Group V and X secretory phospholipase A2s-induced modification of high-density lipoprotein linked to the reduction of its antiatherogenic functions

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Received 30 September 2002; received in revised form 17 June 2003; accepted 24 July 2003

Abstract

The quantitative or qualitative decline of high-density lipoprotein (HDL) is linked to the pathogenesis of atherosclerosis because of its antiatherogenic functions, including the mediation of reverse cholesterol transport from the peripheral cells to the liver. We have recently shown that group X secretory phospholipase A2 (sPLA2-X) is involved in the pathogenesis of atherosclerosis via potent lipolysis of low-density lipoprotein (LDL) leading to macrophage foam cell formation. We demonstrate here that sPLA2-X as well as group V secretory PLA2 (sPLA2-V), another group of sPLA2 that can potently hydrolyze phosphatidylcholine (PC), also possess potent hydrolytic potency for PC in HDL linked to the production of a large amount of unsaturated fatty acids and lysophosphatidylcholine (lysoPC). In contrast, the classical types of group IB and IIA secretory PLA2s evoked little, if any, lipolytic modification of HDL. Treatment with sPLA2-X or -V also caused an increase in the negative charge of HDL with no oxidation and little modification of apolipoprotein AI (apoAI). Modification with sPLA2-X or -V resulted in significant decrease in the capacity of HDL to cause cellular cholesterol efflux from lipid-loaded macrophages. Immunohistochemical analysis revealed significant expression of sPLA2-X in foam cell lesions in the arterial intima of Watanabe heritable hyperlipidemic (WHHL) rabbit. These findings suggest that lipolytic modification of HDL by sPLA2-X or -V causes drastic change of HDL in terms of the production of a large amount of unsaturated fatty acids and lysoPC linked to the reduction of its antiatherogenic functions. These sPLA2-mediated modifications of plasma lipoproteins might be relevant to the pathogenesis of atherosclerosis.

Keywords: Phospholipase A2; High-density lipoprotein; Free fatty acid; Lysophosphatidylcholine; Cholesterol efflux

1. Introduction

The plasma levels of high-density lipoproteins (HDL) are inversely related to the incidence of atherosclerosis and coronary artery disease [1]. The protective effects of HDL from atherosclerosis are attributed, in part, to reverse cholesterol transport, a process by which HDL can remove and deliver excess cholesterol from the peripheral cells to the liver [2,3]. The first and essential step in this transport is the cholesterol efflux from plasma membranes of peripheral cells to HDL, with the phospholipid content and composition in HDL being a major factor determining the capacity of cholesterol efflux [4]. By stimulation of the cellular cholesterol efflux from arterial macrophages, HDL can inhibit the formation of foam cells that play an important role in fatty streak formation as well as in the formation of the complex lesions of plaque rupture [5]. Therefore, the loss of antiatherogenic functions of HDL by either qualitative or quantitative reduction can cause the generation and progression of atherosclerosis.

Abbreviations: HDL, high-density lipoprotein; PLA2, phospholipase A2; sPLA2, secretory PLA2; sPLA2-IB, group IB sPLA2; sPLA2-IIA, group IIA sPLA2; sPLA2-X, group X sPLA2; sPLA2-V, group V sPLA2; PC, phosphatidylcholine; lysoPC, lysophosphatidylcholine; LDL, low-density lipoprotein; apoAI, apolipoprotein AI; WHHL rabbit, Watanabe heritable hyperlipidemic rabbit; Ab, antibody; BSA, bovine serum albumin; TBARS, thiobarbituric acid-reactive substances; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; NZW rabbit, New Zealand White rabbit; apoE, apolipoprotein E; SR-BI, scavenger receptor B type 1

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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 [6,7]. 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$^{2+}$ [8,9], and are now classified into nine different groups in humans (IB, IIA, IID, IIE, IIF, IIB, V, X and XII) [8,10–12]. A variety of circumstantial evidence suggests that sPLA$_2$-induced hydrolysis of phospholipids in HDL plays a role in modulating HDL metabolism and function. For example, snake venom sPLA$_2$ can hydrolyze HDL phospholipids and alter the size and density, as well as the capacity of cholesterol efflux of HDL [4], resulting in increased uptake by rat hepatocytes [13]. Among the endogenous sPLA$_2$s, marked expression of sPLA$_2$-V (sPLA$_2$-IIA) has a potent hydrolyzing activity toward phosphatidylcholine (PC) and can induce the release of fatty acids from HDL more potently than sPLA$_2$-IIA. Although there are few reports on sPLA$_2$-V expression in atherosclerotic lesions in humans [14]. In addition, sPLA$_2$-IIA-transgenic mice exhibit decreased levels of HDL concomitant with increased atherosclerotic lesions [15]. Gesquiere et al. [16] have recently reported that group V sPLA$_2$ (sPLA$_2$-V) has a potent hydrolyzing activity toward phosphatidylcholine (PC) and can induce the release of fatty acids from HDL more potently than sPLA$_2$-IIA. Although these findings prompted us to examine the lipolytic effects of sPLA$_2$-X on HDL functions.

In the present study, we first evaluated the potencies of sPLA$_2$s with respect to the release of fatty acids and the contents of PC and lysophosphatidylcholine (lysoPC) in various cell types, including macrophages, spleen cells and colon cancer cells [18–20]. More recently, we have shown that sPLA$_2$-X can induce potent lipolysis of low-density lipoprotein (LDL) leading to the production of large amounts of unsaturated fatty acids and lysoPC [21]. The sPLA$_2$-X-modified LDL efficiently incorporated into macrophages to induce the accumulation of cellular cholesterol ester and the formation of non-membrane-bound lipid droplets in the cytoplasm [21]. These findings prompted us to examine the lipolytic effects of sPLA$_2$-X on HDL functions.

In the present study, we first evaluated the potencies of sPLA$_2$s with respect to the release of fatty acids and the contents of PC and lysoPC in HDL. We then compared the characteristics of sPLA$_2$-modified HDL with oxidized HDL in terms of phospholipid composition, negative charge and apolipoprotein AI (apoAI) modification. We found that sPLA$_2$-X as well as sPLA$_2$-V can induce potent lipolysis of HDL leading to the reduction of its capacity for cholesterol efflux from lipid-loaded macrophages. We also report on the elevated expression of sPLA$_2$-X in the foam cells in the atherosclerotic arterial wall of Watanabe heritable hyperlipemic (WHHL) rabbit that is commonly used as the animal model of atherogenesis [22].

2. Materials and methods

Purified recombinant proteins of human sPLA$_2$-IB, sPLA$_2$-V and sPLA$_2$-X were prepared as described previously [17,19]. Recombinant human sPLA$_2$-IIA was a generous gift from Dr. Ruth Kramer (Eli Lilly, Indianapolis, USA). Rabbit anti-human sPLA$_2$-X antibody (Ab) was prepared as described previously [19], and anti-sPLA$_2$-IB and anti-sPLA$_2$-IIA Abs were purchased from Cayman Chemicals. Bovine serum albumin (BSA) was obtained from Sigma. Indoxam was synthesized at Shionogi Research Laboratories [23].

2.1. Preparation of human HDL and modification with sPLA$_2$s and CuSO$_4$ oxidation

Human HDL ($d=1.085–1.21$ g/ml) was isolated from the plasma of healthy and fasting donors by sequential ultracentrifugation, as described previously [24]. For modification of HDL with sPLA$_2$s, 1 mg/ml HDL was incubated with various concentrations of sPLA$_2$-IB, -IIA or -X at 37 °C in buffer composed of 1 mM CaCl$_2$, 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 the final concentration of 5 mM. For oxidative modification, 1 mg/ml HDL was incubated with 20 μM CuSO$_4$ at 37 °C, and then dialyzed against 150 mM NaCl containing 0.24 mM EDTA (pH 7.4). HDL prepared by incubation without any modification was used as native HDL.

2.2. Measurement of released fatty acids, PC and lysoPC in sPLA$_2$-treated HDL

Human HDL (1 mg/ml) was preincubated for 10 min at 37 °C and incubated 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 Co.), and then analyzed by reverse-phase HPLC on a LiChroCART 125-4 Supersphere 100 RP-18 column (Merck), as described previously [17,25].

For measurement of the amounts of PC and lysoPC in HDL, lipids were extracted with organic solvent, as described previously [18]. The extracted phospholipids were then separated by normal-phase HPLC on Ultrasphere silica 4.6 × 250 mm (Beckman) connected with a guard column of 4.6 × 45 mm using a solvent of acetonitrile/methanol/sulfuric acid (100:7:0.05, v/v/v) with a flow rate of 1 ml/min at room temperature. Fractions corresponding to authentic PC or 1-α-lysoPC (Sigma), detected at the wavelength of 202
nm, were pooled and subjected to quantitative phosphorus analysis [26].

2.3. Analysis of oxidation in HDL modified with sPLA$_2$s and CuSO$_4$

Following the modification with sPLA$_2$s and CuSO$_4$ oxidation, lipid peroxidation was assessed by the following procedures. The peroxides were quantified in terms of thiobarbituric acid-reactive substances (TBARS) according to the methods of Nagano et al. [27].

2.4. Analysis of electronic charge and apoAI in HDL modified with sPLA$_2$s and oxidation

The electrophoretic mobility of HDL was analyzed with agarose gel electrophoresis (TITAN GEL Lipoproteins; Helena Laboratories, Japan), as described previously [28]. For analysis of apoAI modification, HDL was delipidated and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% acrylamide, as described previously [29].

2.5. Measurement of cholesterol efflux by HDL from macrophage foam cells

Mouse resident peritoneal macrophages were obtained from the peritoneal cavity of female ICR mice (8 weeks). The cells were washed, resuspended in DMEM (Sigma) containing 10% FCS, and plated in 24-well plate (Costar) (5 x 10$^5$ cells/well). Non-adherent cells were removed by washing, and adherent cells were incubated with acetyl LDL at 50 µg/ml for 24 h. After incubation, the foamy macrophages were rinsed and incubated with DMEM containing 0.1% BSA for 24 h. Next, the macrophages were rinsed and incubated with either native or modified HDL at 100 µg/ml for 24 h. The lipid extracts of the macrophages were prepared, evaporated, dissolved with isopropanol and the cholesterol mass was quantified by enzyme fluorometry [30]. The amount of esterified cholesterol was calculated by subtracting the free cholesterol from total cholesterol. The amounts of cellular proteins were quantified with a BCA Protein Assay reagent (PIERCE) after dissolving the cells in 0.2 N NaOH.

2.6. Immunohistochemistry

WHHL rabbits and New Zealand White (NZW) rabbits (8 months old) were sacrificed under pentobarbital anesthesia. Tissue samples were dissected from the aortic arch, thoracic, and abdominal aorta. They were immersion-fixed for 4 h in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2, and embedded in paraffin. Sections of paraffin-embedded tissues were mounted onto polylysine-coated slides, dewaxed in xylene, rehydrated in alcohol, and blocked for endogenous peroxidase (0.3% H$_2$O$_2$ in methanol). For sPLA$_2$-IIA and macrophage immunohistochemistry, sections were reacted with monoclonal mouse anti-sPLA$_2$-IIA Ab (5 µg/ml) (Cayman; Ann Arbor MI) or monoclonal anti-rabbit macrophage Ab (1:200 dilution) (RAM-11, Dako, Carpenteria, CA), and immunoreaction was localized by using a Vectastain ABC kit (Vector Laboratories; Burlingame, CA). For sPLA$_2$-X immunohistochemistry, the sections were subjected to reaction with biotin-conjugated rabbit anti-sPLA$_2$-X Ab (17 µg/ml), and immunoreaction was detected using tyramide signal amplification (TSA Biotin System; NEN Life Science Products, Boston, MA) and visualized with the peroxidase substrate, 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin.

3. Results

3.1. Potent release of unsaturated fatty acids from HDL by sPLA$_2$-X or -V

We first examined the potency of four types of human sPLA$_2$ (sPLA$_2$-IB, -IIA, -V and -X at a concentration of 50 nM) for the release of fatty acids from human plasma HDL within 3 h. As shown in Fig. 1, sPLA$_2$-X induced a marked release of various types of unsaturated fatty acids in human HDL in the following order: linoleic acid (C18:2)>arachidonic acid (C20:4)>oleic acid (C18:1)>docosahexaenoic acid (C22:6). sPLA$_2$-V also released various types of unsaturated fatty acids from HDL, especially linoleic acid and oleic acid. sPLA$_2$-X and -V elicited the release of these unsaturated fatty acids in a dose-dependent manner, and significant release could be detected at values as low as 5 nM (data not shown). Almost all of the released fatty acids are composed of these four types of fatty acids with little production of other types of unsaturated and saturated fatty acids. For sPLA$_2$-IIA and macrophage immunohistochemistry, sections were reacted with monoclonal mouse anti-sPLA$_2$-IIA Ab (5 µg/ml) (Cayman; Ann Arbor MI) or monoclonal anti-rabbit macrophage Ab (1:200 dilution) (RAM-11, Dako, Carpenteria, CA), and immunoreaction was localized by using a Vectastain ABC kit (Vector Laboratories; Burlingame, CA). For sPLA$_2$-X immunohistochemistry, the sections were subjected to reaction with biotin-conjugated rabbit anti-sPLA$_2$-X Ab (17 µg/ml), and immunoreaction was detected using tyramide signal amplification (TSA Biotin System; NEN Life Science Products, Boston, MA) and visualized with the peroxidase substrate, 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin.

![Fig. 1. Profiles of fatty acids released from HDL by the action of human sPLA$_2$s. HDL (1 mg/ml) was incubated with 50 nM purified human sPLA$_2$s for 4 h at 37 °C, and the released fatty acids were quantified as described under Section 2.2. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.](image-url)


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acids (myristic acid, palmitic acid and stearic acid) during the treatments with sPLA₂-V and -X (data not shown).

As shown in Fig. 2A, sPLA₂-X induced a time-dependent release of unsaturated fatty acids from HDL, and the release response was almost saturated within 3 h. sPLA₂-V also induced the release of unsaturated fatty acids with a similar time-dependency, but the total amount was slightly smaller than that of sPLA₂-X especially within 6 h. sPLA₂-IB was found to evoke a small but significant release of fatty acid only at 24 h. In contrast, there was little, if any, release with sPLA₂-IIA and CuSO₄ treatment for 24 h. When focused on the arachidonic acid release (Fig. 2B), sPLA₂-

X induced the most potent release among the sPLA₂s examined. Although sPLA₂-V released almost the same level of total fatty acids from HDL, its potency for the release of arachidonic acid was much lower compared to sPLA₂-X. These results demonstrate that sPLA₂-X and sPLA₂-V could elicit potent release of unsaturated fatty acids from human HDL with different substrate specificities.

3.2. Effects of sPLA₂-X and -V on the PC and lysoPC contents in HDL

Since PC is a major component of phospholipids in HDL, we next examined the PC content in HDL after treatment with sPLA₂s and CuSO₄. As shown in Fig. 3A, the amount of PC in HDL time-dependently decreased after treatment with
sPLA2-X, -V and oxidation. Most of the PC was diminished in HDL by these treatments within 3–6 h, and PC was completely degraded after 24 h treatment. Corresponding to the marked reduction of PC contents, a large amount of lysophosphatidylcholine (lysophosphatidylcholine, lysoPC) was produced in HDL by treatment with sPLA2-X or -V within 3 h (Fig. 2B). Incubation with sPLA2-specific inhibitor Indoxam (10 μM), which can block the enzymatic activities of both sPLA2s [17], resulted in significant suppression of their lipolytic effects on HDL (data not shown). The time-dependency of PC degradation and lysoPC production by sPLA2-X and -V were correlated well with that of fatty acid releases (Fig. 2A). Despite the similar PC degradation, the amount of lysoPC produced during oxidation by CuSO4 was only about 15% of that induced by sPLA2-X or -V. In contrast, treatment with sPLA2-IB or -IIA caused little change in both PC and lysoPC contents in HDL.

Treatment of HDL with CuSO4 caused a dramatic increase in TBARS (Table 1) as well as significant production of conjugated dienes (data not shown), whereas treatment with four types of sPLA2s did not alter these oxidative parameters. These findings demonstrate that sPLA2-X and -V induce PC hydrolysis in HDL leading to the production of a large amount of lysoPC and unsaturated fatty acids without any oxidative modification.

### 3.3. Effect of sPLA2-X and -V on electronic mobility and apoAI in HDL

We next examined the effects of sPLA2 treatment on the electronic charge of HDL with agarose gel electrophoresis. As shown in Fig. 4, increased anodic migration was observed in HDL after 24 h oxidation with CuSO4. Treatment with sPLA2-X or -V also caused enhanced mobility of HDL and marked migration was detected after 3 h of incubation. In contrast, the mobility of HDL after treatment with sPLA2-IB or -IIA was not changed during 24 h of incubation. Addition of anti-sPLA2-X Ab (100 μg/ml), which was proven to inhibit the enzymatic activity of sPLA2-X and -V [17], resulted in complete blockade of sPLA2-X-induced mobilization (data not shown). SDS-PAGE analysis has also revealed little, if any, denaturation of apoAI after sPLA2-X or -V treatments for 24 h, which contrasted with an exaggerated aggregation of apoAI in CuSO4-treated HDL (data not shown). These results demonstrate that sPLA2-X and -V can induce an increase of the negative charge of HDL without any modification of apoAI.

### 3.4. Effect of sPLA2-X and -V on cellular cholesterol efflux by HDL

One of the most important functions for antiatherogenic property of HDL is the mediation of cellular cholesterol efflux, and the characteristics of phospholipids in HDL in terms of contents and composition is a major factor determining the cholesterol efflux capacity from the cells to HDL [4]. Since sPLA2-X or -V treatment cause a dramatic decrease in PC along with the increase in lysoPC, we compared the cholesterol efflux capacity between native and modified HDL from acetyl LDL-pretreated macrophages that accumulated cholesterol ester in the cytoplasm. As shown in Fig. 5, incubation with native HDL (100 μg/ml) resulted in ca. 83% decrease in the mass of cholesterol ester accumulated in the lipid-loaded macrophages, and sPLA2-IB or -IIA-treated HDL possessed a similar efficacy. In contrast, sPLA2-X or -V-modified HDL caused only about 60% reduction, indicating a significant decrease in the capacity to mediate cholesterol efflux. At 50 μg/ml of HDL, similar decrease in cellular cholesterol efflux could be detected in sPLA2-X or -V-modified HDL (data not shown). These results suggest that sPLA2-X and -V can deprive HDL of the capacity to induce cholesterol efflux by causing the alteration of the phospholipid content and composition in HDL.

### 3.5. Expression of sPLA2-X in atherosclerotic lesions of WHHL rabbits

In order to examine the localization of sPLA2-X in the atherosclerotic lesions, we used WHHL rabbits, a familiar model of atherogenesis [22]. Fig. 6A shows atherosclerotic lesions in the aorta of WHHL rabbits. Large foam cells filled with numerous lipid droplets and cholesterol crystals were observed between the middle and deep parts of the intimal...
In the aorta of age- and sex-matched NZW rabbits, no intimal thickening was found (data not shown). Fig. 6B shows the result of immunostaining of WHHL aorta with a monoclonal Ab against rabbit macrophage, RAM-11. Cells with foam cell morphology were intensely stained with RAM-11.

To analyze the expression of sPLA2-X in atherosclerotic lesion by immunohistochemistry, we used biotinylated rabbit anti-human sPLA2-X polyclonal Ab. By ELISA and immunoblotting analysis, we confirmed that this Ab specifically binds to human sPLA2-X with no cross-reactivity for other human sPLA2s (-IB, -IIA, and -V) and also recognizes rabbit sPLA2-X (data not shown). Polyclonal anti-sPLA2-X Ab stained the foam cells in the intima (Fig. 6C), and immunoreaction was diffuse through the middle to deep region of the intima, with most of the foam cells being positive. Moderate staining was also found in the endothelium, but it was non-specific because preincubation of the Ab with 50-fold (in the molar ratio) excess amount of recombinant rabbit sPLA2-X completely abolished the staining of foam cells, but not that of the endothelium (data not shown). No immunoreaction of sPLA2-X was found in atherosclerosis-free aorta from NZW rabbits.

Fig. 6. Immunostaining of atherogenic lesions of WHHL rabbits with anti-sPLA2-X Ab. Paraffin-embedded aorta sections with atherosclerotic lesions from WHHL rabbits were stained with Elastica-van-Gieson (A) or immunostained with mouse anti-rabbit macrophage Ab (B), biotin-conjugated rabbit anti-sPLA2-X Ab (C) or mouse anti-sPLA2-IIA Ab (D). Dark-brown deposits indicated the occurrence of immunoreaction, and the nuclei were counterstained with hematoxylin. The bar indicates 100 μm.

Fig. 5. Measurement of cholesterol efflux by HDL from lipid loaded macrophages. Mouse resident peritoneal macrophages were incubated with acetyl LDL to form foam cells. After incubation with DMEM for 24 h, the macrophages were incubated with either native or modified HDL at 100 μg/ml for 24 h. At the end of the incubation, the cholesterol mass of the macrophages was quantified. The amount of esterified cholesterol was calculated by subtracting the free cholesterol from total cholesterol. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments. Statistical significance was determined by Student’s t-test (*P<0.05 compared to macrophages treated with native HDL).
To examine the localization of sPLA2-IIA, we used mouse anti-human sPLA2-IIA monoclonal Ab. By immunoblotting analysis, we confirmed that this Ab specifically binds to sPLA2-IIA with no cross-reactivity for other human sPLA2s (-IB, -V and -X), as well as rabbit sPLA2-X (data not shown). Prior to immunostaining of aorta, we tested the availability of the Ab using rabbit jejunum sections as a positive control. We detected positive signals in Paneth cells that have been reported to extensively express sPLA2-IIA in rats and humans [31,32], demonstrating that the anti-human sPLA2-IIA Ab we used could cross-react with the counter-part of rabbit origin. As shown in Fig. 6D, positive cells were detected in the middle to deep region of the intima and had the morphology of foam cells. There was great variation in the degree of immunoreactivity among the foam cells in the lesion from intense staining to total absence of staining. These staining patterns of sPLA2-X and -IIA were similar among the aortic arch, thoracic, and abdominal aorta (data not shown), indicating that both sPLA2-X and -IIA are expressed in foamy macrophages in the atherosclerotic lesions of WHHL rabbits.

It has been known that scavenger receptor B type I (SR-BI) can act as the HDL receptor to stimulate cholesterol efflux from foamy macrophages, and the efflux rate is correlated with SR-BI expression levels in various cell lines [39]. In the SR-BI-mediated cholesterol efflux, phospholipids present in the acceptor HDL are essential factors, because the cholesterol efflux is highly correlated with the concentration of HDL phospholipids in serum and stimulated by phospholipid supplementation of serum [40]. Since sPLA2-X and -V induced strong degradation of PC, as a major component of phospholipids in HDL, the reduction of the capacity to induce cellular cholesterol efflux in sPLA2-X or -V-modified HDL (Fig. 6) might be due to a decrease in the SR-BI-mediated responses. Alternatively, the ATP-binding cassette transporter ABCA1 might be involved in this process, as it has been known to mediate the transport of cellular cholesterol to lipid-poor HDL apolipoproteins [41,42]. In fact, mutations in ABCA1 cause Tangier disease [41,43–45], a severe HDL deficiency syndrome characterized by deposition of cholesterol in tissue macrophages and prevalent atherosclerosis [46]. Recent studies have shown that unsaturated fatty acids reduce the macrophage ABCA1 content by enhancing its degradation rate [47]. As sPLA2-X and -V can release a large amount of unsaturated fatty acids from HDL (Fig. 1), these fatty acids might reduce the ABCA1 content in macrophages leading to decline of the cellular cholesterol efflux. Since HDL possesses various antiatherogenic functions, including the protection of LDL from oxidation [48–50], the inhibition of platelet aggregation [51,52] and the protection of endothelial cells from oxidative stress and apoptosis [53], the effects of sPLA2-X or -V modification on these biological events should also be examined in future studies.

Among the human sPLA2s examined, potent lipolysis of HDL was observed only on treatment with sPLA2-X and -V, which may have been due to higher hydrolyzing activity toward PC, the major phospholipid species in HDL [17,54]. Weak fatty acid release was detected during treatment of HDL with higher concentrations of sPLA2-IB (Fig. 2). However, sPLA2-IB is mainly expressed in the pancreas and its expression was not detected in the atherosclerotic lesions of humans [55] and apoE-deficient mice [21], indicating that its role in atherogenesis is quite minor. With regard to sPLA2-IIA, its expression was markedly elevated in human atherosclerotic lesions of WHHL rabbits (Fig. 6C), similar to its distribution in high-fat fed apoE-
deficient mice [21]. The different localizations between sPLA2-IIA and -X suggests their distinct pathophysiological roles in the initiation and progression of atherosclerotic plaques. We found that the potency of sPLA2-IIA in HDL modification in terms of PC degradation and cellular functions is much lower than sPLA2-X, and this type of sPLA2 has been shown to induce the enhanced retention of lipoproteins to human aortic proteoglycans [58,59]. 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-V and -X induce more powerful lipolysis of HDL linked to the reduction of its antiatherogenic effect within the arterial intima. Recent studies have shown that overexpression of sPLA2-IIA resulted in increased rate of catabolism and altered sites of tissue uptake of HDL in mice [60]. The contribution of sPLA2-X to HDL modifications in pathological states should also be clarified in future genetic studies including the generation of mice deficient in sPLA2-X.

In the present study, sPLA2-V was also found to hydrolyze PC in HDL and release a large amount of unsaturated fatty acids. However, as reported by Gesquiere et al. [16], sPLA2-V released more linoleic acid (over 10-fold) than arachidonic acid from HDL (Fig. 1), which corresponded well with its fatty acid specificity using synthetic substrate [61]. In contrast, sPLA2-X can induce potent release of arachidonic acid from HDL with over half the levels of linoleic acid (Fig. 1), and its potency for arachidonic acid release was much higher than sPLA2-V (Fig. 2B), suggesting its greater relevance to the production of proatherogenic eicosanoids [62,63]. Although sPLA2-V is secreted by macrophages and mast cells [64], there have been few reports on its expression in pathological states including atherosclerotic lesions. This may be due to the difficulty of generating sPLA2-V-specific Ab applicable for immunohistochemistry, as many Abs for sPLA2-IIA have been shown to cross-react with sPLA2-V because of their structural similarity [64]. In the present study, we confirmed no cross-reactivity of anti-sPLA2-IIA Ab for human sPLA2-V, -IB, -V and -X, as well as rabbit sPLA2-X. However, there was no data showing the absence of its cross-reactivity with rabbit sPLA2-V, suggesting that positive signals detected in the atherosclerotic lesions in WHHL rabbits might also represent the expression of this enzyme. Nevertheless, these observations strongly suggest that sPLA2 family enzymes may be involved in the development of atherosclerosis via modification of HDL.

In conclusion, we have demonstrated here that sPLA2-X or -V can induce lipolytic modification of HDL leading to a decrease in its ability to mediate the cellular cholesterol efflux. Since sPLA2-X and -V can also evoke potent modification of LDL [16,21], these sPLA2s may be responsible for the generation of atherogenic lipoprotein particles leading to the formation and progression of fatty streaks in atherosclerotic regions. In addition, a variety of lipid mediators including polyunsaturated fatty acids, eicosanoids and lysoPC can be generated during the lipolytic process of lipoproteins, which may also contribute to the progression of atherosclerosis. Further studies regarding the expression of sPLA2-X or -V in human atherosclerotic lesions as well as its circulating levels in patients with atherosclerosis are required for further understanding of its pathological roles in humans as well as the strategies of sPLA2 inhibitors as antiatherogenic drugs.

Acknowledgements

We thank Dr. Ruth Kramer for the generous gift of recombinant human sPLA2-IIA. We are grateful to Kazumi Nakano for her excellent technical assistance. We are also grateful to Dr. Seijiro Hara, Dr. Akihiko Saiga, Dr. Shigeki Kamitani and Dr. Hitoshi Arita for fruitful discussions.

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