/isopropl alcohol (3:2) for 30 min at room temperature. Subsequently, cellular protein was extracted with 0.5 M NaOH for 30 min at 37 °C. The solvent samples containing the extracted cholesterol were evaporated under N2 gas, and the remaining cellular lipids were dissolved in propanol. Cholesterol contents were determined by the cholesterol quantification method described by Heider and Boyett (41). Cellular protein contents were determined by BCA protein assay (Pierce).

Peritoneal cells isolated from mice were seeded in 2 × 10^6 cells/well in 12-well plates (Iwaki) for 2 h in RPMI1640 containing 10% lipoprotein-deficient fetal bovine serum (Sigma), and adherent cells were used as primary macrophages. The cells were then incubated with or without 200 μg/ml LDL or 50 μg/ml Ac-LDL. After 24 h, the cells were stained with oil red O. Replicate cells were washed twice with PBS and lysed in 0.5% SDS to determine protein concentrations. The supernatants were subjected to PGE2 enzyme immune assay (Cayman Chemicals) or interleukin-6 enzyme immune assay (ebioscience).

Separation of Lipoproteins—Lipoproteins in mouse sera (100 μl/run after a 1:10 dilution) were separated by high performance liquid chromatography (HPLC) on TSK gel Lipopropak XL (TOSOH) with TSK Eluent PL1 (TOSOH) as a running buffer at a flow rate of 0.35 ml/min. Total cholesterol, phospholipids, and lysophosphatidylcholine (LPC) levels were determined with Determinar TC2 (Kyowa Medex), a phospholipid C test (Wako), and the LSP assay kit (AZWELL), respectively. Triglyceride levels were measured using the biochemical analyzer Fuji Drychem. Alternatively, LDL and high density lipoprotein (HDL) were separated by discontinuous ultracentrifugation (42), and lipids extracted from them were applied to electrospray ionization-mass spectrometry (ESI-MS).

Lipoprotein Electrophoresis—LDL and HDL obtained from mice were electrophoresed (1-μl aliquot/lane) on TITAN GEL Lipoprotein gels (Helena Laboratories) at 90 V for 25 min. Then the gels were stained with detection reagent for 15 min at 30 °C, followed by incubation with 5% acetic acid for 15 min.

ESI-MS—Recombinant human PLA2G2A, PLA2G2E, PLA2G2F, PLA2G5, PLA2G10 (kindly provided by Dr. M. Gelb, University of Washington, Seattle), or PLA2G3 (see above) (0.2 and 1 μg/ml) was incubated with 1 mg/ml human LDL or HDL (both from Sigma) for 4 h in 100 mM Tris-HCl, pH 7.4, containing 10 mM CaCl2. After incubation, lipids were extracted from the reaction mixtures by the method of Bligh and Dyer (43). The ESI-MS analyses were performed using a 4000Q TRAP, quadrupole-linear ion trap hybrid mass spectrometer (MDS Sciex; Applied Biosystems) with a UltiMate 3000 nano/cap/micro-liquid chromatography system (Dionex Corp., Sunnyvale, CA) combined with an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Phospholipids were subjected directly to ESI-MS analysis by flow injection; typically, 3 μl (3 nmol of phosphorus equivalent) of sample was applied. The mobile phase composition was acetonitrile/methanol/water (6:7:2) (plus 0.1% ammonium formate, pH 6.8) at a flow rate of 10 μl/min. The scan range of the instrument was set at m/z 200–1000 at a scan speed of 1000 Da/s. The trap fill time was set at 1 ms in the positive ion mode and at 5 ms in the negative ion mode. The ion spray voltage was set at 5500 V in the positive ion mode and at −4500 V in the negative ion mode. Nitrogen was used as curtain gas (setting of 10, arbitrary units) and as collision gas (set to “high”). The detailed procedure for ESI-MS analysis was described previously (44, 45). A peak of sphingomyelin (m/z 703 in the positive ion mode) was regarded as an internal standard. The measurement of fatty acids and their oxygenated products in mouse plasma was carried out by TrueMass Profiling (Lipomics Technologies; available on the World Wide Web).

PLA2G3 Tg × apoE−/− Mice—PLA2G3 Tg (PLA2G3g0/0) mice were crossed with ApoE knock-out (apoE−/−) mice (Charles River). PLA2G3g0/0 × apoE−/− mice at the F1 generation were mated again with apoE−/− mice, and PLA2G3g0/0 × apoE−/− mice among six genotypes at the F2 generation thus obtained were crossed with apoE−/− mice. The resulting PLA2G3g0/0 × apoE−/− and littermate PLA2G3g0/0 × apoE−/− mice at the F3 were used in subsequent studies. In the same manner, LNL-PLA2G3 Tg (LNL-PLA2G3g0/0) mice and apoE−/− mice were crossed to obtain LNL-PLA2G3g0/0 × apoE−/− mice. These mice were fed with a high fat/high cholesterol diet (16% fat, 1.14% cholesterol, and 0.4% cholic acid; Oriental Yeast Co.) for 10–14 weeks starting at 10–11 weeks of age. After blood was collected from each mouse, the circulation system was perfused with PBS and fixed with PBS containing 4% paraformaldehyde. The aorta was then excised from the root to the abdominal area, and the connective tissue was carefully removed. The aorta was stained with oil red O, and the positive areas in the specimen were calculated using Photoshop CS2 and Canvas software. The root area of the aorta tissue was fixed using a 4% paraformaldehyde-lysine-sodium periodate
s solution. For histochemistry, the aortas were embedded in paraffin (Wako), and cross-sections (5 μm thick) were then prepared.

LDL fraction was separated by ultracentrifugation as described previously (46). An aliquot of the fraction (10 mg of protein equivalent) was subjected to native PAGE (1.8–13% acrylamide). The particle size was estimated using ferritin (12.2 nm), thyroglobulin (17 nm), and latex beads (30 nm) as standards (47).

RESULTS

Generation of PLA2G3 Tg Mice—The Tg construct for PLA2G3 (Fig. 1A) was microinjected into the pronuclei of fertilized eggs of C57BL/6 females and transferred into the ovi-

ducts of ICR pseudopregnant females. The offspring were examined for expression of the transgene (LNL-PLA2G3) by PCR genotyping. Tail biopsies were taken on day 28, and the genomic DNAs isolated were subjected to PCR genotyping with a CAG forward primer and a reverse primer specific for PLA2G3. A founder male mouse showed the presence of a 2.2 kb band for LNL-PLA2G3 (Fig. 1B). The founder mouse, in which the PLA2G3 transgene was still silent, was mated with female CAG-Cre Tg mice to allow the removal of the neo’ cassette from the LNL-PLA2G3 transgene by the Cre/LoxP reaction. Following this, the transcription of the PLA2G3 transgene was directly regulated by the CAG promoter and was thereby activated in all tissues of the offspring. A representative result of PCR genotyping of the F1 progeny is shown in Fig. 1B. As a result of the Cre/LoxP reaction, the band for the PLA2G3 transgene shifted from 2.2 to 0.7 kb whereas no band was detected in the littermate siblings (Fig. 1B). Mice carrying the active PLA2G3 transgene were bred with C57BL/6 mice. The ratio of transgenic-positive to -negative pups was ~1:1, and these pairs (hereafter designated as PLA2G3 Tg mice (PLA2G3 (g/g)) and non-Tg, wild-type (WT) littermate controls) were used in each experiment. Note that mice carrying the inactive transgene (LNN-PLA2G3) showed no apparent abnormality, implying that the phenotypes detected in PLA2G3 Tg mice described below were indeed caused by overexpression of PLA2G3.

PLA2G3 Tg mice showed no neonatal or postnatal mortality and remained healthy until adulthood. RNA blotting showed expression of human PLA2G3 mRNA in all tissues from PLA2G3 Tg mice (Fig. 1C). When PLA2 enzymatic activities in tissue homogenates were measured with 1-palmitoyl-2-linoleoyl-phosphatidylethanolamine as substrate, the activities in Tg tissues were increased 2–3-fold compared with those in control tissues (which reflect the combined activities of the various PLA2 enzymes intrinsically present in each tissue) (Fig. 1D) and roughly correlated with the result of RNA blotting (Fig. 1C). PLA2 activity in the sera of PLA2G3 Tg mice was >10-fold higher than that of WT mice (Fig. 1E). On the basis of enzyme activity, the concentration of PLA2G3 in PLA2G3 Tg mice appeared to be similar to that of PLA2G5 in the PLA2G5 Tg mice we had generated previously (estimated to be within the range 10–100 ng/ml, depending on the tissue) (22).

Expression of PLA2G3 in the Tg mice was further assessed by subjecting sera from PLA2G3 Tg and WT mice to immunoprecipitation with mouse anti-PLA2G3 monoclonal antibody, followed by immunoblotting with rabbit anti-PLA2G3 polyclonal antibody. As shown in Fig. 1F, a main 18 kDa band was detected in the serum of PLA2G3 Tg but not control mice. As evaluated from molecular sizes, this band appeared to correspond to the S domain-only form (III-S) (19, 20), suggesting that PLA2G3 is processed to a fully processed III-S form in the blood circulation. This is, to our knowledge, the first demonstration that PLA2G3 undergoes proteolytic processing in vivo. Although it has been shown that PLA2G3 can be N-glycosylated in several PLA2G3-transfected cell lines in culture (19), bands corresponding to the N-glycosylated forms were barely detected in the serum of PLA2G3 Tg mice (Fig. 1F), arguing that the majority of PLA2G3 does not undergo N-glycosylation in vivo.
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Unlike PLA2G5 Tg mice, which die shortly after birth from a lung disorder resulting from aberrant hydrolysis of the lung surfactant phospholipids phosphatidylcholine (PC) and phosphatidylglycerol (22), the absence of respiratory disorders in PLA2G3 Tg mice suggests that PLA2G3 hardly hydrolyzes lung surfactant under physiological conditions. Indeed, ESI-MS analysis of lung surfactant phospholipids did not show any appreciable difference between control and PLA2G3 Tg mice (data not shown). Furthermore, although PLA2G2A Tg mice show alopecia (24) as well as male infertility because of impaired spermatogenesis (48), PLA2G3 mice had normal pelage hairs and showed no sign of reproductive defects up to 9 months of age.

Altered Plasma Lipoproteins in PLA2G3 Tg Mice—To identify in vivo effects of Tg expression of PLA2G3, various biochemical markers were analyzed in sera from 8–15-week-old PLA2G3 Tg and control mice. Although most parameters, including serum ions and biomarkers for liver and kidney function, did not differ appreciably between PLA2G3 Tg and WT mice, the level of total cholesterol was significantly lower in PLA2G3 Tg mice than in control mice (Fig. 2A and Table S1). The decrease in cholesterol in PLA2G3 Tg mice relative to WT mice was more prominent after overnight fasting than under normally fed conditions and also more prominent in males than in females; the difference between normally fed female Tg and WT mice was subtle.

Since serum cholesterol is predominantly present in lipoprotein particles, it was anticipated that the decrease in total cholesterol in PLA2G3 Tg mice might result from the action of PLA2G3 on phospholipids in lipoprotein particles. To address this issue, we used HPLC to separate lipoprotein fractions from the sera of male mice after overnight fasting. As shown in Fig. 2B, the major peak corresponding to HDL cholesterol was smaller in PLA2G3 Tg mice than in control mice. Likewise, the HDL of PLA2G3 Tg mice contained less phospholipid than did that of control mice (Fig. 2C). Quantitatively, serum HDL as well as HDL-associated cholesterol and phospholipids were decreased by as much as 25–30% in PLA2G3 Tg mice compared with WT mice (Fig. 2D), whereas phospholipid contents in the VLDL/LDL fraction of PLA2G3 Tg and WT mice were similar (data not shown). There was no appreciable difference in triglyceride level between the genotypes (Fig. 2D). Conversely, the amounts of the PL<sub>2</sub>-hydrolytic products LPC and NEFA in sera of PLA2G3 Tg mice tended to be higher than those in control mice (470 ± 4.7 and 515 ± 46 μM LPC and 890 ± 10 and 1030 ± 40 μeq/liter NEFA in WT and PLA2G3 Tg mice, respectively (n = 5)), although these differences were not statistically significant because of the high background (probably albumin-bound) pools.

Next, we separated the lipoprotein fractions from PLA2G3 Tg and control mice by agarose gel electrophoresis. Consistent with the results from the HPLC analyses (Fig. 2, B and C), significant reduction in the intensity of the band for HDL was apparent in PLA2G3 Tg mice as compared with WT mice (Fig. 2E). Moreover, the HDL of PLA2G3 Tg mice showed faster migration than that of control mice. In addition, the band for LDL in PLA2G3 Tg mice appeared broader and also moved faster than that in control mice (Fig. 2F). These findings suggest increased net negative charge on both HDL and LDL particles in PLA2G3 Tg mice. Since the lipid content of the liver, a tissue that plays a central role in lipoprotein metabolism, of PLA2G3 Tg mice was similar to that of WT mice (Fig. 2F), it is likely that the observed changes in PLA2G3 Tg mice shown above are a result of the direct action of PLA2G3 on phospholipids in HDL and LDL.

Lipoprotein Hydrolysis by Various sPLA<sub>2</sub>s in Vitro—To compare the hydrolytic action of PLA2G3 on lipoprotein phospholipids with that of other sPLA<sub>2</sub>s, recombinant PLA2G3 (III-S), PLA2G5, PLA2G10, PLA2G2A, PLA2G2E, and PLA2G2F were each incubated for 4 h with 1 mg/ml LDL or HDL, and phospholipids extracted from the reaction mixtures were subjected to ESI-MS analyses. Both LDL (Fig. 3) and HDL (Fig. 4) particles contained major PC molecular species (C<sub>16:0-18:2</sub>, C<sub>16:0-18:1</sub>, C<sub>16:0-20:4</sub>, C<sub>18:0-18:2</sub>, and C<sub>18:0-20:4</sub>) and trace levels of LPC molecular species (C<sub>16:0</sub> and C<sub>18:0</sub>). When LDL was treated with...
0.2 μg/ml (Fig. 3A) and 1 μg/ml (Fig. 3B) PLA2G3, PLA2G5, or PLA2G10, there were robust and dose-dependent increases in both LPC species. The release of LPC from LDL was accompanied by concomitant decreases in all PC molecular species, an event that was particularly obvious when each of these three enzymes was added at 1 μg/ml (Fig. 3B). Judging from the peak areas of remaining PC species, the most active enzyme was PLA2G10, with which partial and preferential reduction in PC species with C20:4 was seen at a low concentration (0.2 μg/ml). PLA2G5 and PLA2G3 moderately decreased most PC species; PLA2G2A and PLA2G2E acted only weakly on HDL; and PLA2G2F increased LPC species modestly at a high concentration (1 μg/ml) (Fig. 3B).

Increases in LPC species (C16:0 and C18:0) were also prominent when HDL was incubated with 0.2 μg/ml (data not shown) and 1 μg/ml (Fig. 4A) PLA2G3, PLA2G5, or PLA2G10. As revealed by residual PC species, PLA2G10 was apparently most active on HDL, decreasing all PC species markedly; PLA2G5 reduced PC species with C18:1 or C18:2 in preference to those with C20:4; PLA2G3 moderately decreased most PC species; PLA2G2A and PLA2G2E acted only weakly on HDL; and PLA2G2F caused a modest but substantial increase in LPC species, accompanied by a concomitant and preferential decrease in PC species with C20:4 (Fig. 4A).

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Hydrolysis of lipoproteins by various sPLA2s in vitro, on the basis of ESI-MS analysis, is summarized in Fig. 4, B–D. In general terms, the rank order of hydrolytic potency, as evaluated by increases in LPC-C16:0 (Fig. 4B) and LPC-C18:0 (Fig. 4C), was PLA2G10 > PLA2G5 > PLA2G3 > PLA2G2F > PLA2G2A and PLA2G2E, for both HDL and LDL. In accordance with the preference of PLA2G10 for arachidonate and of PLA2G5 for oleate/linoleate, as noted above and as described recently (49), the release of arachidonic acid from LDL and HDL by PLA2G10 was prominent, and that by PLA2G3 and PLA2G5 was similar (Fig. 4D).

To ascertain whether phospholipids in plasma lipoproteins could indeed be hydrolyzed by PLA2G3 in vivo, lipoprotein fractions separated by discontinuous centrifugation from PLA2G3 Tg and control mice fed a normal diet were analyzed for their LPC content by ESI-MS analyses. Notably, both LPC-C16:0 and LPC-C18:0 were increased in the LDL and HDL of PLA2G3 Tg mice relative to those of control mice (Fig. 4E), further supporting the hydrolysis of lipoprotein-associated PC by PLA2G3 in the Tg mice (also see Fig. 7).

PLA2G3-treated LDL Facilitates Macrophage Foam Cell Formation—Having established that PLA2G3 is able to modify lipoproteins both in vitro and in PLA2G3 Tg mice, we next examined whether PLA2G3-treated LDL could facilitate lipid accumulation in macrophages (i.e. formation of foam cells). To
this end, human LDL was preincubated for 24 h with PLA2G3 as well as with PLA2G5, which was roughly as efficient as PLA2G3 in hydrolyzing LDL-associated PC as assessed by ESI-MS analysis (see above). Quantification of NEFA after overnight incubation showed that there was a massive and quantitatively similar release of NEFA from PLA2G3- or PLA2G5-treated LDL (Fig. 5A). When the PLA2G3- or PLA2G5-treated LDL thus prepared was added to the mouse macrophage cell line J774, cholesteryl ester accumulation in these cells after culture for 24 h was severalfold higher than that in replicate cells after incubation with LDL alone (Fig. 5B). When these cells were stained with oil red O, a marked increase in oil red O-positive lipid droplets was observed in cells incubated with PLA2G3-treated LDL, whereas lipid droplet accumulation was sparse in cells incubated with or without intact LDL (Fig. 5C). Consistent with a previous report (35), PLA2G5-treated LDL or Ac-LDL used as a positive control markedly facilitated the accumulation of oil red O-positive lipid droplets in J774 cells (Fig. 5C).

To confirm the production of atherogenic LDL by PLA2G3, peritoneal macrophages from PLA2G3 Tg mice and control mice were incubated with LDL ex vivo. As shown in Fig. 5D, prominent accumulation of oil red O-positive lipid droplets occurred in macrophages from PLA2G3 Tg mice but not in those from WT mice after incubation for 24 h with LDL (but not with medium alone). We also incubated peritoneal macrophages from PLA2G10 Tg mice (22) with LDL and found that these cells also accumulated oil red O-stained lipid droplets (Fig. 6D), confirming that PLA2G10-exposed LDL facilitates macrophage foam cell formation, as reported previously (23, 31, 34). After treatment with Ac-LDL, formation of foam cells from macrophages of all genotypes was obvious (Fig. 6D). Since it has been reported that foam cell formation by macrophages is accompanied by elevated eicosanoid synthesis (50), we measured PGE2 in the culture supernatants of macrophages from PLA2G3 Tg and PLA2G10 Tg mice relative to that from control mice, with or without 24-h treatment with LDL. Whereas PGE2 generation was increased only modestly in WT macrophages by LDL treatment, a marked augmentation of PGE2 generation by LDL treatment was observed in replicate PLA2G3 Tg or PLA2G10 Tg macrophages (Fig. 5E). Interleukin-6 was also elevated in PLA2G3 Tg macrophages compared with control macrophages after incubation with LDL (Fig. 5F), suggesting that PLA2G3 augments proinflammatory response in macrophages.
To further evaluate the relationship between sPLA₂-mediated LDL hydrolysis and macrophage foam cell formation, peritoneal macrophages were incubated for 24 h with LDL that was pretreated with various concentrations of recombinant PLA2G3 or PLA2G10, and the accumulation of LPC (as an indication of LDL hydrolysis) in medium and the appearance of oil red O-positive cells were examined. Substantial and dose-dependent increases in oil red O-positive cells (Fig. S2A) and LPC (Fig. S2B) were obvious after incubation with >10 ng/ml PLA2G3 or with >1 ng/ml PLA2G10 (Fig. S2B), revealing good correlation with the hydrolysis of LDL and the formation of foam cells by these sPLA₂s (with PLA2G10 being 1 order more potent than PLA2G3). Moreover, these experiments suggest that only a few-fold increase of LPC in LDL particles (reaching a few hundred nmol/mg of protein) can facilitate the accumulation of lipid droplets in macrophages.

**Localization of Endogenous PLA2G3 in Atherosclerotic Lesions**—These observations, together with current reports demonstrating the presence of multiple lipoprotein-active sPLA₂s in atherosclerotic lesions (25–36), prompted us to carry out immunohistochemistry with several distinct anti-PLA2G3 antibodies (see “Experimental Procedures”) to assess if endogenous PLA2G3 is present at foci of atherosclerotic lesions. Atherosclerotic lesions are classified into five categories, namely adaptive intimal thickening and types I, II, III, and IV lesions (40). In this study, we examined the immunohistochemical localization of PLA2G3 in normal aorta (without atherosclerosis and fibrocellular thickening), aorta with fatty streaks (type III lesion), and aorta with atheroma (type IV lesion).

Although immunoreactivity for PLA2G3 was sparse in sections of normal human aorta (Fig. 6A, a and b), staining was prominent in atheroma core and fibrous cap covering the core (Fig. 6A, d and e). Moderate staining for PLA2G3 was detected in intimal cells and stroma with fatty streak lesions (Fig. 6A, g), an initial stage of atherosclerosis, suggesting that expression of PLA2G3 is increased concomitantly with the development of atherosclerosis. Higher magnification of the atheromatous areas revealed marked staining of...
lipid cores and scattered staining of neighboring cells in fibrous caps (Fig. 6A, h) and fatty streaks (g) were immunostained with two distinct anti-PLA2G3 antibodies (S3 (a, d, g, and h) and Nov1 (b and c) and with control antibody (c, f, and l). For double immunostaining, the atheroma sections were stained with anti-HAM (j, k, and m) or anti-SMA (l and n) antibody (brown) in combination with anti-PLA2G3 antibody (blue). In lipid cores, staining of PLA2G3 largely overlapped with that of HAM (j). In fibrous caps covering lipid cores, staining of PLA2G3 co-localized with that of HAM (k and m) and SMA (l and n). Magnified images of double immunostaining are shown in m (HAM + PLA2G3) and n (SMA + PLA2G3). B, human atheroma sections were immunostained with anti-PLA2G2A antibody (a), anti-PLA2G5 antibody (b), and anti-PLA2G10 antibody (c). C, sections of the aortic root in apoE-deficient mice fed a high fat diet were immunostained with control (a) and anti-PLA2G3 (b) antisera. Lipid cores (red arrows), foam cells (blue arrows), adventitia (dark arrows), and smooth muscle cells (asterisk) showed PLA2G3 immunoreactivity.

FIGURE 6. Immunohistochemistry of PLA2G3 and other sPLA$_2$s in human atherosclerosis. A, sections of human aortas with normal histology (a–c) and those with atheroma (d–f and h–l) and fatty streaks (g) were immunostained with two distinct anti-PLA2G3 antibodies (S3 (a, d, g, and h) and Nov1 (b and c) and with control antibody (c, f, and l). For double immunostaining, the atheroma sections were stained with anti-HAM (j, k, and m) or anti-SMA (l and n) antibody (brown) in combination with anti-PLA2G3 antibody (blue). In lipid cores, staining of PLA2G3 largely overlapped with that of HAM (j). In fibrous caps covering lipid cores, staining of PLA2G3 co-localized with that of HAM (k and m) and SMA (l and n). Magnified images of double immunostaining are shown in m (HAM + PLA2G3) and n (SMA + PLA2G3). B, human atheroma sections were immunostained with anti-PLA2G2A antibody (a), anti-PLA2G5 antibody (b), and anti-PLA2G10 antibody (c). C, sections of the aortic root in apoE-deficient mice fed a high fat diet were immunostained with control (a) and anti-PLA2G3 (b) antisera. Lipid cores (red arrows), foam cells (blue arrows), adventitia (dark arrows), and smooth muscle cells (asterisk) showed PLA2G3 immunoreactivity.

Plaque core and scattered staining of neighboring cells in fibrous caps. In lipids core, most PLA2G3-positive cells were also stained with anti-HAM56 antibody (Fig. 6A, j), indicating the localization of PLA2G3 in macrophages. PLA2G3-positive signals in the fibrous caps covering lipid cores overlapped with signals for either anti-HAM56 antibody (macrophages) or anti-SMA antibody (smooth muscle cells), with the latter being more dominant (Fig. 6A, k and l). Magnified views of double immunostaining verified the coexistence of brown (macrophages or smooth muscle cells) and blue (PLA2G3) signals in the same cells (Fig. 6A, m and n). Staining of normal and atherosclerotic aortas with control antibody was negative (Fig. 6A, a, c, f, and i). In comparison, signals for PLA2G2A (Fig. 6B, a), PLA2G5 (Fig. 6B, b), and PLA2G10 (Fig. 6B, c) were also intense in the atheroma lesions of human aortas, in agreement with previous reports (25–36).
We also examined the localization of PLA2G3 immunoreactivity in atherosclerotic plaques in the aortas of apoE-deficient mice fed an atherogenic diet. As in the case of human atherosclerotic lesions (Fig. 6A), PLA2G3 immunoreactivity was located in atheromatous lipid cores and nearby foam cells in the aortic root of these mice (Fig. 6C, b). The adventitia as well as smooth muscle cells of the media just beneath the atheroma were also stained for PLA2G3, whereas control antibody yielded no obvious staining throughout the aortic root sections (Fig. 6C, a and b). None of the anti-PLA2G3 antibodies tested stained normal mouse aorta sections (data not shown).

Development of Atherosclerosis in PLA2G3 Tg Mice in Vivo—
In an effort to evaluate the pathological relevance of PLA2G3 in atherosclerosis in vivo, PLA2G3 Tg mice (C57BL/6 background) were fed an atherogenic, high fat/high cholesterol diet. After 14 weeks, total plasma cholesterol was increased ~3-fold, whereas LDL cholesterol was decreased by 30–60%, in mice fed a high fat diet compared with those fed a normal diet in both genotypes (Fig. S3). Under each diet condition, total and HDL cholesterol levels were consistently lower in PLA2G3 Tg mice than in replicate WT mice. Plasma levels of phospholipids and LPC were minimally affected by feeding conditions, with trends that phospholipids levels were slightly lower and LPC levels were higher in the Tg mice than in replicate WT mice (Fig. S3). Although the development of atherosclerosis in these mice was evaluated by staining the aortic sections with oil red O, accumulation of lipid-rich plaques was barely seen even under high fat-fed conditions (data not shown).

To circumvent this limitation, we next crossed PLA2G3 Tg mice with apoE/−/− mice, a mouse line that is known to be highly susceptible to atherosclerosis. PLA2G3zg/0 × apoE/−/− mice of the F1 generation were mated again with apoE/−/− mice, and the two types of offspring, PLA2G3zg/0 × apoE/−/− and PLA2G3non-Tg × apoE/−/−, were used in subsequent experiments. As another control, LNL-PLA2G3 Tg mice (in which the PLA2G3 transgene was silent) were mated with apoE/−/− mice to obtain LNL-PLA2G3zg/0 × apoE/−/− mice. Under normal diet conditions, plasma cholesterol concentrations of all of these mice reached >350 mg/dl, whereas that of apoE+/+ mice was <100 mg/dl (Fig. 7A), indicating that introducing the PLA2G3 or LNL-PLA2G3 transgene into apoE/−/− mice did not significantly change plasma cholesterol levels. However, fractionation of plasma lipoproteins on gel filtration revealed that there was an increase of VLDL/LDL and a reciprocal decrease of HDL in apoE/−/− mice, as compared with apoE+/+ mice and that Tg expression of PLA2G3 in these mice reduced HDL cholesterol levels (Fig. S4). Plasma PLA2 activity in PLA2G3zg/0 × apoE/−/− mice was >20 times higher than that in PLA2G3non-Tg × apoE/−/− or LNL-PLA2G3zg/0 × apoE/−/− mice (Fig. 7B).

After consumption of a high fat/high cholesterol diet for 10 weeks, apoE−/− mice exhibited hypercholesterolemia, with total cholesterol levels in the plasma reaching 2000–3000 mg/dl regardless of PLA2G3 genotype (data not shown). Concentrations of LPC in plasma (Fig. 7C, left) and in LDL (Fig. 7C, right) were increased 2–3-fold in PLA2G3zg/0 × apoE−/− mice compared with those in PLA2G3non-Tg × apoE−/− or LNL-PLA2G3zg/0 × apoE−/− mice, further implying that introduced PLA2G3 is active on LDL phospholipids under in vivo conditions. Since the formation of aggregated LDL as a result of phospholipid hydrolysis by sPLA2α has been proposed to be a causal factor for atherosclerosis (31, 36), LDL fractions separated from these mice by ultracentrifugation were analyzed on native PAGE to assess their particle sizes. Normally, LDL has a particle diameter around 25 nm (as seen in PLA2G3non-Tg × apoE−/− mice), whereas LDL from PLA2G3zg/0 × apoE−/− mice had a slightly larger diameter and an additional, very large, particle with a diameter of ~45 nm (Fig. 7D). These results suggest that,
in PLA2G3<sup>tg/0</sup> × apoE<sup>−/−</sup> mice, increased LPC levels might cause aggregation of LDL to form large particles.

Atherosclerotic lesion formation following a high fat diet was evaluated by measuring the surface lesion area of the aorta (aortic root to abdominal aorta) after staining with oil red O. Average surface lesion area in the absence of active Tg PLA2G3 (i.e. in both PLA2G3<sup>mon-Tg</sup> × apoE<sup>−/−</sup> and LNL-PLA2G3<sup>tg/0</sup> × apoE<sup>−/−</sup>) mice was about 20% (Fig. 7, E and F). Importantly, the atherosclerotic lesion area was increased about 2-fold on average in PLA2G3<sup>tg/0</sup> × apoE<sup>−/−</sup> mice compared with that in the controls (Fig. 7, E and F), implying that Tg PLA2G3 was able to exacerbate atherosclerosis in vivo. Under a normal diet, oil red O-positive lesions were scarce in apoE<sup>−/−</sup> mice and were not influenced by the overexpression of PLA2G3 (data not shown).

Since it has been reported that treatment of LDL with PLA2G2A renders the particle more susceptible to oxidative modification (51), we measured the plasma concentrations of oxidized LDL in these mice. However, there was no substantial difference in plasma oxidized LDL levels between PLA2G3<sup>tg/0</sup> × apoE<sup>−/−</sup> and PLA2G3<sup>mon-Tg</sup> × apoE<sup>−/−</sup> mice (0.030 ± 0.018 and 0.029 ± 0.019 ng/mg of LDL, respectively; n = 6), suggesting that the atherogenicity of PLA2G3 is not a result of formation of oxidized LDL. The blood pressure (49.8 ± 8.4 to 95.2 ± 12.6 and 47.6 ± 7.4 to 89.1 ± 10.4 mm Hg, respectively (mean ± S.D; n = 7–8)) and the ventricular rate (53.8 ± 60.0 and 54.9 ± 75.9 min<sup>−1</sup>, respectively (mean ± S.D.; n = 7–8)) did not differ significantly between PLA2G3<sup>mon-Tg</sup> × apoE<sup>−/−</sup> and PLA2G3<sup>tg/0</sup> × apoE<sup>−/−</sup> mice fed a high cholesterol diet. Finally, measurement of plasma eicosanoid levels revealed that thromboxane A<sub>2</sub> (TXA<sub>2</sub>), a cyclooxygenase product, and 12-hydroxyeicosanoid acid, a 12-lipoxygenase product, were increased robustly in Tg mice over control mice, whereas other eicosanoids were minimally detected in both genotypes (Fig. S5). These results suggest more pronounced activation of platelets in Tg mice than in control mice, in agreement with the notion that development of atherosclerosis is associated with increased TXA<sub>2</sub> production by platelets (52–54). Detailed plasma lipid profiling (PC and LPC species) of high fat-fed PLA2G3 Tg and control mice is shown in Fig. S6, providing further confirmation that various unsaturated fatty acids are released nonselectively from PC in the plasma of PLA2G3 Tg mice.

**DISCUSSION**

In our ongoing effort to gain new insights into the in vivo functions of sPLA<sub>2</sub> enzymes, in this study we established and analyzed Tg mice for PLA2G3, an atypical mammalian sPLA<sub>2</sub> structurally more similar to bee venom PLA<sub>2</sub> than to other mammalian sPLA<sub>2</sub>s. PLA2G3 Tg mice did not display noticeable phenotypic changes in the skin, which is profoundly affected in PLA2G2A mice (24), or lung, whose architecture is disrupted in PLA2G5 Tg mice (22) and macrophage-specific PLA2G10 Tg mice (23), suggesting that phospholipids in mouse skin and lung surfactant may not be main targets for PLA2G3 in vivo. However, we have obtained evidence that PLA2G3 can target phospholipids in plasma lipoproteins in vivo, leading to a decrease in HDL and an increase in modified and aggregated LDL, which are risk factors for atherosclerosis. The action of PLA2G3 on lipoproteins observed in this study is reminiscent of that of its homolog, bee venom PLA<sub>2</sub>, which also has potent hydrolytic activity against lipoprotein-associated phospholipids in vitro (55). Our finding that the lipolytic action of PLA2G3 renders the LDL particle smaller in size (Fig. 2E) is important, since the smaller size of the LDL particle, the higher its atherogenic potential (31). In addition to the in vitro lipoprotein hydrolysis, we found that PLA2G3, as in the case of PLA2G5 and PLA2G10 (31, 34, 36), induces the accumulation of lipid droplets in LDL-loaded macrophages ex vivo (Fig. 5). Thus, these lipoprotein-acting sPLA<sub>2</sub>s commonly cause LDL particles to be more atherogenic. Moreover, after an atherogenic diet, PLA2G3 Tg mice crossed with apoE<sup>−/−</sup> mice developed more severe aortic atherosclerotic lesions than did control mice (Fig. 7), further supporting an unexplored atherosclerotic property of PLA2G3 in vivo.

Oxidation of LDL is believed to occur in the subendothelial space, where circulating antioxidant defenses are less effective. Oxidized LDL becomes a ligand for the scavenger receptors (e.g. scavenger receptor A and CD36) that contribute to foam cell formation by facilitating uptake of lipoprotein particles (56, 57). Current evidence suggests that sPLA<sub>2</sub>-mediated modification of lipoproteins also plays a role in the development of atherosclerosis (25–36). Hydrolysis of PC in lipoproteins by sPLA<sub>2</sub> produces NEFA and LPC, which can trigger vasoactive and proinflammatory actions leading to the acceleration of atherosclerosis (see below). Hydrolysis of LDL by sPLA<sub>2</sub> correlates with production of the more atherogenic, small-dense, modified LDL with increased net negative charge, whereas hydrolysis of HDL reduces the capacity of this antiatherogenic particle to promote cholesterol efflux from lipid-rich foam cells (58). Modified LDL retained in atherosclerotic lesions contains less PC and more LPC than does circulating LDL, suggesting that arterial LDL undergoes lipolytic modification by sPLA<sub>2</sub> at lesion sites. Further, clinical analyses have shown that elevated plasma sPLA<sub>2</sub> activity is an independent risk factor for cardiovascular disease (59), and a low content of surface phospholipids often characterizes the small-dense LDL and HDL sub-classes (60). Over the past few years, attention initially focused on the potential role of PLA2G2A in atherosclerosis, because this enzyme is enriched in human atherosclerotic plaques and because PLA2G2A Tg mice are susceptible to development of atherosclerosis (25–30). However, the recent discovery of PLA2G5 and PLA2G10 in atherosclerotic foci, plus the fact that these two enzymes hydrolyze lipoprotein phospholipids more readily than does PLA2G2A, has raised the question of which sPLA<sub>2</sub> types are the true contributors to atherosclerosis (31–36). The recent finding that overexpression of PLA2G5 by retrovirus-mediated gene transfer leads to increased lesion area, whereas mice deficient in bone marrow-derived PLA2G5 have reduced lesion area, indicates that PLA2G5 does play a role in atherosclerosis in vivo (33). A tagging single nucleotide polymorphism analysis demonstrating an association of the human PLA2G5 gene haplotype with LDL and oxidized LDL supports this view (61). PLA2G10 also renders lipoprotein particles more proatherogenic to promote macrophage foam cell formation in vitro (31, 34). Taking these understandings together, our results support the idea that PLA2G3 is another sPLA<sub>2</sub> enzyme that is
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capable of producing atherogenic lipoprotein particles and thereby has the potential to promote the development of atherosclerosis.

The lipolytic action of PLA2G3 on LDL and HDL is much superior to that of PLA2G2A and is similar to that of PLA2G5, albeit weaker than that of PLA2G10. The overall modes of sPLA₂ action on phospholipids in lipoproteins are PLA2G10 > PLA2G5 ≥ PLA2G3 > PLA2G2F > PLA2G2A and PLA2G2E (Figs. 4–6), an order that appears broadly consistent with their ability to interact with PC-rich vesicles and with PC-rich cellular plasma membranes (1, 2). Although our present study failed to detect appreciable lipoprotein hydrolysis by PLA2GA, a number of previous studies have shown that PLA2G2A can do so after incubation for longer periods at higher concentrations, after oxidative modification of lipoproteins, or after binding to matrix proteoglycans (25–30). On the basis of our ESI-MS analyses of lipoproteins, PLA2G3 does not discriminate appreciably between fatty acids at the sn-2 position, whereas a trend of preference for oleic and linoleic acids over arachidonic and docosahexaenoic acids is observed for PLA2G5, and vice versa for PLA2G10 (Figs. 3 and 4). This fatty acid selectivity may be relevant in vivo, since PC species containing sn-2 saturated and monounsaturated fatty acids (C₁₆₋₀-C₁₆₋₀ and C₁₆₋₀-C₁₆₋₁) are robustly hydrolyzed in the lungs of PLA2G5 Tg mice (22) and since nonselective release of unsaturated fatty acids from plasma PC was observed in PLA2G3 Tg mice (Fig. S6). Although the physiological importance of these observations is unclear at present, similar fatty acid preferences of each sPLA₂ have also been reported in in vitro enzymatic assays (31, 62, 64) and cellular fatty acid release assays (65), and Pruzanski et al. (49) have also reported a similar fatty acid selectivity of PLA2G5 and PLA2G10 toward lipoprotein PC.

Hydrolysis of lipoprotein-bound phospholipids by sPLA₂ gives rise to the two proatherogenic and proinflammatory lipid products, lysophospholipids and fatty acids. Lysophospholipids function as extracellular signaling molecules in multiple biological processes and also affect insulin metabolism (66) and adipocyte growth and function (67). LPC, the most predominant lysophospholipid, modulates the expression of a number of proteins, such as growth factors, leukocyte adhesion molecules, inducible nitric-oxide synthase, and cyclooxygenase-2 (68). LPC plays an etiologic role in atherosclerosis, is a major constituent of atherogenic lipoproteins (69), and exhibits proinflammatory functions, including activation of macrophages and expression of chemotactic factors and adhesion molecules in endothelial cells (70). Lysophosphatidic acid, an autotaxin-hydrolyzed product of LPC that elicits numerous effects on cells of the cardiovascular system, induces the formation of arterial neointima lesions, a prelude to atherosclerosis, through a PPARγ-dependent mechanism (71). Lysophosphatidic acid accumulates in the lipid-rich core of human carotid atherosclerotic plaques (72). Arachidonate-oxygenated lipid mediators, including prostaglandins and leukotrienes, also have diverse effects on atherosclerosis, as evidenced by studies employing knock-out mice for their receptors or biosynthetic enzymes. For instance, gene ablation of TXA₂ receptor or PGE₂ synthase ameliorates, whereas that of PGI₂ receptor or PGD₂ synthase exacerbates, atherosclerosis in apoE⁻/⁻ or LDLR⁻/⁻ mice (54, 73, 74). Null mice for 5- or 12/15-lipoxygenase are also partially protected from the development of atherosclerosis (75, 76). In this study, a marked decrease in PC and an increase in LPC, which reflect increased lipoprotein hydrolysis, were observed in the plasma and in LDL of PLA2G3 Tg mice fed an atherogenic diet (Fig. 7). Furthermore, we found striking increases in two particular eicosanoids, TXA₂, and 12-hydroxyeicosanoid acid, in the plasma of PLA2G3 Tg mice (Fig. S5), implying that the development of atherosclerosis in these mice is accompanied by accelerated platelet activation. Activated platelets are found in the blood of patients with atherosclerosis (52) and hypercholesterolemia (53), and platelets of apoE⁻/⁻ × TP⁻/⁻ mice, which are highly susceptible to atherosclerosis, have lower reactivity than do those of apoE⁻/⁻ mice (54). Thus, increased LPC and TXA₂ in the circulation could also account for the atherosclerotic phenotype of PLA2G3 Tg mice.

Our results showed that only a few-fold increase in LDL-associated LPC by sPLA₂ could promote the formation of foam cells from macrophages in vitro (Fig. S2). The plasma concentration of PLA2G3 in PLA2G3 Tg mice was estimated to be 10–100 ng/ml, a level above the threshold of inducing macrophage foam cell formation by this enzyme in vitro. In fact, the levels of LPC in plasma and LDL of high-fat-fed PLA2G3 Tg × apoE-null mice were several-fold higher than those of control apoE-null mice (Fig. 7C), agreeing with the development of atherosclerosis in the PLA2G3 Tg × apoE-null mice. However, unlike PLA2G2A, whose plasma levels are highly elevated during inflammation and cardiovascular disease (1, 2), there is no current evidence that either PLA2G3, PLA2G5, or PLA2G10 is present in the circulation. It is thus conceivable that, in pathophysiological settings, the modification of lipoproteins by these sPLA₂s may proceed at local foci of atherosclerosis, where the enzymes are enriched and can interact with matrix-captured lipoprotein particles. This speculation may be true for PLA2G5 and PLA2G10, since the expression of these enzymes are upregulated at local sites of inflammation (e.g. alveolar macrophages and bronchial epithelial cells in asthma) (11, 12), since atherosclerosis is characterized by inflammation in the arterial walls where lipid-loaded activated macrophages accumulate (77, 78) and since the expression of PLA2G5 is in fact increased in atherosclerotic lesions in mice fed a high cholesterol diet and in human subjects (32, 36). The observations that transplantation of bone marrow cells overexpressing PLA2G5 (33) and PLA2G2A (27) into recipient LDL receptor-null mice results in increased atherosclerosis in the absence of alteration in systemic lipoprotein metabolism after a high fat feeding also suggest that increased sPLA₂ activity in macrophages within the vessel wall is sufficient to promote atherosclerosis. In this study, we found that endogenous PLA2G3 is also expressed in macrophages and vascular smooth muscle cells of atherosclerotic lesions but only weakly in normal aorta (Fig. 6). Consistent with our immunohistochemical results, detection of PLA2G3 mRNA in macrophages and vascular smooth muscle cells of human atherosclerotic lesions by means of in situ hybridization has been described recently (40), indicating that PLA2G3 is synthesized in the aortic walls. These observations raise the possibility that PLA2G3 may also act on lipoproteins within the subendothelium at the atherosclerotic foci. It is noteworthy
that the immunoreactive, endogenous PLA2G3 is localized in the microvascular endothelium of various tissues (19). Thus, hydrolysis of circulating lipoproteins by PLA2G3 might occur preferentially on the vascular walls, thereby influencing vascular lipid homeostasis.

As an associated finding, we show that PLA2G2F is also capable of substantial hydrolysis of PC in LDL and HDL in vitro, with a preferential reduction in PC species containing arachidonic acid (Figs. 4 and 5). Reportedly, PLA2G2F shows a ~2-fold preference for arachidonic acid over linoleic acid in vitro (63), and its ability to hydrolyze PC is similar to that of PLA2G3 both in vitro and in cultured cells (18). Although the pathophysiological relevance of these observations is still unclear, a recent study has shown the inducible expression of PLA2G2F (in addition to several other sPLA2s) in human atherosclerotic plaques (40). Therefore, it will be interesting to examine whether alterations in plasma lipoproteins and in the process of atherosclerosis also take place in mice with Tg expression or gene ablation of this unique group II sPLA2 enzyme, of which the behavior and function in vivo are scarcely understood.

Since the oxidation hypothesis of atherosclerosis still remains inconclusive and oxidation alone may not explain the accumulation of LPC in atherosclerotic lesions (34), it is plausible that, in addition to oxidative modification of LDL, lipolytic modification of LDL may represent an alternative pathway for the progression of atherosclerosis. In the arterial wall, multiple sPLA2 isozymes may exert proatherogenic actions by inducing the release of lipid mediators, such as LPC and TXA2, and by lipolytic modification of lipoprotein particles independently of oxidation. Understanding of the roles of sPLA2s in atherosclerosis is thus emerging, and the efficacy of a specific inhibitor of PLA2G3, including the biological mechanisms involved, by conducting studies in mice with targeted disruption of the \textit{plag2g3} gene.

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