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The synergistic inhibition of atherogenesis in apoE$^{-/-}$ mice between pravastatin and the sPLA$_2$ inhibitor varespladib (A-002)[S]

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Abstract Secretory phospholipase A2 (sPLA$_2$) activity promotes foam cell formation, increases proinflammatory bioactive lipid levels, decreases HDL levels, increases atherosclerosis in transgenic mice, and is an independent marker of cardiovascular disease. The effects of the sPLA$_2$ inhibitor A-002 (varespladib) and pravastatin as monotherapies and in combination on atherosclerosis, lipids, and paraoxonase (PON) activity in apoE$^{-/-}$ mice were investigated. Male apoE$^{-/-}$ mice were placed on a 12-week high-fat diet supplemented with A-002 alone or combined with pravastatin. Atherosclerotic lesions were examined for size and composition using en face analysis, Movat staining, anti-CD68, and anti-α actin antibodies. Plasma lipids and PON activity were measured. A-002 decreased atherosclerotic lesion area by ∼75% while increasing fibrous cap size by over 200%. HDL levels increased 40% and plasma PON activity increased 80%. Pravastatin monotherapy had no effect on lesion size but when combined with A-002, decreased lesion area 50% and total cholesterol levels 18% more than A-002 alone. A-002, a sPLA$_2$ inhibitor, acts synergistically with pravastatin to decrease atherosclerosis, possibly through decreased levels of systemic inflammation or decreased lipid levels. A-002 treatment also resulted in a profound increase in plasma PON activity and significantly larger fibrous caps, suggesting the formation of more stable plaque architecture.—Shaposhnik, Z., X. Wang, J. Trias, H. Fraser, and A. J. Lusis. **The synergistic inhibition of atherogenesis in apoE$^{-/-}$ mice between pravastatin and the sPLA$_2$ inhibitor varespladib (A-002).** ***J. Lipid Res.*** **2009.** 50: **623–629.**

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The secretory phospholipase A2 (sPLA$_2$) family of enzymes catalyze the production of fatty acids and lysophospholipids by the hydrolysis of phospholipids on cell membranes and circulating lipoproteins (1). sPLA$_2$ activity has been shown to increase the ability of LDL to aggregate, promote foam cell formation in vitro (2), increase proinflammatory bioactive lipid levels (3), decrease HDL levels, and increase atherosclerosis in transgenic mice (4). Additional mouse studies have shown more specifically that overexpression of human sPLA$_2$ groups IIa and V enzymes resulted in increased lesion size (5, 6), while human group IIa overexpression increased oxidative stress. Human studies have shown that circulating sPLA$_2$ levels and activity are associated with increased risk of coronary artery disease (CAD) in healthy individuals (7) and those with existing disease (8). Expression of sPLA$_2$ enzymes (groups IIa, IId, IIe, IIf, III, V, and X) in atherosclerotic lesions increases with the development of atherosclerosis (9). Additional human studies have identified single nucleotide polymorphisms within the sPLA$_2$ group V gene associated with increased levels of oxidized lipids and CAD risk (10).

Statins are a class of compounds (HMG-CoA reductase inhibitors) known to significantly decrease the risk of CAD by their ability to beneficially alter lipid levels and decrease systemic inflammation (11). However, statins are estimated to address only approximately 1/3 of coronary events (12). A significant need exists to identify additional therapeutic approaches to decrease the risk of CAD while working in association with statins. The majority of mouse studies using statins in apoE$^{-/-}$ mice show a dose-response effect on prevention of atherosclerosis while the effect on lipid levels is less pronounced (13); this model has also been used to determine synergism between statins and an apolipoprotein A-I mimetic peptide with cardioprotective properties (14).

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary Methods, Results, and Figures.

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A-002 (varespladib methyl/LY333013/S-3013) is the oral prodrug of A-001 (varespladib sodium/LY315920/S-5920), which was discovered using structural-based drug design using $sPLA_2$ IIa structure (15) and takes advantage of the unique catalytic site His-Asp dyad not present on the related $cPLA_2$ or Lp-PLA_2 enzymes to specifically and selectively inhibit the target enzyme (16). A-001 inhibits both and murine $sPLA_2$ activity with IC_{50}s between 9 and 15 nM when measured using recombinant forms of the group IIa, V, or X enzymes, or when measured in serum from various species (17). The oral or parenteral administration of A-001 abolished PLA_2 activity in a dose-dependent fashion in a transgenic mouse overexpressing human $sPLA_2$ IIa(17) and in sepsis patients expressing high levels of $sPLA_2$(17). Additional in vivo studies in several animal models of lung injury in rats and rabbits (18) demonstrated that administration of A-001 or A-002 inhibited $sPLA_2$ activity, lowered the production of eicosanoids downstream of $sPLA_2$, and prevented injury, which are consistent with the specific inhibition of $sPLA_2$ enzymes rather than downstream enzymes or $cPLA_2$.

Based on the combined animal and human data implicating $sPLA_2$ enzymes in atherosclerosis and the known protective effect of statins in human CAD, the potential synergistic effect between A-002 and pravastatin was explored in an apo$E^{-/-}$ mouse model of atherogenesis. In addition to atherosclerotic lesions, plasma lipids and paraoxonase (PON) activity were also measured. There was a significantly larger decrease in atherosclerosis and total cholesterol levels with the combined treatment compared with either treatment alone, and there was a significant increase in PON activity associated with A-002 treatment. Thus, the combination of the $sPLA_2$ inhibitor, A-002, with statins is an effective strategy to increase the antiatherosclerotic potential of statin therapy.

MATERIALS AND METHODS

Animal husbandry

Apo$E^{-/-}$ null male mice on a C57BL/6J genetic background were purchased from the Jackson Laboratory (Bar Harbor, MI). At approximately 8 weeks of age the mice were divided into six groups (n = 12 per group) and placed on a 12-week ad libitum Western-type diet (TD 88137, Teklad, Madison, WI) in which the diet was supplemented with A-002 and/or pravastatin. See Supplemental Methods for more details.

Plasma lipid analysis and PON activity levels

Animals were fasted overnight before being bled from the retro-orbital sinus. Plasma was collected using heparin as an anticoagulant and used to determine total cholesterol, HDL cholesterol levels, and PON activity. Plasma lipids were determined as described previously (19). See Supplemental Methods for more details.

Quantitation of atherosclerosis, plaque composition, and immunohistochemistry

Methods for the quantitation of atherosclerotic lesions in the aortic root were as previously reported (20). See Supplemental Methods for more details.

Statistical tests

Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using ANOVA (StatView, SAS Institute, Cary, NC) except for comparisons of lesion area, where a nonparametric Kruskal-Wallace test was used to determine differences followed by a Mann-Whitney test to identify synergistic effects of A-002 and pravastatin on lesion area.

RESULTS

Dietary drug delivery

The anti-inflammatory and antiatherosclerotic potential of a specific small molecule $sPLA_2$ inhibitor, A-002, was tested alone and in combination with the widely used statin, pravastatin. Further, a dose-response effect of A-002 and the potential for a synergistic effect of the $sPLA_2$ inhibitor combined with pravastatin was evaluated. The pravastatin dose used was previously demonstrated to not show an effect on lesion size or lipoprotein profiles in apo$E^{-/-}$ mice when administered as monotherapy (14) but was effective when combined with a complementary anti-inflammatory agent. Male apo$E^{-/-}$ mice were placed for 12 weeks on Western-type diets formulated to deliver 2 mg/kg per day of pravastatin and 15 mg/kg (low dose A-002) or 150 mg/kg (high dose A-002) per day of A-002. The high dose was selected to maintain serum levels of A-002 at least 10-fold over the concentration required to inhibit $sPLA_2$ enzyme activity by 50% (IC_{50}s for murine $sPLA_2$ groups V and X are 17 nM and 1.8 nM, respectively; data not shown). The low dose was selected to partially inhibit $sPLA_2$ enzyme activity based on earlier efficacy studies using transgenic mice overexpressing human $sPLA_2$ group IIa that showed that doses in the 0.3–3 mg/kg range exhibited dose-dependent inhibition of $sPLA_2$ activity (data not shown). The formulations were based on an empirically determined average food intake of 2.5 g/day per mouse. No significant differences were detected between any of the groups with respect to behavior, the rate of weight gain or food intake (see Supplemental Results).

Atherosclerotic lesion formation

A-002 decreased atherosclerotic lesion formation in a dose-dependant manner and demonstrated synergistic effects when combined with pravastatin. As previously described, pravastatin alone demonstrated no effect on lesion size, either in the aortic root or in the ascending and thoracic aorta. The low dose of A-002 decreased lesions 40% ($P = 0.019$) while the high dose decreased lesions by 75% ($P = 0.0006$), as measured by en face analysis. When the low dose of A-002 was combined with pravastatin, en face measured lesions decreased 75%, a significant decrease as compared with the low dose A-002 alone ($P = 0.048$) (**Fig. 1A**). Although the high dose of A-002 combined with pravastatin suppressed en face measured lesion area 86% to levels about half as much as high dose A-002 alone, this was not significantly smaller than the 74% reduction in lesion size of mice treated only with high dose A-002. Aortic root lesions decreased more modestly, by

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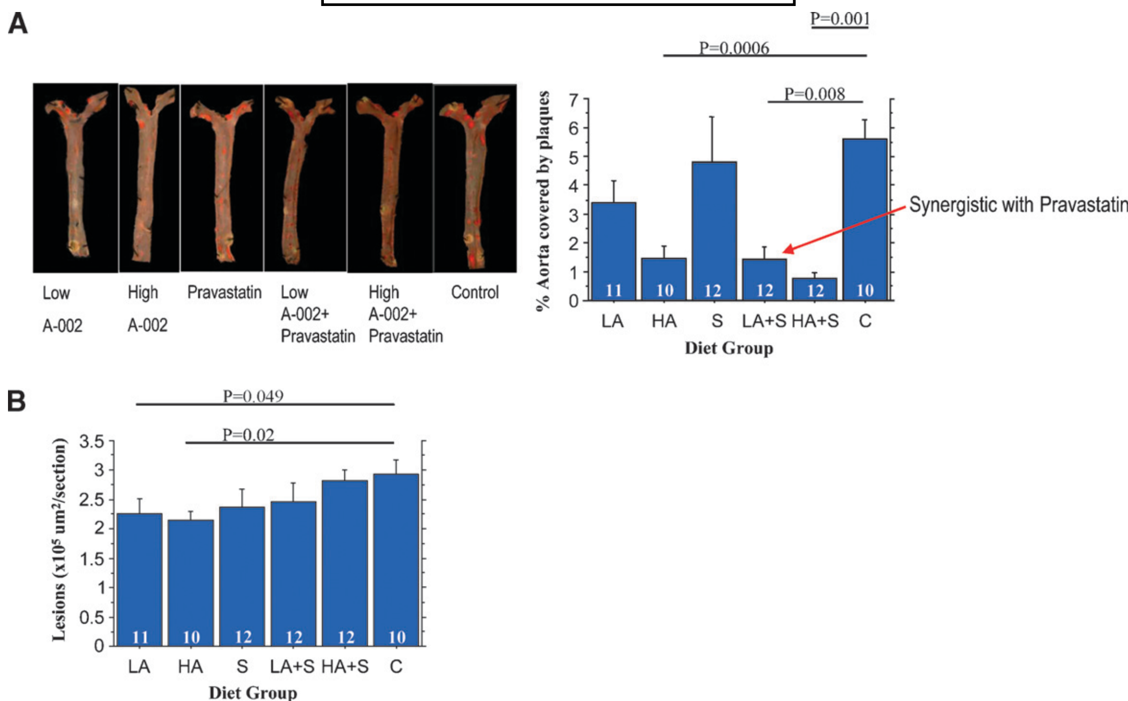

Fig. 1. Effect of A-002 on atherosclerotic lesions in the proximal, ascending, thoracic, and abdominal aorta. The ascending and thoracic aorta was removed and cleared of all connective tissue before being fixed and pinned out. Lipids were stained with Oil Red O (red). En face staining of the thoracic and abdominal aorta from such animals is shown in A. Lesions in aortic root sections are shown in B. Error bars represent ± SEM. C control; HA: high dose A-002; HA+S: high dose A-002 plus pravastatin; LA: low dose A-002; LA+S: low dose A-002 plus pravastatin; S: Pravastatin.

23% ($P = 0.049$), with low dose A-002 and 27% ($P = 0.02$) with high dose A-002 (Fig. 1B). There were no significant differences in aortic root lesion area between any of the other groups of mice.

Atherosclerotic lesion composition

Lesion composition was examined in the aortic root by staining sections for macrophage content, smooth muscle cell (SMC) content, and by Movat staining, which allows visualization of the fibrous cap as well as collagen and proteoglycans. The most striking effect of A-002 was a substantial increase in the size of the fibrous cap (**Fig. 2A–D**) (see supplementary Figure IIA–D). Movat staining revealed that mice treated with the high dose of A-002 exhibited approximately a 2- to 3-fold increase in the percentage of lesion area consisting of fibrous caps (Fig. 2D). There was no change in fibrous cap size in mice treated with low dose A-002. Increased fibrous cap area was also observed after examining the SMC content of the fibrous cap (Fig. 2B). No change was detected in the overall percentage of lesion area consisting of collagen and proteoglycans (Fig. 2C); and there was no effect of pravastatin alone on any parameter of lesion composition or any synergistic effect of treating mice with pravastatin and A-002. Macrophages, which constituted approximately 70% of the aortic root lesion area across all groups, did not significantly decrease as a percentage of lesion area (Fig. 2E). A small increase of approximately 10% in the macrophage positive area was noted in low dose A-002 treated mice. This increase was not noted in any other treatment group and is not statistically different from the statin-treated group. Also, this treatment group had dramatically smaller lesion area when examined by an en face approach. It appears to be an anomaly not indicative of any larger significant trends. The sum of the macrophage and collagen/proteoglycan area appears to exceed 100% because the matrix components overlap with the macrophage positive area and are not mutually exclusive markers of plaque composition.

Plasma lipid composition

Overexpression of $sPLA_2$ in mice has been shown to modify LDL and HDL, presumably by hydrolysis of phospholipids. Therefore, various parameters of plasma lipids and lipoprotein particle size distribution were measured. A synergistic effect of combining A-002 and pravastatin was observed in decreasing total cholesterol levels 18% ($P = 0.015$) in the high dose A-002 plus pravastatin group as compared with the control group (**Table 1**). HDL cholesterol levels trended higher in A-002 treated mice and increased 20% to 40% in both groups treated with A-002 and pravastatin. There was no effect of pravastatin or a synergistic effect between pravastatin and A-002 on HDL cholesterol levels.

Fast protein liquid chromatograpy analyses did not show that the sizes and relative levels of the various lipoprotein classes were substantially affected by A-002 (**Fig. 3**). There

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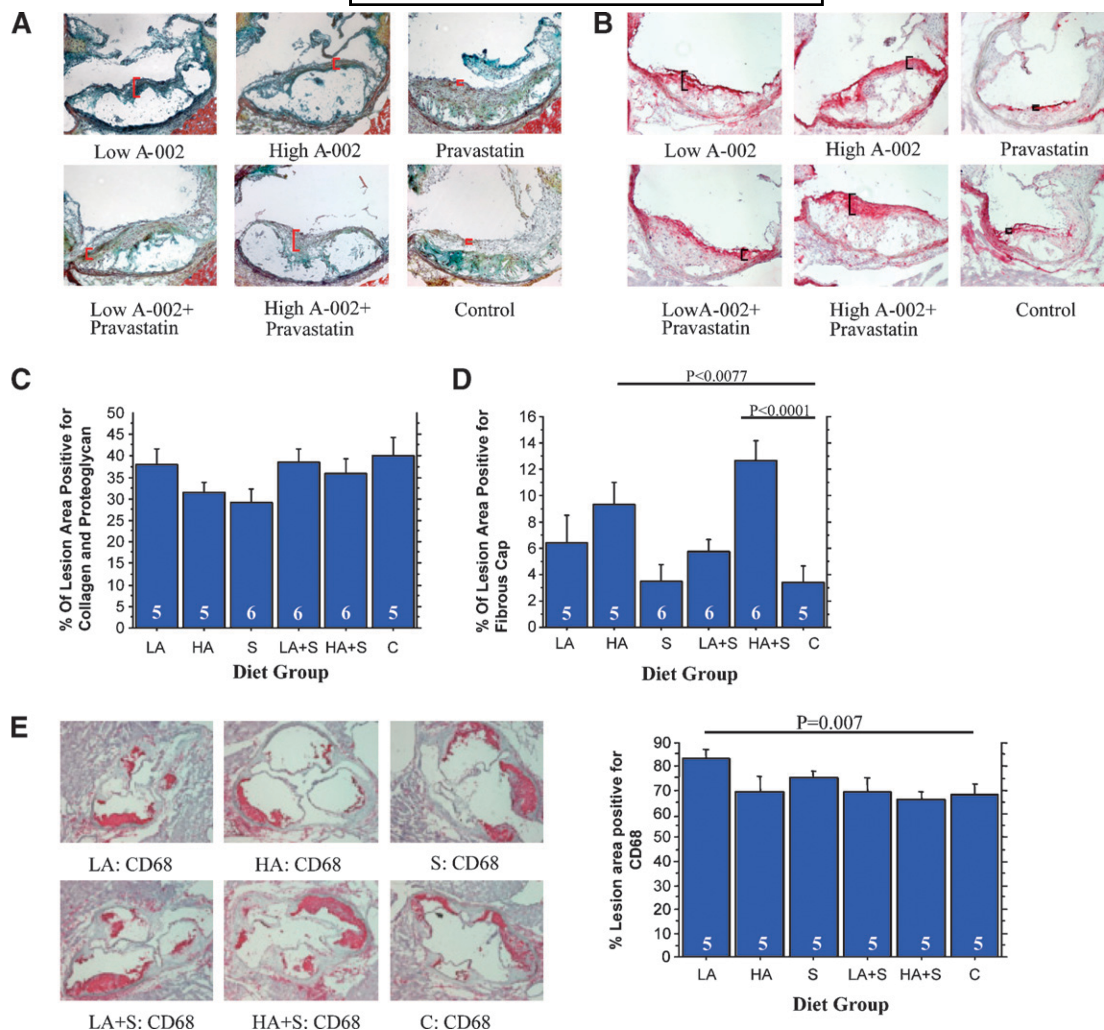

Fig. 2. Effect of A-002 on atherosclerotic lesion composition in the aortic root. Representative lesion sections from male apo$E^{-/-}$ treated with A-002 were stained with Movat Pentachrome showing collagen in yellow, smooth muscle cells (SMCs) in red, and proteoglycans in blue (A) (100×). The thickest portion of the fibrous cap is highlighted in red brackets. SMC α actin content of lesions was stained in red, and the thickest portion of the fibrous cap is highlighted in black brackets (B) (100×). The percentage of lesion area consisting of collagen and proteoglycans (C), or fibrous caps (D) were quantitated. Lesional macrophages, stained red, were detected with an anti-CD68 antibody. Atherosclerotic lesion area positive for macrophages was measured and expressed as a percentage of intimal thickening area in each respective section (E) (50×). Error bars represent ± SEM. C: control; HA: high dose A-002; HA+S: high dose A-002 plus pravastatin; LA: low dose A-002, LA+S: low dose A-002 plus pravastatin, S: Pravastatin.

were no significant differences in glucose (data not shown) or triglyceride levels (Table 1) between any groups.

A-002 increases PON activity

PON1 is an enzyme carried on HDL that exerts antioxidant effects. Plasma PON activity was elevated approximately 90% ($P < 0.0001$) in both groups treated with A-002 and in the group treated with low dose A-002 plus pravastatin (Table 1). Pravastatin treatment alone increased PON activity approximately 38% ($P = 0.02$) but did not enhance the ability of A-002 to increase PON activity.

TABLE 1. Plasma lipid parameters

	Low A-002 (n = 11)	High A-002 (n = 12)	Pravastatin (n = 12)	Low A-002 + Pravastatin (n = 10)	High A-002 + Pravastatin (n = 10)	Placebo (n = 12)
Total cholesterol (mg/dl)	1,355 ± 103	1,301 ± 46	1,405 ± 39	1,399 ± 97	1,219 ± 70[a]	1,478 ± 68
HDL cholesterol (mg/dl)	21 ± 1.1[b]	17 ± 1.0	14 ± 0.9	21 ± 1.4[b]	18 ± 1.0[a]	15 ± 0.7
Triglycerides (mg/dl)	100 ± 4	95 ± 6	90 ± 3	100 ± 8	74 ± 6	84 ± 5
Relative PON activity	46 ± 5[a]	46 ± 1[a]	33 ± 3[a]	45 ± 3[a]	38 ± 2[a]	24 ± 2

PON, paraoxonase.
[a] $P < 0.05$.
[b] $P < 0.0001$.

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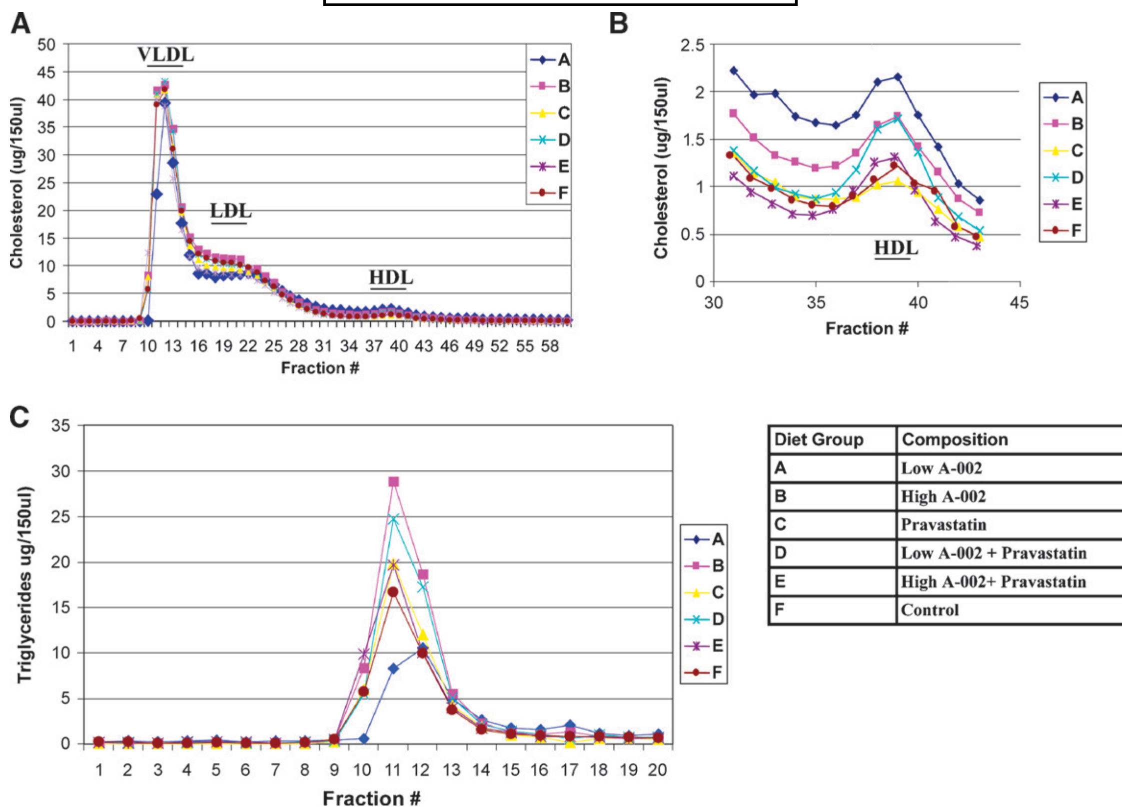

Diet Group	Composition
A	Low A-002
B	High A-002
C	Pravastatin
D	Low A-002 + Pravastatin
E	High A-002+ Pravastatin
F	Control

Fig. 3. Effect of A-002 on plasma lipoprotein fractionation by size exclusion chromatography. A: Pooled plasma samples from 10 male apoE$^{-/-}$ of each group were fractionated by fast protein liquid chromatography (FPLC). Individual 0.5 ml fractions were collected and used to determine the relative cholesterol and triglyceride content as described in Methods. B: HDL fraction peaks are highlighted. C: Triglyceride levels from each plasma sample fraction are plotted.

DISCUSSION

The ability of the specific small molecule $sPLA_2$ inhibitor A-002 to inhibit the development of atherosclerosis in apoE$^{-/-}$ mice on a Western type diet was tested. A-002 treatment resulted in a substantial decrease in atherosclerotic lesion size and a remodeling of the plaque toward a lesion with a more prominent fibrous cap. These effects of A-002 were enhanced by pravastatin in a synergistic manner with respect to atherosclerosis lesion content and plasma total cholesterol. Lipoprotein metabolism was altered, resulting in elevated HDL levels and an even larger increase in plasma antioxidant PON activity.

Profound effects of the combined $sPLA_2$/statin treatment were observed, particularly on lesion area in the ascending and thoracic aorta as measured by en face. The low dose of A-002 decreased lesion size by 40% alone and further decreased lesion size by 75% in the presence of pravastatin. A similar trend was observed with the higher dose of A-002, which decreased lesion area 75% alone. This decreased further to an 86% reduction with the addition of pravastatin (P = NS). These data strongly suggest synergy of A-002 with pravastatin on inhibition of atherosclerotic lesion size.

In contrast to en face data, a maximum 27% lesion size reduction was observed when examining aortic root atherosclerotic lesions from mice treated with high dose A-002 with no significant difference in lesion size noted in mice treated with A-002 and pravastatin. This observation is similar to results reported from LDLR$^{-/-}$ mice that received group V $sPLA_2$ deficient bone marrow and demonstrated no significant difference in aortic root lesion size but a 36% reduction in lesion area within the aortic arch and thoracic aorta (5). In light of large variability in plaque size and the number of mice examined in this study, we estimated that we could reliably detect a 40–50% difference in lesion size. Smaller differences, as observed in this study, can be detected, but not in all cases. Therefore, the lack of our ability to detect an effect in the groups receiving both agents could be due to a lack of power in our study to detect modest changes in plaque size. Also, variation in lesion formation between these two compartments could be due to $sPLA_2$ having a more dramatic effect on amount of aortic surface area covered by plaque by promoting lateral plaque growth rather than the total amount of plaque volume. Human studies (21) have indicated that carotid plaques grow approximately 2.5 times more rapidly in width than in thickness along the vessel in the direction of flow. Overall, these results suggest that $sPLA_2$ has a much larger effect on lesion size in the ascending and thoracic aorta compared with the aortic root.

Increased $sPLA_2$ expression has been demonstrated to enhance arterial collagen deposition (22). While no clear mechanistic explanation was provided or careful examina-

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tion of the fibrous cap conducted, Oestvang and Johansen (23) speculate that this increased collagen formation could be due to some alteration in eicosanoid production as a result of $sPLA_2$ activity. However, because the mice used in this study are deficient in group II $sPLA_2$, no direct comparisons can be made to those results.

Work by Bostrom et al. (5) examining the effect of macrophage specific group V $sPLA_2$ expression on plaque formation is more relevant to this study. That report noted increased collagen content only within the ascending aorta and not within the aortic sinus, the region where we examined lesion size and composition. Although plaque size and $sPLA_2$ distribution was also examined in mice deficient in macrophage specific group V $sPLA_2$, no mention was made of collagen content within the intima.

No change was detected in collagen content within the lesions of the A-002 treated mice using a Movat staining method but a 2- to 3-fold increase in fibrous cap area and lesional SMC content was observed, suggesting increased smooth muscle proliferation and/or decreased protease or matrix metalloproteinase activity within the lesion. It is still possible that increased $sPLA_2$ activity could enhance collagen deposition while $sPLA_2$ inhibition would have no effect on collagen deposition. Also it is possible that a decreased inflammatory state exists in this model as a result of $sPLA_2$ inhibition resulting in increased SMC content, which is manifested in the larger fibrous caps. Extended treatment of SMCs with oxidized lipids led to the formation of oxidized adducts of the platelet-derived growth factor β receptor, decreasing cell proliferation (24). Platelet-derived growth factor is an important factor regulating SMC proliferation in vitro and in the fibrous cap (25). $sPLA_2$ inhibition could be suppressing the formation of such oxidized adducts and result in increased SMC proliferation and larger fibrous caps. A 3-fold increase in lesional SMC content was observed in mice deficient in the serine protease Serp-1 gene, suggesting that altered protease activity may also have a role in the fibrous cap size increase (26). However, due to the fact that there was no change in the proteoglycan or collagen content of the lesion, it seems more likely that $sPLA_2$ inhibition has specifically altered SMC proliferation or survival and not changed the matrix degrading potential of macrophages or other cells within the lesion. No change was detected in the macrophage content of the lesions except for the group receiving low dose A-002. It appears that treatment with the $sPLA_2$ inhibitor A-002 promotes the development of a smaller and more stable lesion encased in a thicker fibrous cap.

It is interesting to speculate on the role of group V $sPLA_2$ in atherosclerosis. Group V $sPLA_2$ is most frequently expressed by macrophages in mouse lesions but is also expressed in aortic SMC (5). We did observe a large increase in the fibrous cap size with A-002 treatment, indicating that perhaps group V enzyme activity negatively regulates SMC proliferation or migration. The group V enzyme has approximately a 3-fold greater enzymatic activity with purified HDL as a substrate compared with LDL and can modify lipoproteins in the presence of serum, properties that the group IIa enzyme lacks. Also, the group V gene is induced by a high-fat Western diet while the group IIa gene is not affected (27). This suggests that in this study A-002 could be inhibiting the group V enzyme's ability to modify plasma LDL into a more inflammatory species and to prevent the degradation of plasma HDL function that normally occurs in $apoE^{-/-}$ mice on a high-fat diet.

$sPLA_2$'s effects on lipid metabolism have been studied primarily in transgenic mice. Such mice were more prone to atherosclerosis and showed increased plasma levels of apoB-containing lipoproteins combined with suppressed HDL cholesterol and PON activity (4). A-002 did not significantly change the VLDL/LDL fraction of lipoproteins based on the fast protein liquid chromatograpy analysis, but there were increased levels of HDL cholesterol. In fact, plasma HDL cholesterol increased up to 40% with A-002 treatment. It is interesting to note that $sPLA_2$ expression appears to increase HDL catabolism while simultaneously increasing hepatic SR-BI cholesterol ester uptake (28). Thus, A-002 may increase both HDL production and turnover, but the former to a greater extent.

A-002 treatment not only increased plasma HDL concentration but also mediated up to a 90% increase in plasma PON activity, suggesting that the increased HDL is functionally active and perhaps more significantly anti-inflammatory on a per-particle basis than prior to treatment. PON1 expression can be suppressed in HepG2 liver cells by the addition of oxidized-LDL or inflammatory cytokines and atherogenic diets reduce PON activity in mice (29). It is therefore possible that inhibiting $sPLA_2$ activity results in decreased oxidized lipid or inflammatory cytokine production, as indicated by previous studies (3, 4) that in turn leads to increased expression of hepatic PON1 and the subsequent increase in plasma PON activity.

$PON1^{-/-}$ $apoE^{-/-}$ mice showed a 2-fold increase in lesion size (30) that was attributed to increased lipid oxidation and production of bioactive phospholipids while human PON1 transgenic mice on an $apoE^{-/-}$ background showed a 20% reduction in aortic root lesions in the context of no increased HDL levels (31). Therefore, treatment with the $sPLA_2$ inhibitor A-002 could be inhibiting lesion formation by decreasing the levels of bioactive oxidized phospholipids through increased PON activity. Increased PON activity could also be important in regulating fibrous cap size by suppressing the formation of oxidized adducts of the platelet-derived growth factor β receptor. A link between elevated HDL levels and substantially increased SMC content was previously demonstrated within lesions from apoA1 transgenic mice (32), again supporting the idea that a component of HDL can increase SMC proliferation and plaque stability by decreasing inflammation.

Overall, the $sPLA_2$ inhibitor A-002 can synergize with statins to decrease lesion size via different mechanisms. A-002 treatment results in certain changes in lipid metabolism that lead to elevated HDL levels and increased HDL protective capacity. This perhaps leads to a suppression of inflammatory lipid generation, decreased systemic inflammation, and/or increased reverse cholesterol transport. Interestingly, we detected a synergistic effect between high dose A-002 and pravastatin on decreasing total cholesterol

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levels. These data also suggest that inhibiting $sPLA_2$ in human disease, particularly when combined with the activity of statins, could retard atherogenesis. Unlike most direct interventions that target one vulnerable plaque at a time, A-002 promises to broadly improve plaque stability and decrease CAD by increasing the size of the fibrous cap and decreasing plaque size while increasing plaque SMC content.

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