Research article

# DIFFERENCES BETWEEN GROUP X AND GROUP V SECRETORY PHOSPHOLIPASE A$_2$ IN LIPOLYTIC MODIFICATION OF LIPOPROTEINS

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**Abstract:** Secretory phospholipases A$_2$ (sPLA$_2$s) are a diverse family of low molecular mass enzymes (13-18 kDa) that hydrolyze the *sn*-2 fatty acid ester bond of glycerophospholipids to produce free fatty acids and lysophospholipids. We have previously shown that group X sPLA$_2$ (sPLA$_2$-X) had a strong hydrolyzing activity toward phosphatidylcholine in low-density lipoprotein (LDL) linked to the formation of lipid droplets in the cytoplasm of macrophages. Here, we show that group V sPLA$_2$ (sPLA$_2$-V) can also cause the lipolysis of LDL, but its action differs remarkably from that of sPLA$_2$-X in several respects. Although sPLA$_2$-V released almost the same amount of fatty acids from LDL, it released more linoleic acid and less arachidonic acid than sPLA$_2$-X. In addition, the requirement of Ca$^{2+}$ for the lipolysis of LDL was about 10-fold higher for sPLA$_2$-V than sPLA$_2$-X. In fact, the release of fatty acids from human serum was hardly detectable upon incubation with sPLA$_2$-V in the presence of sodium citrate, which contrasted with the potent response to sPLA$_2$-X. Moreover,

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$sPLA_2$-X, but not $sPLA_2$-V, was found to specifically interact with LDL among the serum proteins, as assessed by gel-filtration chromatography as well as sandwich enzyme-immunosorbent assay using anti-$sPLA_2$-X and anti-apoB antibodies. Surface plasmon resonance studies have revealed that $sPLA_2$-X can bind to LDL with high-affinity ($K_d = 3.1$ nM) in the presence of $Ca^{2+}$. Selective interaction of $sPLA_2$-X with LDL might be involved in the efficient hydrolysis of cell surface or intracellular phospholipids during foam cell formation.

**Key words:** Secretory phospholipase $A_2$, Low-density lipoprotein, High-density lipoprotein, Phospholipids, Calcium ion

## INTRODUCTION

Phospholipase $A_2$s ($PLA_2$s) are a diverse family of lipolytic enzymes that hydrolyze the *sn*-2 fatty acid ester bond of glycerophospholipids to produce free fatty acids and lysophospholipids [1, 2]. Over the past three decades, a number of $PLA_2$s have been identified and classified into different families based on biochemical features and primary structure [3, 4]. Among them, secretory $PLA_2$s ($sPLA_2$s) have several characteristics including a low molecular mass (13-18 kDa) and an absolute catalytic requirement for millimolar concentrations of $Ca^{2+}$ [4, 5]. At present, nine different groups of $sPLA_2$s have been identified in humans (IB, IIA, IID, IIE, IIF, III, V, X and XII) [4, 6-8]. Group IB $sPLA_2$ ($sPLA_2$-IB) has been thought to act as a digestive enzyme, given its abundance in digestive organs [9]. Besides a role in lipid digestion, this sPLA2 has been shown to play a role in cell proliferation, lipid mediator release, acute lung injury, and endotoxic shock through binding to a specific receptor known as the phospholipase $A_2$ receptor ($PLA_2$R) [10-13]. Group IIA $sPLA_2$ ($sPLA_2$-IIA) is thought to play a pivotal role in the progression of inflammatory conditions, since its local and systemic levels are elevated in numerous inflammatory diseases [14, 15]. It was previously shown that $sPLA_2$-IIA is expressed in the atherosclerotic arterial intima and is associated with extracellular matrix structures and lipid droplets [16-19]. Group V $sPLA_2$ ($sPLA_2$-V) was identified as a homologue of another group II family $PLA_2$ [20]. However, Bingham *et al.* [21] have unraveled the difference in the subcellular location of these $sPLA_2$s in bone-marrow-derived mast cells and demonstrated that these enzymes exert different functions and thus are not redundant. It was also reported that exogenously added $sPLA_2$-V was internalized and localized to perinuclear membranes and induced fatty-acid release leading to leukotriene synthesis [22]. Secretory $PLA_2$-X has 16 cysteine residues located at positions characteristic of the classical types of $sPLA_2$-IB and $sPLA_2$-IIA, and also has an amino acid C-terminal extension that is typical of group II $sPLA_2$ subtypes [23]. We have shown that $sPLA_2$-X can induce the release of arachidonic acid leading to cyclooxygenase (COX)-dependent prostaglandin formation, as well as marked production of lysophosphatidylcholine (lysoPC) in various cell types, including macrophages, spleen cells, and colon cancer cells [24-26]. Moreover, we

demonstrated that sPLA$_2$-X, as well as sPLA2-IB, is also an endogenous ligand of PLA2R and the soluble form of the receptor could regulate the biological functions of secretory phospholipase A$_2$ [27].

Although some of the biological roles of these sPLA$_2$s are expressed through binding to PLA$_2$R, most of their functions are exerted by their lipid-digesting property to produce free fatty acids and lysophospholipids. Notably, sPLA$_2$-V and X were shown to induce the release of fatty acids from various types of cells as expected from their higher specificity for phosphatidylcholine (PC), which is a major lipid component in the outer membrane of cells [28, 29, 30-32]. Moreover, it was reported by us and other groups that PLA$_2$-V and X efficiently hydrolyze PC, which is a major component in serum lipoproteins, as well as that on cell membrane [28, 33]. For example, we have shown that sPLA$_2$-X can induce potent lipolysis of LDL, leading to the production of large amounts of unsaturated fatty acids and lysoPC [34]. The sPLA$_2$-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 [34]. Furthermore, sPLA$_2$-X was found to induce lipolytic modification of HDL linked to the loss of its anti-atherogenic property [35]. Moreover, we have found that sPLA$_2$-X is expressed markedly in foam cell lesions in the arterial intima of high fat-fed apolipoprotein E-deficient mice. Meanwhile, Gesquiere *et al.* [33] have reported that sPLA$_2$-V shows strong hydrolyzing activity toward PC and can induce the release of fatty acids from both LDL and HDL more potently than sPLA$_2$-IIA. These observations strongly suggest that these sPLA$_2$s play important roles in the development of atherosclerosis through their modification of lipoproteins. However, the difference in enzymatic and biological properties of these sPLA$_2$s in lipolysis of lipoproteins has not been well characterized.

Therefore, we compared here the potency and characteristics of human sPLA$_2$-V and PLA$_2$-X in the hydrolysis of LDL. Although both sPLA$_2$s efficiently hydrolyzed PC in LDL to the same extent, sPLA$_2$-X released more arachidonic acid and less linoleic acid than sPLA$_2$-V. We also found a difference of Ca$^{2+}$ dependency on the hydrolysis of phospholipids between these two sPLA$_2$s. Finally, we found a specific association of sPLA$_2$-X, but not sPLA$_2$-V, with LDL in human serum by means of gel filtration and a specific sandwich enzyme-linked immunosorbent assay (ELISA) system. High-affinity interaction between sPLA$_2$-X and LDL was also confirmed by analysis with BIAcore instruments.

## MATERIALS AND METHODS

### Materials

Purified recombinant human sPLA$_2$-IB, sPLA$_2$-X and sPLA$_2$-X-HisTag proteins were prepared as described previously [28, 25]. The recombinant human sPLA$_2$-V protein was also prepared as described previously [23]. Recombinant human sPLA$_2$-IIA was generously provided by Dr. Ruth Kramer (Eli Lilly, USA).

Mouse anti-human sPLA$_2$-X was prepared by the standard method. Rabbit anti-human sPLA$_2$-X antibody (Ab) was prepared as described previously [28], and anti-sPLA$_2$-IIA Ab was purchased from Cayman Chemicals (Ann Arbor, MI). Bovine serum albumin (BSA) and other chemicals were purchased from Sigma (St. Louis, MO). The sensor chip NTA was purchased from BIAcore AB (Uppsala, Sweden).

## Preparation of human LDL and HDL

Very low-density lipoprotein (VLDL; density less than 1.006 g/ml), LDL (d = 1.019-1.063 g/ml), and high-density lipoprotein (HDL; d = 1.085-1.210 g/ml) were isolated from plasma of healthy and fasting donors by sequential ultracentrifugation, as described previously [36]. LDL and HDL were used as a substrate from a plasma pool. This study was approved by our institutional review committee and the procedures followed were in accordance with our institutional guidelines. All subjects gave informed consent with this study.

## Measurement of released fatty acids, PC and lysoPC in sPLA$_2$-treated lipoproteins

Human LDL (1 mg/ml) was pre-incubated for 10 min at 37°C, and stimulated with various concentrations of sPLA$_2$ enzymes in a final volume of 40 μl. The reaction was stopped by the addition of 160 μl of Dole's reagent, and the released fatty acids were extracted, labeled with 9-anthryldiazomethane (Funakoshi, Japan), and then analyzed by reverse-phase high-performance liquid chromatography (HPLC) on a LiChroCART 125-4 Superspher 100 RP-18 column (Merck), as described previously [36, 37]. In some experiments, total amounts of released fatty acids were measured using NEFA C-test Wako (Wako, Japan). For measurement of the amounts of PC and lysoPC in LDL, lipids were extracted with organic solvent as described previously [23].

## Gel-filtration analysis of human serum modified with sPLA$_2$s by FPLC

For the modification of human serum (or plasma) with sPLA$_2$s, human serum was incubated with 50 nM sPLA$_2$-IB, IIA, V or X at 37°C in a buffer composed of 12.5 mM Tris-HCl (pH 8.0), 0.25 M NaCl and 0.0125% BSA. The reaction was stopped by addition of EDTA at a final concentration of 5 mM. The sPLA$_2$ modified human serum was analyzed with Superose HR 6 10/30 (Amersham-Pharmacia, Uppsala, Sweden) by fast protein liquid chromatography (FPLC). A degassed and filtered solution of 0.15 M sodium chloride, 0.3 mM disodium-EDTA and 3.1 mM sodium azide, pH 7.3, was used for pre-equilibration of the columns. Samples were chromatographed with the same solution at a flow rate of 0.5 mL/min, and fractions of 500 μl each were collected. Individual fractions were assayed for PLA$_2$ activity and immunoblotting as described below.

## Immunoblotting analysis

Each fraction was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using either a 4-20% gradient gel (Daiichi

Chemical Co., Ltd.) for detection of Apolipoprotein B-100 (ApoB-100) or a 15-25% gradient gel for detection of sPLA$_2$s. The separated proteins were electroblotted onto nitrocellulose membranes (Atto, Japan) using a semidry blotter (Atto, Japan), according to the manufacturer's instructions. The membranes were probed with the relevant antibodies and visualized using a chemiluminescent detection reagent (ECL Western blotting detection reagents, Amersham Pharmacia Biotech) according to the manufacturer's instructions and analyzed using a Fluor-S MAX MultiImager (Bio-Rad).

## PLA$_2$ activity assay by chromogenic assay

Secretory PLA$_2$ activity was measured using diheptanoyl thio-PC as a substrate according to the method of Reynold *et al.* [38]. Briefly, mixed micelles consisting of 1 mM diheptanoyl thio-PC and 0.3 mM Triton X-100 were used as a substrate. The assay mixture contained 25 mM Tris-HCl buffer (pH 7.5), 0.12 mM DTNB, 10 mM CaCl$_2$, 0.1M KCl, and 1 mg/ml BSA. The reactions were initiated by addition of each gel filtration fraction to the assay mixture containing the substrate in a final volume of 200 μl. The reaction was monitored at an absorbance wavelength of 405 nm with a microplate reader.

## PLA$_2$ activity assay by the PG/Chol method

Phospholipase A$_2$ activity in each gel filtration fraction was measured by the method of Tojo *et al.* [39] with some modifications. Briefly, the substrate used was the mixed micelles of 1 mM 1-palmitoyl-2-olelyl-sn-glycero-3-phosphoglycerol (Avanti Polar Lipid, Inc., AL) and 2 mM sodium cholate, and each gel filtration fraction was incubated at 40ºC for 30 min in 100 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 10 mM CaCl$_2$, 1 mg/ml fatty acid-free BSA (Sigma) and substrate (final volume of 100 μl). The reaction was almost linear during this incubation. The enzymatic reaction was stopped by the addition of 400 μl of Dole's reagent (2-propanol : heptane : 2N H$_2$SO$_4$ = 40:10:1 (v/v/v)), and 6 nmol of margaric acid (Nu-Chek Prep, Inc., Elysian, MN) was added as an internal standard. Fatty acids were extracted by a modified Dole's extraction procedure [40]. The heptane layer containing fatty acids was dried *in vacuo* and the residue was dissolved in 50~100 μl of 0.05% anthryldiazomethane (Funakoshi Co., Tokyo, Japan) in 10% ethyl acetate and 90% methanol followed by incubation at room temperature for 15 min. An aliquot (10~20 μl) of the sample was injected into a reverse-phase HPLC system. A LiChroCART 75-4 Superspher 60 RP-8 column was employed (Merck, Darmstadt, Germany). Sample components were eluted isocratically at a flow rate of 1.1 ml/min with a mobile phase of acetonitrile-water (93:7, v/v) and UV absorbance was monitored at a wavelength of 254 nm. The column oven temperature was 26ºC.

## Detection of sPLA$_2$-X/LDL complexes with sandwich ELISA

ELISA plates (NUNK  MAXISORP #442404) were coated with 100 μl/well of anti-human sPLA$_2$-X IgG dissolved in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). The plates were incubated for 18 h at 4°C and then

blocked with 25% Block Ace (Dainippon Pharmaceutical) in $H_2O$ for 2 h at room temperature. The wells were washed with TBS containing 2 mM $CaCl_2$ or 2 mM EDTA and incubated with samples diluted with 1% BSA/TBS containing 2 mM $CaCl_2$ or 2 mM EDTA. The plate was washed as above, followed by incubation with a 1:1000 dilution of peroxidase-conjugated anti-human ApoB antibody (The Binding Site). After washing, the plate was developed with 100 µl/well of tetramethylbenzidine, and the reaction was stopped by adding 100 µl/well of 1N $H_2SO_4$. Absorbance was read at 450 nm.

**Surface plasmon resonance (SPR) analysis**
The binding of sPLA$_2$-X to LDL and HDL was analyzed on a Biacore 3000 surface plasmon resonance system (Uppsala, Sweden) using an NTA chip. All the binding experiments were carried out at 25$^o$C and a flow rate of 20 µl/min except nickel loading (5 µl/min). SPR buffers and solutions were as follows: eluent buffer: 10 mM HEPES, 0.15 M NaCl, 0.05 mM EDTA, 0.005% Tween-20 pH 7.4; nickel solution: 0.5 mM $NiCl_2$ in eluent buffer; regeneration solution: 10 mM HEPES, 0.15 M NaCl, 0.35 M EDTA, 0.005% Tween-20 pH 8.3. Secretory PLA$_2$-X-HisTag and lipoproteins were diluted with eluent buffer. If the analyses were performed in the presence of $CaCl_2$, all the buffers contained 1 mM $CaCl_2$ other than the nickel solution. The NTA sensor chip was loaded with 20 µl of the nickel solution to saturate the NTA surface with $Ni^{2+}$ after washing with the regeneration solution followed by eluent buffer. Forty microliters of 200 nM sPLA$_2$-X-HisTag was injected to immobilize the ligand. Subsequently, 50 µl of each lipoprotein (LDL or HDL) at 0.2 µM was injected to analyze the interaction between sPLA$_2$-X and lipoproteins, and the sensorgram was allowed to run for up to 300 s, following which the chip was regenerated. For kinetic analysis, the anaylte concentration of LDL were 10, 25, 50, 100, and 125 µg/ml. The kinetic parameters for lipoproteins were estimated by BIAevaluation 3.0 software (BIAcore AB) using A + B = AB (kinetic 1:1 binding with drifting baseline) by separate fitting to fit the data.

**RESULTS**

**Hydrolysis of the phospholipids in LDL by sPLA$_2$-V and sPLA$_2$-X**
We have demonstrated that sPLA$_2$-X markedly induced the lipolysis of LDL [34] and another group reported that sPLA$_2$-V caused the lipolysis of both LDL and HDL [33]. As shown in Fig. 1A, a large amount of free fatty acid was dose-dependently released upon the incubation of LDL with both sPLA$_2$-V and sPLA$_2$-X, while little was released on the incubation of LDL with sPLA$_2$-IB and IIA. Total amounts of fatty acids released by sPLA$_2$-V were comparable to those released by sPLA$_2$-X at the same time point (Fig. 1B). To dissect the profiles of lipolysis by both sPLA$_2$-V and sPLA$_2$-X, we first examined the phosphatidylcholine (PC) content in LDL after treatment since both sPLA$_2$s have been shown to have higher specificity to hydrolyze PC, which is a major component of phospholipids in LDL [34, 33]. PC content decreased with time after treatment

with 50 nM of both sPLA$_2$s, to an equal extent (data not shown). Corresponding to this reduction, the production of lysoPC was detected in LDL at 3 h and increased up to 24 h after both treatments (data not shown). We next determined the composition of free fatty acids (FFA) released from LDL with the respective sPLA$_2$s at a concentration of 50 nM. As shown in Fig. 1C, sPLA$_2$-X elicited the 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) > linolenic acid (C18:3), whereas little palmitic acid (C16:0), a saturated fatty acid, was observed at 3 h (Fig. 1C) though slightly more was released at 24 h (data not shown). The amount of fatty acid increased time-dependently. Secretory PLA$_2$-V released various types of unsaturated fatty acids from human LDL in the following order: linoleic acid (C18:2) > oleic acid (C18:1) > arachidonic acid (C20:4) = docosahexaenoic acid (C22:6) > linolenic acid (C18:3) (Fig. 1C). Again there was little release of saturated fatty acids such as palmitic acid (C16:0) from LDL after the sPLA$_2$-V treatment. Moreover, much less arachidonic acid than linoleic acid was released from LDL by sPLA$_2$-V, and the amount of arachidonic acid was only about 30% of that in
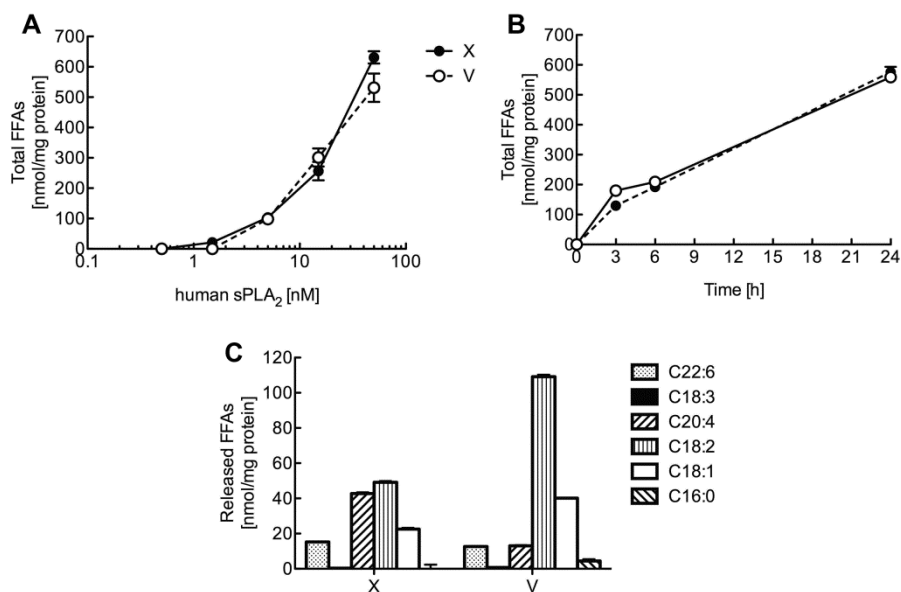


Fig. 1. Fatty acids released from LDL by human sPLA$_2$s. A – LDL (1 mg/ml) was incubated with the indicated concentration of purified human sPLA$_2$s at 37°C for 24 hours. B – LDL (1 mg/ml) was incubated with 50 nM purified human sPLA$_2$s at 37°C for the period indicated, C – LDL (1 mg/ml) was incubated with 50 nM purified human sPLA$_2$-V or X for 3 hours at 37°C. The released fatty acids were quantified as described in Materials and Methods. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

the case of sPLA$_2$-X (Fig. 1C). Secretory PLA$_2$-V was shown to release about 7-fold more linoleic acid than arachidonic acid from LDL (Fig. 1C), which was consistent with its fatty acid specificity using a synthetic substrate [41]. In contrast, sPLA$_2$-X induced marked release of arachidonic acid from LDL with almost the same level of linoleic acid (Fig. 1C). No dose-dependent difference in the released fatty acid profile was observed on treatment with the two sPLA$_2$s (data not shown). Also, longer incubation increased the amount released, but did not change the profile (data not shown). These findings showed that although both sPLA$_2$s release unsaturated fatty acids from LDL effectively, there is an obvious difference in the fatty acids they prefer, especially arachidonic acid.

**Release of fatty acids from whole human serum and plasma by sPLA$_2$-V and X**
Next we investigated the effect of both sPLA$_2$s on whole serum or plasma prepared using sodium citrate to clarify the differences between the lipolysis by these sPLA$_2$s under physiological conditions. Both whole human serum and plasma were incubated with either sPLA$_2$-V or sPLA$_2$-X at 37ºC for 24 h. A large amount of fatty acid was released from human serum and plasma on treatment with sPLA$_2$-X (Fig. 2). By contrast, much less fatty acid was released by sPLA$_2$-V from human plasma than from human serum (Fig. 2). Whole human serum contained a sufficient (>1 mM) concentration of calcium cations (Ca$^{2+}$), while human plasma prepared using sodium citrate (0.38%) buffer contained much less calcium due to quenching. Then human serum containing the same concentration of sodium citrate was prepared and incubated with both sPLA$_2$s and the release of fatty acids was determined. The amount released by sPLA$_2$-X was almost the same in human plasma and human serum with or without sodium citrate. However, sodium citrate decreased the amount of fatty acid released by sPLA$_2$-V from human serum to near that released from plasma.
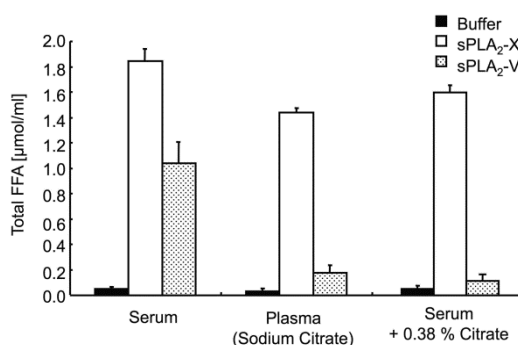


Fig. 2. The release of fatty acids from whole human serum and plasma by sPLA$_2$-V or sPLA$_2$-X. Whole human serum and plasma were incubated with 50 nM purified human sPLA$_2$-V or sPLA$_2$-X for 24 hours at 37°C. The total amount of fatty acids released was quantified as described in Materials and Methods. In the case of human serum, incubation was performed in the absence and presence of 0.38% sodium citrate. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

**Difference of calcium dependency between sPLA$_2$-X and sPLA$_2$-V**
The difference in the release of fatty acids by these two sPLA$_2$s in Fig. 2 was mostly caused by the concentration of Ca$^{2+}$ within reaction mixtures and implied a difference in Ca$^{2+}$ dependency between these two sPLA$_2$s. Therefore, we examined the PLA$_2$ activity using PLPC (1-palmitoyl, 2-linoleoyl-phosphatidyl choline) liposomes and LDL in various Ca$^{2+}$ concentrations. Under our experimental conditions, these sPLA$_2$s showed almost the same maximum. As shown in Fig. 3A, the activity of sPLA$_2$-X to lyse PLPC reached a maximum at 1 mM and was still about 20% at 0.03 mM. The activity of sPLA$_2$-V did not reach a plateau even at 10 mM of CaCl$_2$, and was slight at less than 0.1 mM. The difference in PLA$_2$ activity between them was more remarkable for LDL hydrolysis (Fig. 3B). After incubation at 37ºC for 24 h, sPLA$_2$-X displayed about 40% activity even at 0.1 mM and reached maximum activity at 1 mM. Even if CaCl$_2$ was not contained in the reaction mixture, sPLA$_2$-X still exhibited residual PLA$_2$ activity compared to that in the presence of EDTA (data not shown). In contrast, sPLA$_2$-V hardly showed any PLA$_2$ activity at low concentrations though sPLA$_2$-V activity was increased to about 60% at 1 mM and sPLA$_2$-V did not reach a plateau even at more than 1 mM. Accordingly, sPLA$_2$-V requires about a 10-fold concentration of calcium cation compared to sPLA$_2$-X, and sPLA$_2$-X is capable of acting at low concentration of calcium.
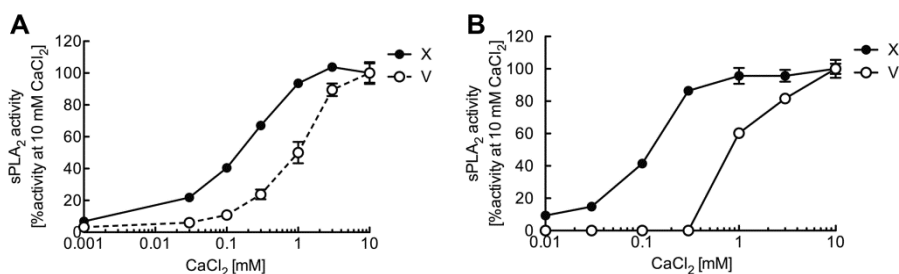


Fig. 3. The difference of calcium dependency between sPLA$_2$-V and X for PLA$_2$ activity. PLPC (A) and LDL (B) were incubated with sPLA$_2$-V or X (50 nM) at 40°C for 30 min (A) and 37°C for 24 hours (B) respectively under various concentrations of CaCl$_2$. Enzymatic activity was respectively measured as described in Materials and Methods. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

**Selective association of sPLA$_2$-X with LDL in human serum**
Under physiological conditions such as in vascular vessels, concentration of calcium cations in the extracellular space is considered as more than 1 mM. Thus, it is unknown which sPLA$_2$ hydrolyzes lipoproteins *in vivo*. It has been shown that some enzymes such as platelet activating factor acetylhydrolase bind to lipoproteins [42-44]. In our study we tried to determine the interactions between lipoproteins and sPLA$_2$s. To investigate whether sPLA$_2$s associate with lipoproteins in physiological conditions, they were incubated with human serum

at a physiological temperature. After 24 h of incubation, each reaction mixture was subjected to a gel filtration analysis. As shown in Fig. 4A, for the reaction mixture of human serum/sPLA$_2$-X, PLA$_2$ activity was concentrated between fraction 17 and 25 where authentic purified LDL also eluted. In contrast, the same fractions chromatographed from human serum incubated with either sPLA$_2$-IB or IIA did not show any PLA$_2$ activity (Fig. 4A). As shown in Fig. 4A, sPLA$_2$-IIA as well as sPLA$_2$-IB was eluted in the fraction corresponding to low molecular weight proteins including serum albumin. Activity of sPLA$_2$-V ranged broadly from the LDL fraction to the HDL fraction. Also, the peak activity of sPLA$_2$-V was not consistent with the LDL fraction. Further, the existence of sPLA$_2$-X in the LDL fraction was confirmed by immunoblotting using antibody
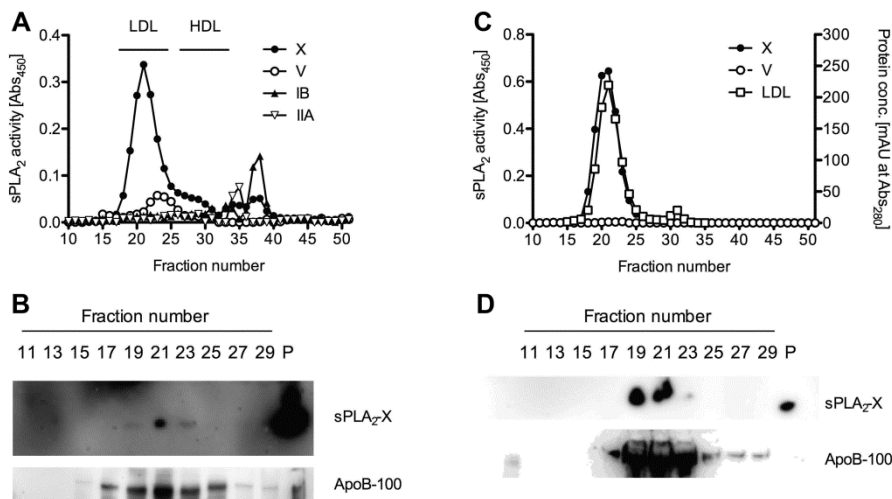


Fig. 4. Human sPLA$_2$-X selectively binds to LDL on incubation with human serum. Human serum (A, B) or 1 mg/ml isolated LDL (C, D) was incubated with various types of 50 nM purified human sPLA$_2$ at 37°C for 24 hours. After 24-h incubation, the reaction mixture was separated by gel filtration and subsequently each fraction was subjected to a PLA$_2$ activity assay (A, C) and western blotting (B, D) using anti-ApoB-100 antibody or anti-sPLA$_2$-X antibody as described in Materials and Methods. The protein concentration of each fraction was monitored by Abs$_{280}$ with Pharmacia FPLC (C). The data are representative of three experiments.

against sPLA$_2$-X and apoB protein, which is a major component of LDL particles (Fig. 4B). Immunoblotting also showed that no other isotypes of sPLA$_2$s including sPLA$_2$-IIA were detected in the LDL fraction (data not shown). Moreover, these results were ascertained in the case of human plasma (data not shown). Next, we examined the interaction between sPLA$_2$s and the isolated LDL. After 24-h incubation of LDL with either sPLA$_2$-X or -V each reaction mixture was subjected to gel filtration analysis. As shown in Fig. 4CD, PLA$_2$ activity of sPLA$_2$-X, not sPLA$_2$-V, was detected in the LDL fraction,

determined by protein concentration. Western blotting by using anti-sPLA$_2$-X and anti-apoB antibodies also revealed the coexistence of sPLA$_2$-X with LDL.

Taken together, these results indicated that among these sPLA$_2$s, only sPLA$_2$-X selectively binds to LDL in human blood. To confirm that only sPLA$_2$-X binds specifically to LDL, purified LDL was incubated with the sPLA$_2$s and chromatographed and analyzed as above. PLA$_2$ activity was detected in the LDL fraction from the reaction mixture incubated with sPLA$_2$-X but not other subtypes including sPLA$_2$-IB, IIA, and V (data not shown). Further, it was confirmed that sPLA$_2$-X existed in the LDL fraction by immunoblotting (data not shown). Secretory PLA$_2$-X from the reaction without LDL was chromatographed around fraction 35 where low molecular weight proteins were eluted (data not shown). Therefore sPLA$_2$-X, but not other sPLA$_2$s, specifically binds to LDL retaining its enzymatic activity.

**Detection of sPLA$_2$-X/LDL complexes with sandwich ELISA**

To confirm directly that sPLA$_2$-X associated with LDL to form sPLA$_2$-X/LDL complexes, we set up a sandwich ELISA system. We immobilized anti-human sPLA$_2$-X antibodies on the plate to trap sPLA$_2$-X/LDL complexes, and detected the trapped complexes by recognition of apolipoprotein B (ApoB) on the surface of LDL particles with peroxidase-conjugated anti-ApoB antibody. In the presence of CaCl$_2$, we detected strong signals in LDL treated with sPLA$_2$-X by sandwich ELISA (Fig. 5A). In contrast, in native LDL alone or in sPLA$_2$-X alone, we did not detect any signals (data not shown). These results show that we could specifically detect sPLA$_2$-X/LDL complexes by sandwich ELISA.
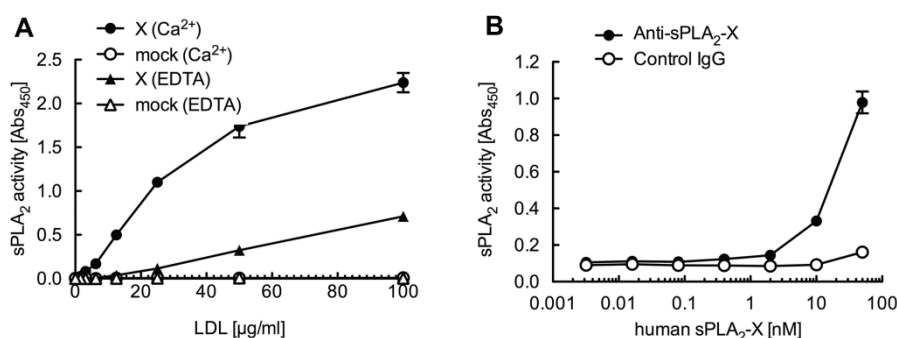


Fig. 5. Detection of the binding between sPLA2-X and LDL by sandwich ELISA system. A – LDL (1 mg/ml) was incubated with (sPLA$_2$-X) or without (mock) 50 nM human sPLA$_2$-X in the presence of 1 mM CaCl$_2$ (Ca$^{2+}$) or 5 mM EDTA (EDTA) for 24 hours at 37°C and diluted to various concentrations as described in "Experimental Procedures". The binding between sPLA$_2$-X and LDL was detected by sandwich ELISA with anti-human sPLA$_2$-X IgG and peroxidase-conjugated-anti-human ApoB antibody. B – Human serum was incubated with various concentrations of human sPLA$_2$-X for 24 hours at 37°C and diluted 5 times with 1% BSA/TBS containing 2 mM CaCl$_2$. The binding between PLA$_2$-X and LDL was detected by sandwich ELISA. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

However, in the absence of $Ca^{2+}$ condition during both the pre-incubation and ELISA procedure, we detected weak but significant signals in LDL incubated with sPLA2-X (Fig. 5A). This result suggests that sPLA2-X associated weakly with LDL under $Ca^{2+}$-free conditions, and $Ca^{2+}$ acted to strengthen this association. Furthermore, we treated human serum with various concentrations of sPLA2-X, and tried to detect the binding of sPLA2-X to LDL in serum. As shown in Fig. 5B, the binding of sPLA2-X to LDL was found dose-dependently with an increasing amount of sPLA2-X, and the binding of sPLA2-X to LDL could be detected even at 2 nM sPLA2-X. This result shows that the binding of sPLA2-X to LDL is found in human serum, and that when endogenous sPLA2-X exists in the bloodstream, sPLA2-X could exist in the LDL-bound state.

**Kinetic analysis of the interaction between sPLA2-X and LDL**
To investigate the fashion in which sPLA2-X and LDL associate, a SPR analysis was performed. A Recombinant sPLA2-X-His Tag protein was immobilized to the nickel ion-activated NTA sensor chip, and LDL at various concentrations was applied as described in the materials and methods. In the absence of $CaCl_2$, the sensorgram of LDL was increased but rapidly decreased to baseline soon after the injection of LDL (Fig. 6A). This suggested that LDL could be associated with but easily dissociated from immobilized sPLA2-X-HisTag protein. In contrast, in the presence of 1 mM $CaCl_2$, the sensorgram of LDL increased but decreased very slowly after the injection of LDL (Fig. 6B). The sensorgrams of HDL using as a negative control of the binding to sPLA2-X were hardly increased and decreased to baseline and were not changed regardless of either the absence or presence of $CaCl_2$ (data not shown). Further, the association and dissociation constants were calculated by the BIAevaluation 3.5 program (Table 1). The apparent
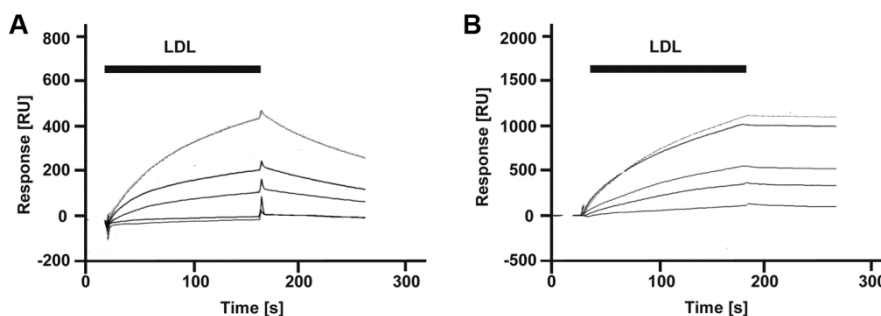


Fig. 6. Study of interaction between human sPLA2-X and LDL by surface plasmon resonance (SPR) analysis. Recombinant human sPLA2-X-HisTag at 200 nM was immobilized to a nickel-activated NTA sensor chip, and then 10, 25, 50, 100 and 125 μg/ml of LDL was respectively injected in the absence (A) and presence (B) of 1 mM $CaCl_2$ without control. Nonspecific interaction was excluded by subtracting the sensorgram of the control cell (without immobilized sPLA2-X). Thick lines show the injection periods of LDL. The data are representative of three independent experiments.

dissociation constant of LDL for sPLA$_2$-X was about 1.10 x 10$^{-7}$ M in the absence of CaCl$_2$, but became two- orders of magnitude lower in the presence of CaCl$_2$ (Kd = 3.10 x 10$^{-9}$ M), following tight binding. The association constant of LDL was not significantly changed in the the presence of CaCl$_2$. These data indicated that Ca$^{2+}$ plays an important role in the binding of LDL to sPLA$_2$-X.

Table 1 . Kinetic binding constant of interaction between sPLA$_2$-X and lipoprotein.

| Lipoprotein | CaCl$_2$ (mM) | k$_a$ (1/Ms) | k$_d$ (1/s) | K$_d$ (M) |
|---|---|---|---|---|
| LDL | 0 | 8.25 ± 2.41 x 10$^4$ | 8.86 86 ± 5.98 x 10$^{-3}$ | 1.10 ± 0.62 x 10$^{-7}$ |
| | 1 | 6.54 ± 3.44 x 10$^4$ | 0.24 ± 0.23 x 10$^{-3}$ | 0.031 ± 0.017 x 10$^{-7}$ |

The data shows the average ± SD of calculated value from each analyte concentration.

## DISCUSSION

The present study demonstrated differences between sPLA$_2$-V and sPLA$_2$-X in substrate specificity in the lipolysis of lipoprotein, Ca$^{2+}$ dependency on PLA$_2$ activity, and specificity to bind LDL. We have previously shown that sPLA$_2$-X can induce the 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 [24-26]. Also, we have recently shown that sPLA$_2$-X was expressed in various pathogenic states including atherogenic plaques in some animal models of atherosclerosis [34]. In this study, both sPLA$_2$-X and sPLA$_2$-V induced lipolytic modifications of LDL to the same extent. However, sPLA$_2$-X released arachidonic acid from LDL more effectively than did sPLA$_2$-V (Fig. 1). This result indicated that the substrate specificity of both sPLA$_2$s was preserved even on LDL, as found previously with synthetic phospholipids as substrates [45]. Eicosanoid production following the arachidonic acid release plays pivotal roles in various pathological conditions such as inflammation and atherosclerosis [28, 46, 47]. Therefore, sPLA$_2$-X might play a more important role than sPLA$_2$-V by inducing the production of proinflammatory lipid mediators, such as prostaglandins (PGs) and leukotrienes (LTs). When whole human serum and plasma were treated with sPLA$_2$-V and sPLA$_2$-X, the amount of fatty acids released by sPLA$_2$-X from whole human plasma was 10-fold higher than that by sPLA$_2$-V. Generally, human plasma is prepared with sodium citrate or EDTA, which is a chelating reagent of divalent cations, especially Ca$^{2+}$, and therefore the concentration of Ca$^{2+}$ in the reaction mixture using plasma is very low. In accordance with these results, activation of sPLA$_2$-V required a higher concentration of Ca$^{2+}$ than sPLA$_2$-X for the lipolysis of synthetic substrates and LDL. Secretory PLA$_2$-X can be activated at 0.03 mM up to about 60%, while sPLA$_2$-V remained inactivated at similar concentrations. In previous reports [34, 33], the concentration of Ca$^{2+}$ in the reaction mixture was mostly more than 1 mM, which is possibly why no distinct difference in

$Ca^{2+}$ dependency between these sPLA$_2$s was observed. This clarified difference in $Ca^{2+}$ dependency for enzymatic activity between sPLA$_2$-V and sPLA$_2$-X could be important for considering their biological roles *in vivo*. In this study, $Ca^{2+}$ played an important role in the interaction between sPLA$_2$-X and LDL *in vitro*. We showed that $Ca^{2+}$ facilitated the binding of sPLA$_2$-X to the LDL. Surface plasmon resonance (SPR) analysis revealed that sPLA$_2$-X immediately associated with LDL and gradually dissociated in the absence of CaCl$_2$ (Fig. 6). The Kd value was $1.1 \times 10^{-7}$ M. In the presence of CaCl$_2$, the apparent association rate of sPLA$_2$-X for LDL was almost the same as that in the absence of CaCl$_2$, while the apparent dissociation rate remarkably decreased. Thus, the binding of sPLA$_2$-X to LDL became more rigid in the presence than the absence of CaCl$_2$, and the Kd value was about $3.1 \times 10^{-9}$ (an increase of more than 30-fold). Generally, sPLA$_2$s are considered to change their conformation as calcium cations bind to their $Ca^{2+}$ loop. Therefore calcium-dependent conformational change of sPLA$_2$-X may lead to tight binding to LDL.

It was previously reported that some kinds of phospholipase bind to serum proteins [30, 31]. For instance, sPLA$_2$-IIA bound to human factor Xa and that interaction was largely dependent on the basic properties of sPLA$_2$-IIA [30]. Another study has shown that human sPLA$_2$-IIA was selectively associated with HDL by a gel-filtration analysis of serum in transgenic mice overexpressing human sPLA$_2$-IIA and apoA-I [48]. From the results in Fig. 4, however, sPLA$_2$-IIA was detected not in the HDL fraction but in the lower molecular weight fraction. To identify the difference in eluted fractions among sPLA$_2$s, we measured PLA$_2$ activity for each fraction from each enzyme reaction mixture by another method (PG/Chol method). Then sPLA$_2$s without sPLA$_2$-IIA were found to have the same profiles of PLA$_2$ activity in Fig. 4A, but sPLA$_2$-IIA was mainly detected in the HDL fraction (data not shown). Thus sPLA$_2$-IIA probably associated with HDL in our experiments. Our results show that sPLA$_2$-IB and IIA were not bound to LDL in human whole serum (Fig. 4) and isolated LDL (data not shown). As for sPLA$_2$-V, broad PLA$_2$ activity was found from the LDL fraction to the HDL fraction (Fig. 4A). Although sPLA$_2$-V was not detected by western blotting using commercially available anti-human sPLA$_2$-V antibody, we found that sPLA$_2$-V did not associate with the purified LDL by measuring PLA$_2$ activity (Fig. 4CD). These findings indicated that only sPLA$_2$-X was selectively associated with LDL in our experimental conditions (Fig. 4CD). The specificity with which sPLA$_2$-X bound to LDL was very different from that of sPLA$_2$-V. Meanwhile, it has been shown that LDL binds to various proteins other than membrane receptors [49-53]. Among them, lipoprotein lipase (LPL) is well characterized [49, 54]. Each lipoprotein is composed of specific apoproteins and lipid molecules such as phospholipids and cholesterol and triglyceride. Boren *et al.* [54] concluded that the LPL-apoB interaction is dependent on lipids but not on apoB because chemical modification of apoB did not abolish the interaction, and partial delipidation of LDL markedly decreased the binding to LDL. In contrast, Goldberg *et al.* proposed that protein-protein

interaction between LPL and apoB is more important than the interaction between LPL and LDL lipids [55-59] using mutant apoB proteins. Thus, whether apoB or lipids contribute to the interaction with LPL has been controversial. Likewise, whether the interaction between sPLA$_2$-X and LDL is primarily mediated by the apoprotein (perhaps apoB-100) or by lipids is an important subject. We did not detect specific binding between sPLA$_2$-X and HDL which did not contain Apo B-100 [60]. These results suggest the importance of Apo B-100 in the association of these molecules. Whether ApoB-100 or phospholipid interacts with sPLA$_2$-X is unclear in the present study. The mature sPLA$_2$-X protein is acidic (pI 5.3) in contrast to the basic properties of group IIA and V sPLA$_2$s. If the interaction is ascribed to this acidic characteristic, basic residues including lysine or arginine within ApoB-100 might be essential. In the future, what is bound to sPLA$_2$-X should be clarified by means of LDL including mutational recombinant ApoB-100 or other methods. The biological roles of the association between sPLA$_2$-X and LDL remain unclear. One hypothesis is that LDL acts as a carrier protein for binding to sPLA$_2$-X, and such concentrated sPLA$_2$-X rapidly hydrolyzes LDL. Further, we have shown that LDL modified by sPLA$_2$-X was efficiently incorporated into macrophages to induce the accumulation of cellular cholesterol ester and formation of non-membrane-bound lipid droplets in the cytoplasm [34]. Therefore, sPLA$_2$-X itself was also possibly incorporated due to the high affinity of sPLA$_2$-X for LDL. Because sPLA$_2$-X showed substantial activity even in the absence of $Ca^{2+}$ (Fig. 3), it could be active after incorporation into cells, where the concentration of $Ca^{2+}$ is much lower than in the extracellular space. Thus, sPLA$_2$-X in the modified LDL may also elicit the release of arachidonic acid from the cell surface and/or cytoplasm in macrophages.

In conclusion, we have demonstrated here marked differences between sPLA$_2$-V and sPLA$_2$-X compared to other sPLA$_2$s: sPLA$_2$-X introduces large amounts of arachidonic acid following eicosanoid biosynthesis, is activated less dependently on the calcium cation concentration, and specifically and tightly binds to LDL. These results indicate sPLA$_2$-X to play a pivotal role in the modification of lipoproteins related to atherosclerosis.

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