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# Deficiency in $sPLA_2$ does not affect HDL levels or atherosclerosis in mice

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**Abstract**

Secretory non-pancreatic phospholipase $A_2$ ($sPLA_2$) has been implicated in inflammation and has been found in human atherosclerotic lesions. To test the effect of $sPLA_2$ deficiency on atherosclerosis, C57BL/Ks mice ($apoE^{+/+}$ and $sPLA_2^{+/+}$) were bred with C57BL/6 apoE knockout mice which are $sPLA_2^{-/-}$ due to a spontaneous mutation. Sibling pairs of mice ($apoE^{-/-}/sPLA_2^{+/+}$ and $apoE^{-/-}/sPLA_2^{-/-}$) on high fat Western diets were dissected at 22 weeks. In vitro enzyme assays confirmed higher serum $sPLA_2$ activity in the $sPLA_2^{+/+}$ compared to $sPLA_2^{-/-}$ for both sexes, while $sPLA_2^{-/-}$ males had slightly higher serum cholesterol and phospholipids. Analysis of lipoprotein profiles by FPLC showed no effect of $sPLA_2$ genotype on any measured parameters. Atherosclerosis was quantitated by assaying cholesterol in aortic extracts. Male $sPLA_2^{-/-}$ trended slightly higher than $sPLA_2^{+/+}$ with no statistical significance. Female $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ mice showed no significant differences in any of the measured parameters. These results suggest that the endogenous mouse $sPLA_2$ gene does not significantly affect HDL or atherosclerosis in mice. 

*Keywords:* $sPLA_2$; ApoE; Aortic lesion; Cholesterol; Phospholipids

Secretory non-pancreatic type II phospholipase $A_2$ or synovial phospholipase $A_2$ ($sPLA_2$) (EC 3.1.1.4) is a 14-kDa calcium-dependent enzyme that hydrolyzes phospholipid specifically at the sn-2 acyl ester bond position [1,2]. The enzyme is synthesized and secreted by several tissues including smooth muscle cells [3], liver, spleen, and lung [4,5]. Vadas et al. [6] have shown that $sPLA_2$ hydrolyzes phospholipids in HDL particles but not those in membranes of living cells, and therefore $sPLA_2$ levels might influence lipoprotein levels or atherosclerosis.

It is now widely accepted that inflammation plays an integral role in the development of atherosclerosis [7]. $sPLA_2$ is a pro-inflammatory marker [8], an acute-phase reactant whose levels rise dramatically during infection [6]. Furthermore, numerous groups have identified this enzyme in atherosclerotic lesions [9–12]. Transgenic mice overexpressing human $sPLA_2$ exhibit low HDL levels and increased incidence of atherosclerosis [13].

Recent work by Kennedy and colleagues [14] has shown that several strains of inbred laboratory mice carry spontaneous null mutations in the $sPLA_2$ gene. One of these strains is the C57BL/6J apoE knockout mouse, used extensively as a model for atherosclerosis. The C57BL/Ks strain has the intact $sPLA_2$ gene and its genome is 84% homologous to that of C57BL/6J [15]. In the present work, we have crossed these two strains, identified sibling pairs of ($apoE^{-/-}/sPLA_2^{+/+}$) or ($apoE^{-/-}/sPLA_2^{-/-}$) mice, and compared the development of atherosclerotic lesions in both groups. Our results indicate little effect of the endogenous mouse $sPLA_2$ gene on HDL levels and atherosclerosis.

## Materials and methods

*Mice*. All mice were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (revised 1996, National Academy Press, Washington, DC). They were housed in a specific pathogen free (SPF) barrier and provided ad libitum with autoclaved food and water. The animal experimental protocol was reviewed and

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approved by the Institutional Animal Care and Use Committee at Merck Research Laboratories (Rahway, NJ).

Male C57BL/Ks ($sPLA_2^{+/+}$/$apoE^{+/+}$) mice (Taconic Farms, Germantown, NY) were mated with female C57BL/6J apoE KO ($sPLA_2^{-/-}$/$apoE^{-/-}$) mice (Jackson Labs, Bar Harbor, ME) and their double heterozygote ($sPLA_2^{+/-}$/$apoE^{+/-}$) F1 progeny were subsequently mated. The F2 progeny were typed for the apoE KO phenotype by determining their plasma cholesterol levels [16]. F2 male and female mice showing the $apoE^{-/-}$ phenotype were then genotyped by PCR for the $sPLA_2$ trait [14]. Male and female apoE KO mice with a heterozygous $sPLA_2$ genotype were then mated and their F3 progeny were weaned at 4 weeks onto a high fat, Western-type diet containing 21.22% (g/100 g) fat, 17.01% protein, 48.48% carbohydrate, and 0.15% cholesterol (TD88137, Harlan Teklad, Madison, WI). From these F3 progeny, male and female apoE KO mice with either $sPLA_2^{+/+}$ or $sPLA^{-/-}$ genotypes were recruited for the study. These animals were sib paired wherever possible. Some animals in these studies were F4 progeny of $sPLA_2^{+/-}$ F3 mice.

*Analysis of serum lipids*. Serum cholesterol, triglyceride, and phospholipid measurements were made using standard enzymatic kits (Wako Diagnostics, Richmond, VA). For a subset of animals, serum was used for lipoprotein analysis by FPLC size exclusion chromatography using the BioLogic Chromatography System (Bio-Rad Life Science, Hercules, CA). Some of the samples subjected to FPLC lipoprotein analysis were pools of serum from multiple animals. In all cases, 200 μl of filtered serum was chromatographed on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated and run in phosphate-buffered saline containing 1 mM EDTA, pH 7.4. The column was run at a flow rate of 0.2 ml/min and 0.27 ml fractions were collected. Each fraction (100 μl volumes or appropriate dilutions) was assayed for cholesterol (Cholesterol CII Kit) and for phospholipids (Phospholipids B Kit).

*Aortic cholesterol measurements*. From a subset of animals, the aortas were harvested and the lipids extracted and assayed, as described previously [17].

*$sPLA_2$ enzyme assay*. The level of $sPLA_2$ enzyme activity was determined in the serum of each animal following a modification of a method described previously [18,19]. Briefly, $^3$H-oleate-labeled *Escherichia coli* disrupted membrane was used as substrate [20–22] at 10 μg protein/ml with serum (15% (v/v) final concentration) in a solution containing 5 mg/ml fatty acid-free bovine serum albumin, 100 mM Tris, 5 mM calcium (pH 8.0) for 6 h at 37 °C both with and without EDTA (10 mM). The $sPLA_2$ enzyme activity was quenched by addition of EDTA and lipids were extracted [23,24]. The lipids were separated by thin layer chromatography, and the percentage of free fatty acid was determined from the amount of $^3$H associated with the fatty acid region of the thin layer plate relative to the total $^3$H in the reaction mix. The release of $^3$H-oleate was linear with time for 6 h, and EDTA effectively quenched the enzyme activity. For animals in the atherosclerosis study, results are expressed as the percentage of free fatty acid released in the absence of EDTA minus the percentage released in its presence.

*Statistical analyses*. Student's unpaired, two-tailed $t$ test was used for the assessment of effects of genotype on the various parameters and on atherosclerosis. Further analyses were performed using the two-way analysis of variance (ANOVA) method with factors' gender, genotype, and their interaction. When there was a violation of normal assumption, which is required by the ANOVA method, data were transformed to Tukey's normal ranks first, then analyzed by ANOVA.

## Results

### *In vitro assay of $sPLA_2$ activity confirms lower levels of enzyme in $sPLA_2^{-/-}$ mice*

Mice were bred to obtain $apoE^{-/-}$, $sPLA_2^{-/-}$ and $apoE^{-/-}$, $sPLA_2^{+/+}$ animals (see Materials and methods). The genotyping at the $sPLA_2$ locus was confirmed by enzyme assay. Aufenanger et al. [19] have demonstrated that *E. coli* membranes used as substrate for phospholipase $A_2$ activity in serum are resistant to other lipases, including lipoprotein lipase. In addition, despite the abundance of type II $PLA_2$ in mouse platelets, its secretion in response to thrombin stimulation is insignificant [25]. Taken together, these two facts affirm the validity of the enzyme assay results on serum from the mice in this study. The EDTA-sensitive phospholipase activity was decreased in the $sPLA_2^{-/-}$ male ($3.4 \pm 0.9\%$ vs $1.2 \pm 0.3\%$, $p = 0.04$) and female ($4.6 \pm 1.4\%$ vs $1.1 \pm 0.2\%$, $p = 0.02$) mice (Fig. 1). Since ANOVA analysis of the data showed no significant gender effects and no gender/genotype interaction, all the data were pooled for assessment of the genotype. A highly significant difference ($p < 0.001$) was seen between $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ genotypes.

The enzyme activity in the $sPLA_2^{+/+}$ mice was similar to that in normal human serum. Human serum obtained from four different sources was pooled and assayed for phospholipase enzyme activity in the same system (data not shown). The human serum response ($n = 3$ for each timepoint) was linear for 3 h with a steeper slope than seen with mouse serum (3.8 compared to 2.0). The phospholipase activity in human serum was completely blocked (<2% fatty acid released) in the presence of 10 mM EDTA.

### *Total serum lipid levels are not affected by $sPLA_2$ gene expression*

Total serum lipid levels of cholesterol, triglycerides, and phospholipids were compared in the $sPLA_2^{+/+}$ and

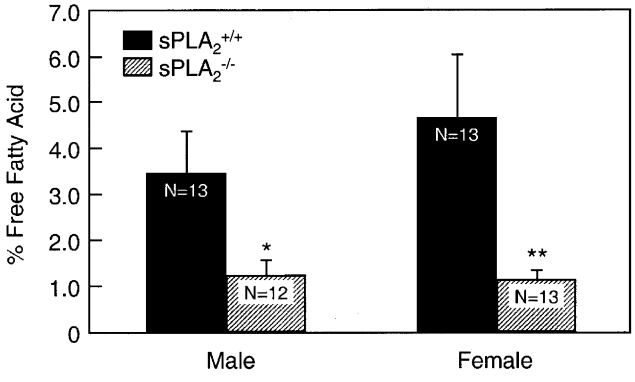


Fig. 1. Calcium-dependent phospholipase enzyme activities in $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE KO mice. Serum from mice in the atherosclerosis study was assayed for EDTA-sensitive phospholipase activity. The serum (15%) was incubated at 37 °C for 6 h in 100 mM Tris (pH 8.0) with 5 mM calcium, and 5 mg/ml fatty acid-free bovine serum albumin with $^3$H-oleate-labeled *E. coli* membrane substrate (10 μg protein/ml) in the presence and absence of 10 mM EDTA. Extracted lipids (Dole) were separated on silica gel thin layer in petroleum ether:ethyl ether:acetic acid (50:50:1) and the percent of $^3$H-oleate calculated as described in Materials and methods. *, $p = 0.04$; **, $p = 0.02$.

$sPLA_2^{-/-}$ groups (Fig. 2). Cholesterol levels were similar in the females ($814 \pm 85$ vs $848 \pm 62$ mg/dL) for $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ groups, respectively, while in the males, the $sPLA_2^{-/-}$ group ($1102 \pm 73$ mg/dL) was higher than the $sPLA_2^{+/+}$ ($913 \pm 52$ mg/dL, $p = 0.04$).

Phospholipid levels tracked similarly between the $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ genotypes with females showing no difference ($536 \pm 47$ vs $605 \pm 38$ mg/dL), but with male $sPLA_2^{-/-}$ ($769 \pm 37$ mg/dL) being higher than the $sPLA_2^{+/+}$ ($652 \pm 36$ mg/dL, $p = 0.04$).

Triglyceride levels in male ($501 \pm 65$ vs $374 \pm 47$ mg/dL) and female ($380 \pm 44$ vs $391 \pm 45$ mg/dL) $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE knockout mice showed no differences.

### *FPLC fractionation of serum lipoproteins reveals minor differences only in male apoE$^{-/-}$ mice for the sPLA$_2^{+/+}$ and sPLA$_2^{-/-}$ genotypes*

Lipoprotein separation on Superose 6/HR revealed only minor differences in cholesterol (Fig. 3) or phospholipid (not shown) profiles. Despite the higher mean levels of cholesterol in the VLDL fractions for the male $sPLA_2^{-/-}$ group, the standard errors were large and therefore the differences were insignificant.

Comparison of the HDL-cholesterol and HDL-phospholipid contents for $sPLA_2^{+/+}$ versus $sPLA_2^{-/-}$ showed no impact of the $sPLA_2$ gene on either HDL-associated lipid class (see Table 1).

### *Aortic cholesterol content is not altered by sPLA$_2$ genotype*

We assessed the extent of atherosclerosis in mice by quantifying the aortic content of total cholesterol, free cholesterol, and cholesteryl ester. This biochemical approach has been used previously to quantify atherosclerosis in mice [26,27]. A recent study involving over 150 mice showed that aortic cholesterol content correlates very well with other measures of atherosclerosis, including morphometry where $r = 0.855$, $p < 0.001$ for free cholesterol; and $r = 0.857$, $p < 0.0001$ for esterified cholesterol [28]. In the rabbit model, many studies have shown good correlations between aortic cholesterol content and atherosclerosis measured by other criteria, including intima/media ratio [29,30] and lesion area/total aortic surface area ratio [30–37]. Similar correlations have been reported for apoE KO mice [38]. Furthermore, there are reports in both rabbits [34,35] and apoE$^{-/-}$ mice [39] that drug interventions show larger effects in the thoracic aorta than in the arch, presumably because the limited area available in the arch for lesion development provides a smaller signal-to-noise ratio to measure changes.

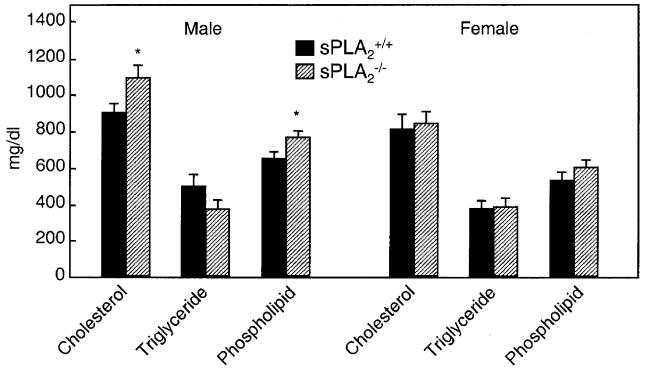


Fig. 2. Total serum lipids in $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE KO mice. Total serum cholesterol, triglycerides, and phospholipids were measured using commercial kits (Wako Chemicals) with appropriate commercially available controls (Sigma Accutrol). Bars represent the means ± SEM. For male $sPLA_2^{+/+}$, $n = 15$; for male $sPLA_2^{-/-}$, $n = 13$. For female $sPLA_2^{+/+}$, $n = 16$; for female $sPLA_2^{-/-}$, $n = 15$. *, $p = 0.04$ vs wild type.

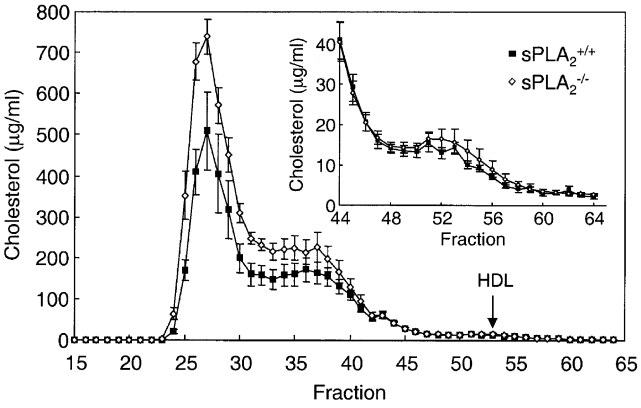


Fig. 3. FPLC lipid profiles for male $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE KO mice. Filtered mouse serum was fractionated by size on a Superose 6 HR column (Pharmacia) in PBS with 1 mM EDTA running at constant flow rate of 0.2 ml/min. Fractions (0.27 ml) were collected and assayed for cholesterol and phospholipid content (Wako Diagnostic Kits). Graphs represent the means ± SEM for 10 male $sPLA_2^{+/+}$ and 9 male $sPLA_2^{-/-}$ mice. No significant differences were found. Serum from female $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ mice had overlying chromatograms with similar levels of VLDL, LDL, and HDL (data not shown).

Table 1
HDL-lipid levels in $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE KO mice

| | HDL-cholesterol (mg/dL) | HDL-phospholipids (mg/dL) |
|---|---|---|
| Male $sPLA_2^{+/+}$ ($n = 10$) | $27.6 \pm 2.5$ | $32.8 \pm 2.5$ |
| Male $sPLA_2^{-/-}$ ($n = 9$) | $31.3 \pm 3.9$ | $33.8 \pm 4.5$ |
| Female $sPLA_2^{+/+}$ ($n = 10$) | $23.9 \pm 2.0$ | $27.0 \pm 2.9$ |
| Female $sPLA_2^{-/-}$ ($n = 10$) | $24.0 \pm 1.7$ | $30.3 \pm 3.5$ |

Fractions collected from FPLC were assayed for cholesterol and phospholipid content as described in Materials and methods. Results for those fractions corresponding to the HDL region (fractions 48–64) were combined to determine the total cholesterol and phospholipid levels. There are no statistically significant effects of $sPLA_2$ genotype on either parameter. Values are means mg/dL ± SEM calculated by totaling the area under the curve of the HDL peak for each parameter.

The $sPLA_2$ gene product had no effect on aortic cholesterol content (Fig. 4). In males the values for the $sPLA_2^{+/+}$ versus $sPLA_2^{-/-}$ genotypes were: total cholesterol ($52.4 \pm 3.4$ vs $54.2 \pm 8.0$ nmoles/mg wet wt aorta); free cholesterol ($32.5 \pm 2.6$ vs $31.6 \pm 4.2$ nmoles/mg wet wt aorta); and cholesteryl ester ($19.9 \pm 1.8$ vs $22.6 \pm 3.9$ nmoles/mg wet wt aorta). Similarly, for the female $sPLA_2^{+/+}$ vs $sPLA_2^{-/-}$ mice, the values were: total cholesterol ($64.1 \pm 3.8$ vs $73.3 \pm 4.6$ nmoles/mg wet wt aorta); free cholesterol ($40.6 \pm 2.8$ vs $47.0 \pm 3.2$ nmoles/mg wet wt aorta); and cholesteryl ester ($23.5 \pm 1.3$ vs $26.3 \pm 1.6$ nmoles/mg wet wt aorta).

Aortic weight has been proposed as a valid surrogate for atherosclerosis [40]. Similarly, we have found that aortic weights typically reflect the extent of lesion seen visually during dissection, as well as correlate with the cholesterol content determined biochemically. In the present work, we found no effect of $sPLA_2$ genotype on aortic weights normalized to body weight. Male $sPLA_2^{-/-}$ mice had a ratio of $0.27 \pm 0.01$ mg wet wt aorta/g body weight which was not significantly different ($p = 0.27$) from that of male $sPLA_2^{+/+}$ mice ($0.25 \pm 0.01$). Similarly, these ratios were not different in female mice between $sPLA_2^{-/-}$ ($0.34 \pm 0.02$)) and $sPLA_2^{+/+}$ ($0.38 \pm 0.07$) genotypes ($p = 0.55$).

*Analysis of sibling pairs shows no effect of $sPLA_2$ genotype on aortic cholesterol accumulation*

Fig. 4 shows that there was no effect of $sPLA_2$ genotype on aortic cholesterol accumulation. The mice used in this study, however, were not congenic strains. To minimize the possibility that minor genetic variations hid a subtle effect of $sPLA_2$ genotype, we analyzed a subset of the animals that were true sibling pairs. Animals were analyzed as sibling pairs if three criteria were met:

1. Both of the animals were derived from the same breeding pair;
2. The animals were the same sex;
3. One animal was $sPLA_2^{+/+}$ and the other was $sPLA_2^{-/-}$.

There were nine pairs of animals that met these criteria. The difference between $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ mice was calculated for each of these nine pairs, for each of the three parameters of aortic cholesterol content. None of these differences were statistically significantly different from zero ($0.62 < p < 0.97$). Therefore, even within sibling pairs, $sPLA_2$ genotype did not alter the progression of atherosclerosis. The sex of the animals did significantly affect atherosclerosis in the study, consistent with previously published work [41,42]. Female animals showed increased aortic content of total cholesterol ($p = 0.002$), free cholesterol ($p = 0.0016$), and cholesteryl ester ($p = 0.09$).

Since there was no quantitative difference in the content of free cholesterol or cholesteryl ester between $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$, histological assessment of the lesions in the aortic root was limited to a qualitative assessment. The overall morphology of the lesions was similar between mice with and without $sPLA_2$ (data not shown). Both contained prominent lesions with foam cells, and necrotic cores were easily detectable in each. Staining with Oil Red O revealed a similar distribution of lipid content within the lesions from both types of mice.

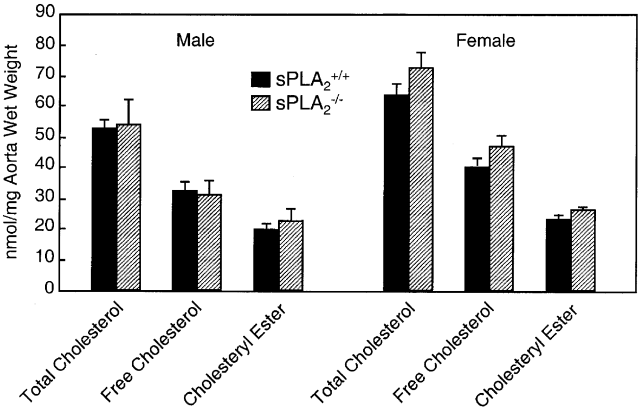


Fig. 4. Aortic cholesterol content of male and female $sPLA_2^{+/+}$ and $sPLA_2^{-/-}$ apoE KO mice. At dissection, the aorta from each mouse was perfused in situ with PBS/5 mM EDTA, carefully cleaned of external fat deposits, and removed from the animal taking the entire aortic root down to the right renal artery and cutting each branch off cleanly at the junction point. The aorta was then extracted in chloroform–methanol and assayed fluorimetrically for total and free cholesterol. Cholesteryl ester was calculated by difference. Bars represent the means ± SEM of 10 mice in each group ($n = 9$ for male $sPLA_2^{-/-}$ group). There was no significant effect of $sPLA_2$ genotype.

## Discussion

Examination of the pathology of atherosclerotic lesions has led to the conclusion that atherosclerosis is an inflammatory disease [7]. Recent epidemiological investigations have shown that certain indices of inflammatory tone correlate with disease progression, including C-reactive protein [43], lipoprotein-associated phospholipase $A_2$ [44], and $sPLA_2$ [45]. The mechanisms that lead to these correlations, however, are unclear. These protein markers may reflect other disease processes such as enhanced cytokine secretion, or these molecules may directly accelerate disease. The interpretation of these observations is further complicated by the fact that many of the inflammatory markers correlate positively with each other. For example, plasma $sPLA_2$ levels correlate with CRP levels in patients with coronary artery disease [45]. Furthermore, Yudkin et al. [46] showed that plasma levels of CRP correlate with indices of insulin resistance, which is known to be a risk factor for atherosclerosis progression.

In the present work, we have used a genetic approach to examine whether the endogenous $sPLA_2$ gene influences lipoprotein levels and/or atherosclerosis in apoE knockout mice. C57BL/6 mice, including the standard apoE knockout strain, carry a spontaneous null mutation in $sPLA_2$ [14]. Therefore, we bred apoE knockout mice with C57BL/Ks mice, which are 84% identical to C57BL/6 but carry a wild type $sPLA_2$ gene. The resulting sib pairs ($apoE^{-/-}/sPLA_2^{-/-}$ vs $apoE^{-/-}/sPLA_2^{+/+}$) were analyzed for lipoprotein levels and aortic cholesterol content. Enzyme assays showed that the $sPLA_2^{-/-}$ genotypes generated have essentially no calcium-dependent sPLA2 activity (see Fig. 1). Therefore the type IIA $sPLA_2$ gene described by Kennedy et al. [14] encodes the predominant $sPLA_2$ activity in mouse serum. Yet, neither lipoprotein levels nor aortic cholesterol were influenced by the presence or absence of endogenous levels of mouse $sPLA_2$. Supporting evidence that the mouse $sPLA_2$ locus is not important for atherosclerosis comes from studies of various mouse strains that are susceptible or resistant to atherosclerosis. The C57BL/6 strain is susceptible to atherosclerosis [47], despite carrying a null mutation in $sPLA_2$ [14]. Conversely, the C3H/HEJ strain is resistant to atherosclerosis [47], despite being wild type for $sPLA_2$ [14]. Although many genes may influence the difference in disease susceptibility between these two strains, it is unlikely that the mouse $sPLA_2$ gene has a dramatic effect on atherosclerosis.

Previous investigators reported that transgenic overexpression of human $sPLA_2$ decreased HDL levels and increased atherosclerosis in mice [13]. There are at least two possible explanations for the difference between the present work and these previous studies. One possibility is that transgenic overexpression often leads to extremely high protein levels in plasma. The human $sPLA_2$ transgene results in serum $sPLA_2$ activity that is approximately eightfold greater than the activity in wild-type mice [4]. Therefore, it is possible that the normal level of $sPLA_2$ in wild-type mice does not play a significant role in atherosclerosis, whereas transgenic overexpression of the enzyme accelerates progression of the disease. We found that the enzyme activity in wild-type mice was comparable to the enzyme activity in normal human serum. This would imply that patients most at risk for $sPLA_2$-mediated disease would be those patients with the highest $sPLA_2$ levels in plasma.

Possible species differences between mouse $sPLA_2$ and human $sPLA_2$ may also explain the difference between our work on the endogenous mouse $sPLA_2$ gene and the previous studies that used mice transgenic for the human enzyme. Important species differences have previously been described for other proteins important to lipoprotein biology such as apoA-I [48–50] and hepatic lipase [51–53]. Because of the many examples of important species differences between mice and humans, it is reasonable to speculate that species differences in the $sPLA_2$ enzyme may explain the difference between the present work and previous studies. It remains possible that $sPLA_2$ plays a larger role in human atherogenesis than in murine atherogenesis. However, the complete lack of an effect of $sPLA_2$ in the mouse model suggests that any contribution of $sPLA_2$ to human atherosclerosis is likely to be slight.

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