Potent Modification of Low Density Lipoprotein by Group X
Secretory Phospholipase A<sub>2</sub> Is Linked to Macrophage Foam Cell Formation*

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The deposition of cholesterol ester within foam cells of the artery wall is fundamental to the pathogenesis of atherosclerosis. Modifications of low density lipoprotein (LDL), such as oxidation, are prerequisite events for the formation of foam cells. We demonstrate here that group X secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-X) may be involved in this process. sPLA<sub>2</sub>-X was found to induce potent hydrolysis of phosphatidylcholine in LDL leading to the production of large amounts of unsaturated fatty acids and lyso-phosphatidylcholine (lyso-PC), which contrasted with little, if any, lyso-lipid modification of LDL by the classic types of group IB and IIA secretory PLA<sub>2</sub>s. Treatment with sPLA<sub>2</sub>-X caused an increase in the negative charge of LDL with little modification of apolipoprotein B (apoB) in contrast to the excessive aggregation and fragmentation of apoB in oxidized LDL. The sPLA<sub>2</sub>-X-modified LDL was efficiently incorporated into macrophages to induce the accumulation of cellular cholesterol ester and the formation of non-membrane-bound lipid droplets in the cytoplasm, whereas the extensive accumulation of multilayered structures was found in the cytoplasm in oxidized LDL-treated macrophages. Immunohistochemical analysis revealed marked expression of sPLA<sub>2</sub>-X in foam cell lesions in the arterial intima of high fat-fed apolipoprotein E-deficient mice. These findings suggest that modification of LDL by sPLA<sub>2</sub>-X in the arterial vessels is one of the mechanisms responsible for the generation of atherogenic lipoprotein particles as well as the production of various lipid mediators, including unsaturated fatty acids and lyso-PC.

Initiation of atherosclerosis is characterized by the appearance of fatty streaks underlying the endothelium of large arteries. Recruitment of macrophages and their subsequent uptake of low density lipoprotein (LDL)<sup>1</sup>-derived cholesterol are the major cellular events contributing to fatty streak formation (1, 2). Oxidative modifications in the lipid and apolipoprotein B (apoB) components of LDL are thought to drive the formation of fatty streaks (2, 3), because oxidized LDL can be incorporated into the macrophages via scavenger receptors leading to the formation of foam cells that contain massive amounts of cholesterol esters. In addition, there is substantial evidence that LDL oxidation occurs in both animals and humans during the progression of atherogenesis (4). However, prospective clinical trials with antioxidants, such as vitamin E and beta carotene, in patients with pre-existing atherosclerosis, have thus far been disappointing (5). These findings suggest that other types of LDL modifications, such as that resulting from lyso-lipid enzymes (6), also play pivotal roles in the formation of foam cells.

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) are a diverse family of lyso-lipid enzymes that hydrolyze the sn-2 fatty acid ester bond of glycerophospholipids to produce free fatty acids and lysophospholipids (7, 8). Over the past two decades, a number of PLA<sub>2</sub>s have been identified and classified into different families based on their biochemical features and primary structures (9, 10). Among them, secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) have several characteristics, including a low molecular mass (13–18 kDa) and an absolute catalytic requirement for millimolar concentrations of Ca<sup>2+</sup> (10, 11). At present, nine different groups of sPLA<sub>2</sub>s have been identified in humans (IB, IIA, IID, IIE, IIF, III, V, X, and XII) (10, 12–14). Recent studies have shown that group IIA sPLA<sub>2</sub> (sPLA<sub>2</sub>-IIA) is expressed in the atherosclerotic arterial intima and is associated with extracellular matrix structures and lipid droplets (15–18). In addition, sPLA<sub>2</sub>-IIA was shown to induce the lipolysis of LDL leading to enhanced retention of LDL to human aortic proteoglycans (19, 20), suggesting a potential role of sPLA<sub>2</sub>-IIA in the accumulation of LDL in the proteoglycan matrix on the subendothelial layer of the arterial intima. However, potent modifications of LDL leading to increased uptake by macrophages were reported with the type III bee venom sPLA<sub>2</sub> but not with sPLA<sub>2</sub>-IIA (21). The differences in the potency of LDL modification might be due to discrepancies in substrate specificity in the mixed micelle assay, because sPLA<sub>2</sub>-IIA preferably hydrolyzes anionic phospholipids (22) such as phosphatidylglycerol and phosphatidylserine and has a very low enzymatic activity toward phosphatidylcholine (PC), a major phospholipid component of LDL (23). Recently, we and other groups (24) have shown that, among the endogenous sPLA<sub>2</sub>s in mammals, group X sPLA<sub>2</sub> (sPLA<sub>2</sub>-X) is one of the enzymes with a potent hydrolyzing activity toward PC. sPLA<sub>2</sub>-X has 16 cysteine residues located at positions characteristic of the classic types of group IB sPLA<sub>2</sub> (sPLA<sub>2</sub>-IB) and sPLA<sub>2</sub>-IIA and has an amino acid C-terminal extension that is typical of group II sPLA<sub>2</sub> subtypes (25). We have shown that

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* The abbreviations used are: LDL, low density lipoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; sPLA<sub>2</sub>, secretory PLA<sub>2</sub>; sPLA<sub>2</sub>-IIA, group IIA sPLA<sub>2</sub>; FC, phosphatidylcholine; sPLA<sub>2</sub>-X, group X sPLA<sub>2</sub>; sPLA<sub>2</sub>-IB, group IB sPLA<sub>2</sub>; COX, cyclooxygenase; lyso-PC, lyso-phosphatidylcholine; FCS, fetal calf serum; BSA, bovine serum albumin; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; VLDL, very low density lipoprotein; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; TBARS, thiorbarbituric acid-reactive substances; PBS, phosphate-buffered saline; Ab, antibody.
sPLA2-X can induce potent release of arachidonic acid leading to cyclooxygenase (COX)-dependent prostaglandin formation, as well as marked production of lysophosphatidylcholine (lyso-PC) in various cell types, including macrophages, spleen cells, and colon cancer cells (26–28). During the process of these cell-based experiments, we found that sPLA2-X elicits potent release of unsaturated fatty acids from the culture medium containing fetal calf serum (FCS) in cell-free systems. These observations prompted us to examine its potential role in lipolysis of human serum lipoproteins.

In the present study, we first evaluated the potencies of three types of human sPLA2 (sPLA2-IB, -IIA, and -X) with respect to the release of fatty acids and the contents of PC and lyso-PC in LDL. We then compared the characteristics of sPLA2-X-modified LDL with oxidized LDL in terms of phospholipid composition, negative charge, and apoB aggregation as well as for the efficacy in uptake into macrophages. We found that sPLA2-X induced potent lipolysis of LDL leading to the formation of numerous lipid droplets in the macrophages. Finally, we showed elevated expression of sPLA2-X in the foam cells in the atherosclerotic arterial wall in high-fat-fed mice deficient in apolipoprotein E (apoE).

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified recombinant human sPLA2-IB, sPLA2-X, and mouse sPLA2-X proteins were prepared as described previously (24, 27). Recombinant human sPLA2-IIA was a generous gift from Dr. Ruth Kramer (Eli Lilly, Indianapolis, IN). Rabbit anti-human sPLA2-X Ab was prepared as described previously (24), and anti-sPLA2-IB and anti-sPLA2-IIA Abs were purchased from Cayman Chemicals. Bovine serum albumin (BSA), indomethacin (COX inhibitor), and nordihydroguaiaretic acid (NDGA, lipoxigenase (LOX) inhibitor) were obtained from Sigma Chemical Co. Indoxam (sPLA2 inhibitor) was synthesized at Shionogi Research Laboratories (29).

**Preparation of Human LDL and Modification with sPLA2s and CuSO4 Oxidation**—Very low density lipoprotein (VLDL, density less than 1.006 g/ml), LDL (density 1.019–1.063 g/ml), and high density lipoprotein (HDL, density 1.055–1.210 g/ml) were isolated from plasma of healthy and fasting donors by sequential ultracentrifugation, as described previously (30). For modification of LDL with sPLA2s, 1 mg/ml LDL was preincubated for 10 min at 37°C in a final concentration of 5 mM. For oxidative modification, 1 mg/ml LDL was incubated with 20 μM CuSO4 at 37°C and then dialyzed against 150 mM NaCl containing 0.24 mM EDTA (pH 7.4). LDL prepared by incubation without any modification was used as native LDL.

**Measurement of Released Fatty Acids, PC, and Lyso-PC in sPLA2-treated LDL**—Human LDL (1 mg/ml) was preincubated for 10 min at 37°C and stimulated with various concentrations of sPLA2 enzymes in a final volume of 40 μl. The reaction was stopped by the addition of 160 μl of Dele’s reagent, and the released fatty acids were extracted, labeled with 9-α-antilinolizomethane (Funakoshi, Japan), and analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a Lichrospher 125-4 Superphere 100 RP-18 column (Merck), as described previously (24, 31).

For the measurement of the amounts of PC and lyso-PC in LDL, lipids were extracted with organic solvent as described previously (26). The extracted phospholipids were then separated by normal-phase HPLC on Ultrasphere silica 4.6 × 250 mm (Beckman) with a guard column of 4.6 × 45 mm using a solvent of acetoniitrile/methanol/0.05% phosphoric acid (100:7.9:0.5, v/v) with a flow rate of 1 ml/min at room temperature. Fractions corresponding to authentic PC or lyso-PC (Sigma Chemical Co.), detected at the wavelength of 202 nm, were pooled and subjected to quantitative phosphorus analysis (32).

**Analysis of Oxidation, Electrophoretic Mobility, and ApoB Modification in LDL Modified with sPLA2s and CuSO4 Oxidation**—Following modification with sPLA2s and CuSO4 oxidation, lipid peroxidation was assessed by the following procedures. The peroxides were quantified in terms of thiobarbituric acid-reactive substances (TBARS) according to the method of Nagano et al. (33). Conjugated dienes were determined by monitoring the changes in absorbance of A436 at a final concentration of 200 μg/ml LDL, as reported previously (34). The electrophoretic mobility of LDL was analyzed by agarose gel electrophoresis (Titan Gel Lipoproteins, Helena Laboratories, Japan), as described previously (35). For analysis of apoB modification, LDL was delipidated and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% acrylamide, as described previously (36).

**Measurement of the Amount of Cholesterol Ester in Macrophages**—Mice peritoneal macrophages were obtained from the peritoneal cavity of male C57BL/6J mice (8 weeks) 5 days after injection of 3% thioglycollate (Difco Laboratories). The cells were washed, resuspended in serum-free medium, X-VIVO 15 (BioWhittaker), and plated in 24-well plates (Costar) (5 × 105 cells/well). Non-adherent cells were removed by washing and adherent macrophages were incubated with native LDL or modified LDL (200 μg/ml) for 48 h. The lipid extracts of adherent cells were prepared, extracted, dissolved with isopropanol, and the cholesterol mass was quantified by enzyme fluorometry (37). The amount of esterified cholesterol was calculated by subtracting the free cholesterol from total cholesterol. The amount of cellular proteins were quantified with BCA Protein Assay Reagent (Pierce) after dissolving the cells in 0.2 N NaOH.

**Oil Red O Staining of Macrophages Incubated with sPLA2-treated and Oxidized LDL**—Macrophages were prepared as described above and cultured in four-well glass slides (Lab-Tek II chamber slide, Nalge Nunc International Corp.). Acetylation of LDL was performed as described previously (38). After incubation with native LDL or modified LDL (200 μg/ml) for 24 or 48 h at 37°C, the cells were fixed with 0.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Samples were rinsed in 7% sucrose in 0.1 M phosphate buffer, post-fixed in 1% OsO4, dehydrated, and then embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEOL 1200EX microscope.

**Immunohistochemistry—ApoE-deficient mice (8 weeks) were obtained from The Jackson Laboratory, and age-matched C57BL/6J mice were obtained from Clea Japan. They were fed a high fat and high cholesterol diet (15.8% cocoa butter, 1.25% cholesterol, 0.5% sodium cholate) for 9 weeks then flushed with PBS via the abdominal aorta under pentobarbital anesthesia and perfused with 4% paraformaldehyde in PBS. Segments of the proximal aorta and the portions of the heart containing the aortic arch were dissected into small pieces, which were then immersed in the same fresh fixative at 4°C overnight. Fixed samples were thoroughly rinsed with PBS and subsequently dehydrated by passage through an alcohol series diluted with double-distilled water followed by n-butyl alcohol. The tissue preparations were then passed into paraaffin at 56°C. Transversal tissue sections (6-μm thickness) were cut from embedded paraaffin blocks and mounted on slides freshly thin-coated with 3-aminopropyltriethoxysilane. Immunohistochemistry was performed after paraaffin dewaxing. The tissue slides were incubated in methanol containing 0.3% H2O2 for 30 min and then treated with 5% normal rabbit serum for 20 min. The slides were incubated with anti-sPLA2-X Ab (6 μg/ml), anti-sPLA2-IB Ab (5 μg/ml), or anti-sPLA2-IIA Ab (7 μg/ml) in PBS containing 0.1% BSA for 1 h at room temperature. After rinsing with PBS, they were incubated with goat-conjugated rabbit-anti-guinea pig IgG (30 min) followed by treatment with horsedarish peroxidase avidin-biotin complex reagent (Vector Laboratories). After washing, the peroxidase activity was visualized by 0.25% 3,3′-diaminobenzidine and 0.006% H2O2. After counterstaining of the nuclei with 0.4% hematoxylin, the preparations were mounted in Malinol resinous medium (Muto Pure Chemicals, Japan). Immunohistochemistry was performed after paraaffin dewaxing. The tissue slides were incubated in methanol containing 0.3% H2O2 for 30 min and then treated with 5% normal rabbit serum for 20 min. The slides were incubated with anti-sPLA2-X Ab (6 μg/ml), anti-sPLA2-IB Ab (5 μg/ml), or anti-sPLA2-IIA Ab (7 μg/ml) in PBS containing 0.1% BSA for 1 h at room temperature. After rinsing with PBS, they were incubated with biotin-conjugated goat-anti-rabbit IgG for 30 min followed by treatment with horseradish peroxidase avidin-biotin complex reagent (Vector Laboratories). After washing, the peroxidase activity was visualized by 10-min incubation in 50 mM Tris-HCl (pH 7.6) containing 200 μg/ml 3,3′-diaminobenzidine and 0.006% H2O2. After counterstaining of the nuclei with 0.4% hematoxylin, the preparations were mounted in Malinol resinous medium (Muto Pure Chemicals, Japan). Positive signals were detected as dark brown diaminobenzidine deposits of the nuclei with 0.4% hematoxylin, the preparations were mounted in Malinol resinous medium (Muto Pure Chemicals, Japan). Positive signals were detected as dark brown diaminobenzidine deposi-
RESULTS

Potent Release of Unsaturated Fatty Acids from LDL by sPLA2-X—We first examined the potency of three types of human sPLA2 for the release of fatty acids in human plasma at a concentration of 50 nM and found that sPLA2-X induced the most potent release of unsaturated fatty acids (data not shown). We then prepared three types of lipoprotein fraction (VLDL, HDL, and LDL) from freshly isolated human plasma and then examined the potencies of sPLA2s for the release of fatty acids. As shown in Fig. 1, sPLA2-X elicited marked release of various types of unsaturated fatty acids from human LDL in the following order: linoleic acid (C18:2) > arachidonic acid (C20:4) > oleic acid (C18:1) > docosahexaenoic acid (C22:6), whereas sPLA2-IB and -IIA caused little release. In contrast, there was little, if any, release of saturated fatty acids, including myristic acid, palmitic acid, and stearic acid, from LDL after sPLA2-X treatment. The profiles of free fatty acids released by sPLA2-X were almost identical among LDL, HDL, and VLDL. In addition, there were no significant changes in the contents of sphingomyelin in LDL and HDL (data not shown). As shown in Fig. 2A, sPLA2-X induced time-dependent release of arachidonic acid from LDL. In contrast, no significant release was observed during treatment with sPLA2-IB or -IIA for 4 h. Fig. 2B shows the dose-dependent release of arachidonic acid by three types of sPLA2 during 1-h incubation. sPLA2-X induced significant release at 5 nM, whereas sPLA2-IB evoked slight but significant release at 500 nM. There was little, if any, release with sPLA2-IIA treatment even at 500 nM, demonstrating that sPLA2-X elicits more potent release of unsaturated fatty acids from human LDL as compared with sPLA2-IB and -IIA.

Effect of sPLA2-X on the PC and Lyso-PC Contents in LDL—Because PC is a major component of phospholipids in LDL (23), we next examined the PC contents in LDL after treatment with sPLA2s or CuSO4. As shown in Fig. 3A, PC contents were time-dependently decreased after treatment with 50 nM sPLA2-X and oxidation. Over half of the PC was diminished in LDL by sPLA2-X within 3 h, and PC was completely degraded after 24-h treatment. Corresponding to the reduction of PC contents, the amounts of lyso-PC in LDL was increased up to 24 h after sPLA2-X treatment (Fig. 3B). Incubation with the sPLA2-specific inhibitor indoxam (10 μM) or anti-sPLA2-X Ab (100 μg/ml), both of which have been shown to block the enzymatic activity of sPLA2-X (24), resulted in significant suppression of sPLA2-X-induced lipolysis of LDL (data not shown). In contrast, treatment with sPLA2-IB or -IIA caused little change in either PC or lyso-PC contents in LDL. Oxidation of LDL with CuSO4 also caused significant production of lyso-PC. However, the amount of lyso-PC produced during 24-h oxidation was about 30% of that induced by sPLA2-X, although PC was degraded similarly by both treatments. In addition, there was no significant release of long-chain unsaturated fatty acids examined during oxidation of LDL (data not shown). Treatment of LDL with CuSO4 caused an increase in TBARS (Table I) as well as the production of conjugated dienes (data not shown), whereas treatment with three types of sPLA2 did not alter these oxidative parameters. Taken together, these findings demonstrate that sPLA2-X induces PC hydrolysis in LDL leading to the production of large amounts of lyso-PC and unsat-
Effects of human sPLA₂ and Cu²⁺-oxidation on PC and lyso-PC contents in LDL. A, effects on PC contents in LDL. LDL (1 mg/ml) was incubated with 50 nM purified human sPLA₂ or 20 µM CuSO₄ for various times at 37°C, and the amounts of PC were quantified as described under “Experimental Procedures.” The results are expressed as the percentage of the amount present in the LDL before the incubation (1400 nmol/mg of protein). B, effects on lyso-PC contents. After incubation, the amounts of lyso-PC were quantified as described under “Experimental Procedures.” Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

**TABLE I**

**Amounts of TBARS in LDL treated with human sPLA₂ or CuSO₄**

Human LDL was incubated with 50 nM human sPLA₂ or 20 µM CuSO₄ for 3 h at 37°C, and the amounts of TBARS were measured as described under “Experimental Procedures.” Each value for TBARS represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments. Statistical significance was determined by Student’s t test.

| Treatment | TBARS (nmol/mg protein) |
|-----------|-------------------------|
| None      | 5.70 ± 1.40             |
| sPLA₂-IB  | 8.09 ± 3.08             |
| sPLA₂-IIA | 4.15 ± 0.89             |
| sPLA₂-X   | 5.60 ± 2.40             |
| CuSO₄     | 36.5 ± 8.56*            |

* p < 0.01 compared to no treatment.

Effects of sPLA₂-X on Electrophoretic Mobility and ApoB in LDL—Modifications of apolipoproteins or surface lipids in LDL were shown to affect the cellular uptake of LDL and hence the formation of foam cell macrophages (2). We then examined the effects of sPLA₂-X treatment on the electronic charge of LDL by agarose gel electrophoresis. As shown in Fig. 4, oxidized LDL was characterized by increased anodic migration compared with native LDL. Treatment with sPLA₂-X also caused enhanced mobility of LDL. Small but significant migration was detected after 3 h of treatment, and marked migration was observed after 24 h of treatment with sPLA₂-X. In contrast, the mobility of LDL after treatment with sPLA₂-IB or -IIA was not changed during 24-h incubation. Addition of anti-sPLA₂-X Ab (100 µg/ml) resulted in complete blockade of sPLA₂-X-induced mobilization (data not shown), demonstrating that the increase of negative charge in LDL is dependent on the enzymatic activity of sPLA₂-X.

Next, we examined the effects of sPLA₂-X on the modification of apoB by SDS-PAGE analysis. As shown in Fig. 5, excessive aggregation and proteolytic fragmentation of apoB was detected in oxidized LDL even at 3 h of incubation. In contrast, apoB in the sPLA₂-X-treated LDL was almost intact with slight aggregation at 24 h of treatment compared with native LDL. There were no changes in apoB of LDL treated with sPLA₂-IB and -IIA even at 500 nM (data not shown). These findings demonstrate that sPLA₂-X induced an increase in the negative charge of LDL with little modification of apoB.

**Cellular Uptake of sPLA₂-X-treated LDL by Macrophages—**

Next, we examined the potency of sPLA₂-X-treated LDL for uptake into macrophages. After exposure of mouse peritoneal macrophages to modified LDL for 48 h, the cellular levels of free and esterified cholesterol were measured. As shown in Fig. 6, the esterified cholesterol mass was significantly increased in the macrophages after incubation with sPLA₂-X-treated LDL, and its level was about 6-fold higher than that induced by native LDL but half that evoked by oxidized LDL. The cellular lipid droplets were then stained with oil red O and analyzed by light microscopy. As shown in Fig. 7A, there was little staining in the macrophages treated with native LDL. In contrast, the formation of numerous intracellular lipid droplets was observed in macrophages incubated with sPLA₂-X-modified LDL (Fig. 7B), and lipid droplets were obviously larger than those observed in macrophages treated with oxidized LDL (Fig. 7C). Lipid droplets could not be detected after incubation with sPLA₂-IB or sPLA₂-IIA-treated LDL (Fig. 8, A and B). When anti-sPLA₂-X Ab (100 µg/ml) was added during the modification of LDL with sPLA₂-X followed by incubation with macrophages, the formation of lipid droplets was completely abolished (Fig. 8C). In contrast, lipid droplet formation was not affected when anti-sPLA₂-X Ab was added to block the enzymatic activity of sPLA₂-X only during incubation with macrophages (Fig. 8D). Furthermore, pretreatment of macrophages...
with sPLA2-X alone did not cause lipid droplet formation after incubation with native LDL, and the COX inhibitor indomethacin and LOX inhibitor NDGA did not affect lipid droplet formation in the macrophages treated with sPLA2-X-treated LDL (data not shown). These findings suggest that sPLA2-X-induced formation of intracellular lipid droplets is completely dependent on the modification of LDL and is not related to either the activation of scavenger functions of macrophages or the action of eicosanoid metabolites.

**Electron Microscopic Analysis of Lipid Droplets in Macrophages Incubated with Modified LDL**—To clarify the morphological differences in oil red O staining studies (Fig. 7), we further examined the cellular lipid droplets by electron microscopy. In contrast to the macrophages incubated with native LDL (Fig. 9A), numerous non-membrane-bound lipid droplets with about 1-μm profile diameters were detected in the cytoplasm of macrophages incubated with sPLA2-X-treated or acetylated LDL (Fig. 9, B and C). Especially, accumulation of cholesterol crystals was detected in the lysosomes of the cells incubated with acetylated LDL for 48 h (data not shown). In contrast, there were few non-membrane-bound lipid droplets in the macrophages incubated with oxidized LDL (Fig. 9D). Instead, extensive accumulation of multilayered structures was found in their cytoplasm (Fig. 9E). Similar structures have been reported in the lipid-laden cells of atherosclerotic lesions or in the oxidized LDL-treated macrophages, and these have been designated as lysosomal lipid bodies (39) or multilamellar lipid structures containing large amounts of cholesterol (40). However, these multilamellar structures were not found in cells incubated with sPLA2-X-treated or acetylated LDL, thus suggesting that sPLA2-X-modified LDL undergo similar intracellular processing to acetylated LDL.

Expression of sPLA2-X in Atherosclerotic Lesions of ApoE-deficient Mice—To examine the localization of sPLA2-X in atherosclerotic lesions, we used apoE-deficient mice, a commonly used rodent model of atherogenesis (41). Although apoE-deficient mice develop atherosclerotic lesions with normal diet, an atherogenic diet dramatically accelerates the development of atherosclerotic lesions (41). Therefore, we examined the expression profiles of sPLA2 enzymes in apoE-knockout mice fed with a high fat and high cholesterol diet for 9 weeks. In the vessels isolated from age-matched normal C57BL/6J mice, there were no atherosclerotic lesions and few, if any, positive signals for sPLA2-X expression (Fig. 10A). In the sections prepared from apoE-deficient mice, positive signals were detected in the multilayered foam cell lesions present in the arterial intima as well as in smooth muscle cells in the medial layer of the artery wall (Fig. 10B). These signals were specific for sPLA2-X, because pretreatment of the Ab with a 10-fold excess of mouse sPLA2-X protein abolished the signals (Fig. 10C), and no signals were detected in parallel control samples with non-immune IgG (data not shown). To identify the sPLA2-X-expressing cell types, the macrophages were stained with rat anti-mouse macrophage F4/80 Ab. The sPLA2-X signals in the arterial intima were found to coincide well with the locations of the macrophages (data not shown), similar to the location in mouse splenic macrophages (27). There were no detectable signals with anti-sPLA2-IB Ab (Fig. 10D), although its availability for immunohistochemical analysis had been confirmed in the pancreas. Because apoE-deficient mice have a mixed genetic background derived from the two inbred strains with naturally disrupted sPLA2-IIA genes (42), there was no signal with anti-sPLA2-IIA Ab (data not shown).

**Discussion**

The development of foam cells is a hallmark of both early and late atherosclerotic lesions, and cholesterol accumulation in macrophages is mediated primarily by uptake of modified forms of LDL (2). The present study demonstrated that sPLA2-X induces lipolytic modification of LDL leading to the enhanced accumulation of cholesterol ester in macrophages. In addition, the elevated expression of sPLA2-X was detected in atherogenic lesions in apoE-deficient mice. These findings suggest that, in addition to oxidative modifications, enzymatic modification by sPLA2-X is one of the mechanisms for the generation of atherogenic LDL. sPLA2-X-modified LDL shows features in common with oxidized LDL in terms of the reduction of PC associated with increased lyso-PC production as well as the increase in negative charge. However, marked differences were observed in several aspects between both modifications. The increase in lyso-PC production during sPLA2-X treatment was accompanied with release of large amounts of unsaturated fatty acids, which was in contrast with the lack of release responses during LDL oxidation. Because oxidation causes lipid peroxidation and oxidized fatty acids are efficiently
cleaved by lipoprotein-associated PLA₂ (43), the polyunsaturated fatty acids could not be released during this process. Higher levels of lyso-PC production were detected in LDL with sPLA₂-X treatment than with oxidized LDL despite the similar PC degradation (Fig. 3), which might also be due to differences in the PLA₂ subtypes involved. Because lyso-PC is believed to play an important role in atherosclerosis and inflammatory diseases by altering various cellular functions, such as the induction of various chemokines and cell adhesion molecules (44, 45), sPLA₂-X-modified LDL might evoke proatherogenic cellular events similarly to oxidized LDL (3, 46).

Another difference between LDL modifications with sPLA₂-X and oxidation is the modification of apoB (Fig. 5). Oxidation with cupric ions caused exaggerated aggregation and fragmentation of apoB in contrast to few, if any, changes seen following sPLA₂-X treatment. It has been shown that LDL oxidation elicits the production of fatty acid hydroperoxides that can directly cause oxidation of apoB associated with an increase in the negative charge of LDL as well as the generation of structures recognized by the scavenger receptors (47). The absence of chemical modification of apoB during sPLA₂-X treatment suggests the existence of different mechanisms for the increase in the negative charge of LDL. We found that the majority of unsaturated fatty acids released during sPLA₂-X treatment are nonspecifically bound to LDL. Furthermore, the addition of 4% BSA resulted in the removal of fatty acids from sPLA₂-X-modified LDL leading to a decrease in its negative charge. However, the accumulation of cholesteryl ester as well as the formation of intracellular lipid droplets in macrophages (Figs. 7 and 8) were also observed in LDL treated with sPLA₂-X in the presence of 4% BSA.² These findings strongly suggest that the negative charge of LDL due to the bound fatty acids does not

² S. Yamamoto, Y. Ishimoto, and K. Hanasaki, unpublished data.
account for the increased uptake into the macrophages. Although the precise mechanisms remain uncertain, potent lipolysis of LDL might alter the conformation of apoB leading to the increased uptake into macrophages, as reported for LDL treated with bee venom PLA2 (48). Alternatively, the released unsaturated fatty acids might be incorporated into the macrophages leading to the production of bioactive lipid mediators. For example, linoleic acid could be metabolized by 12/15-LOX leading to the production of 13-hydroxyoctadecadienoic acid that can act as an endogenous ligand for peroxisome proliferator-activated receptor γ involved in foam cell formation (49). Because fatty acids and oxidized LDL were reported to induce the expression of adipocyte fatty acid-binding protein, αP2, one of the key molecules involved in foam cell formation (50), sPLA2-X might also regulate αP2 expression in macrophages via modification of LDL. Further studies are required to understand the cellular mechanisms underlying the potent uptake of sPLA2-X-modified LDL.

In the atherosclerotic lesions in vivo, three types of intracellular lipid deposits have been reported in macrophage foam cells: non-membrane-bound lipid droplets, lysosomal lipid bodies and cholesterol crystals (39, 51–54). In the present study, numerous non-membrane-bound lipid droplets and cholesterol crystals were detected in the cytoplasm of macrophages incubated with acetylated LDL (Fig. 9), whereas numerous multilamellar structures were found in the cytoplasm of the cells treated with oxidized LDL. This observation was consistent with previous reports demonstrating that acetylated and oxidized LDL are distributed in different intracellular compartments after internalization into macrophages (55–57). The differences in compartmentalization may be attributable to the inefficient degradation of oxidized LDL in macrophage lysosomes in ways that lead to lysosomal lipid accumulation (58) or the differences in uptake pathways between acetylated and oxidized LDL (56). The formation of non-membrane-bound lipid droplets by sPLA2-X-treated LDL (Fig. 9B) suggests a degradation pathway similar to that of acetylated LDL. The large lipid droplets having more than 0.4-μm profile diameters have been observed in the fatty streak regions in human arteries (59). In addition, the non-membrane-bound lipid droplets have been demonstrated to be the main form of lipid accumulation at the early stage of the lesions, whereas the lysosomal lipid accumulation found in oxidized LDL treatment was more prominent in the advanced stage of atherosclerotic lesions in animal models (51, 52). In this context, sPLA2-X might be one of the endogenous molecules involved in the initiation of atherogenesis, because acetylated LDL is an artificial product.

In the present study, we focused on the differences between sPLA2-X-treated and oxidized LDL in various aspects. However, these modifications can work synergistically in vivo, because sPLA2-IIA has been shown to cause oxidative modification of LDL by the cooperative action with 12/15-LOX that is present in the atherogenic lesions (60–62). Because abrogation of the 12/15-LOX gene caused marked reduction in lipid peroxidation and atherogenesis in apoE-deficient mice (63, 64), the synergistic action of oxidation and sPLA2 enzymes in LDL modification deserves further examination. In apoE-deficient mice, the expression of sPLA2-X was elevated in foam cells in the arterial intima as well as in vascular smooth muscle cells (Fig. 10). Because sPLA2-X is a secreted enzyme (24, 25), it could be released from foam cells and smooth muscle cells to act in the circulation and/or in the local area in atherogenic lesions. Although we analyzed the expression profiles of sPLA2-X in apoE-deficient mice after the extreme treatment by feeding a high fat and high cholesterol diet, we also detected its expression in foam cell lesions of the arterial intima in Watanabe heritable hyperlipidemic rabbits. Further studies of the expression of sPLA2-X in human atherosclerotic lesions as well as its circulating levels in patients with atherosclerosis are required to understand its pathological roles in humans.

Among the human sPLA2s examined, potent lipolysis of LDL was observed only on treatment with sPLA2-X, which may have been due to its higher hydrolyzing activity toward PC, the major phospholipid species in LDL (23, 24). Weak fatty acid release was detected during treatment of LDL with higher concentrations of sPLA2-IB (Fig. 2), which was consistent with previous reports describing the hydrolyzing potency of porcine sPLA2-IB on LDL (65). However, sPLA2-IB is mainly expressed in the pancreas, and its expression was not detected in the atherosclerotic lesions of humans (15) or apoE-deficient mice (Fig. 9), indicating that its role in atherogenesis is quite minor. With regard to sPLA2-IIA, significant modification of LDL was not detected even at 500 nM during 24-h incubation. However, Hakala et al. (19) have recently shown that incubation of LDL with proteoglycan-bound sPLA2-IIA for a longer time (48 h).

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results in weak lipolysis of LDL leading to particle fusion and enhanced retention of LDL to human aortic proteoglycans. It has also been reported that the expression of sPLA₂-IIA is markedly elevated in human atherosclerotic arterial intima, where the majority of sPLA₂-IIA is localized along the extracellular matrix, associated with collagen fibers and other structures (16, 18). These findings suggest that sPLA₂-IIA is involved in the accumulation of extracellular lipoproteins in the proteoglycan-rich subendothelial layer of the arterial intima, whereas sPLA₂-X induces more powerful lipolysis of LDL linked to foam cell formation within the arterial wall. Most recently, Gesquiere et al. (66) have reported that sPLA₂-V, another type of sPLA₂ with a potent hydrolyzing activity toward PC (10), can also hydrolyze lipoprotein PC with about 30 times more efficient than sPLA₂-IIA. Our preliminary studies also revealed the potent lipolytic modification of LDL and HDL, although further studies are required to characterize the potency of sPLA₂-V in lipoprotein modifications linked to the lipid droplet formation in macrophages. However, sPLA₂-V was shown to release more linoleic acid (over 10-fold) than arachidonic acid from lipoproteins (66), which was consistent with its fatty acid specificity using synthetic substrate (67). In contrast, sPLA₂-X can induce potent release of arachidonic acid from LDL with on the level of more than half of linoleic acid (Fig. 1), suggesting its relevance to the eicosanoid biosynthesis. Although sPLA₂-V is known to be secreted by macrophages (72), Sartipy et al. (67) have recently shown that type III sPLA₂ from bee venom can cause similar modifications of LDL, although the identities of the endogenous sPLA₂ enzymes involved remain uncertain. In the present study, sPLA₂-X showed powerful lipolytic activity on LDL to induce modification of LDL leading to enhanced uptake into macrophages. Although morphological changes of LDL during sPLA₂-X treatment have not yet been studied, the contribution of this sPLA₂ subtype to the formation of small, dense LDL deserves further examination. Potent release of unsaturated fatty acids was also observed in HDL and VLDL treated with sPLA₂-X. Our preliminary experiments revealed that sPLA₂-X can induce modification of HDL similarly to LDL in terms of the reduction of PC contents and increases in lyso-PC and negative charge. These findings suggest that sPLA₂-X is a key molecule that induces the alternation of native lipoproteins into the atherogenic phenotype. Recent studies have shown that sPLA₂-IIA transgenic mice have lower plasma levels of HDL compared with wild-type littermates (74), because overexpression of this sPLA₂ increased the rate of catabolism and altered sites of tissue uptake of HDL (75). The contribution of sPLA₂-X to lipoprotein modifications should also be clarified in future genetic studies, including the generation of knockout mice. Because sPLA₂-X elicits potent lipolytic effects in human plasma as well as FCS in vitro, previous reports concerning sPLA₂-X-induced lipid mediator production in cultured cell systems, such as transfection experiments (76, 77), should be re-evaluated with respect to the contribution of lipolysis of serum lipoproteins.

In conclusion, we have demonstrated here that sPLA₂-X induces modification of LDL leading to foam macrophage formation without oxidation or proteolysis of LDL. In addition, a variety of lipid mediators are produced during lipolysis of LDL by sPLA₂-X, which might also contribute to the progression of atherosclerosis. Given the atherogenic features of oxidized LDL, prospective clinical trials with antioxidants have been performed but to date have been unsuccessful (5). Because sPLA₂ inhibitors, but not COX or LOX inhibitors, suppressed the sPLA₂-X-induced lipolysis of LDL, the availability of sPLA₂ inhibitors as anti-atherogenic drugs should be evaluated in future studies.

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