Expression of Type IIA Secretory Phospholipase A₂ Inhibits Cholesteryl Ester Transfer Protein Activity in Transgenic Mice

Eva Hurt-Camejo,* Thomas Gautier,* Birgitta Rosengren, Arne Dikkers, Margareta Behrendt, David S. Grass, Daniel J. Rader, Uwe J.F. Tietge

Objective—High circulating levels of group IIA secretory phospholipase A₂ (sPLA₂-IIA) activity and mass are independent cardiovascular risk factors. Therefore, inhibition of sPLA₂-IIA may be a target for the treatment of atherosclerotic cardiovascular disease. The present study evaluated the effects of sPLA₂-IIA inhibition with varespladib acid in a novel mouse model, human apolipoprotein B (apoB)/human cholesteryl ester transfer protein (CETP)/human sPLA₂-IIA triple transgenic mice (TTT) fed a Western-type diet.

Approach and Results—sPLA₂-IIA expression increased atherosclerotic lesion formation in TTT compared with human apoB/human CETP double transgenic mice (P<0.01). Varespladib acid effectively inhibited plasma sPLA₂-IIA activity. Surprisingly, however, administration of varespladib acid to TTT had no impact on atherosclerosis, which could be attributed to a proatherogenic plasma lipoprotein profile that appears in response to sPLA₂-IIA inhibition because of increased plasma CETP activity. In the TTT model, sPLA₂-IIA decreased CETP activity by reducing the acceptor properties of sPLA₂-IIA–modified very low-density lipoprotein specifically because of a significantly lower apoE content. Increasing very low-density lipoprotein-apoE content by means of adenovirus-mediated gene transfer in sPLA₂-IIA transgenic mice restored the acceptor properties for CETP.

Conclusions—These data show that in a humanized triple transgenic mouse model with hypercholesterolemia, sPLA₂-IIA inhibition increases CETP activity via increasing the very low-density lipoprotein-apoE content, resulting in a proatherogenic lipoprotein profile. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: apolipoproteins E ■ atherosclerosis ■ cholesteryl ester transfer proteins ■ cholesterol, VLDL ■ high-density lipoprotein cholesterol

Atherosclerotic cardiovascular disease (CVD) is a leading cause of morbidity and mortality in developed countries. Although high-density lipoproteins (HDL) represent a protective factor, both increased plasma levels of cholesterol within apolipoprotein B-containing lipoproteins and inflammation causally contribute to the pathogenesis of atherosclerosis. The latter is further illustrated by various acute phase proteins having a predictive value for future cardiovascular events. Among these, plasma levels and activity of the acute phase protein type IIA secretory phospholipase A₂ (sPLA₂-IIA) have been demonstrated to predict coronary events in selected patients with coronary artery disease and in the general population. In mouse models, transgenic (tg) human sPLA₂-IIA expression has several proatherogenic properties, such as decreased plasma HDL levels because of increased catabolism, formation of aggregated/fused low-density lipoprotein (LDL), increased LDL oxidation, and induction of severe endothelial dysfunction. These effects actually translate into significant atherosclerotic lesion formation in human sPLA₂-IIA tg mice, even on a chow diet. Taken together, these proatherogenic features of sPLA₂-IIA suggest that pharmacological inhibition of this enzyme might represent a therapeutic strategy against atherosclerotic CVD targeting both the lipoprotein component and the inflammatory component of atherogenesis. Indeed, in animal models, sPLA₂-IIA inhibition showed a reduction in atherosclerotic lesion formation. However, in contrast to the consistent results in preclinical models, clinical trials using sPLA₂ inhibitors...
reveal no HDL-raising effect. The underlying mechanistic basis for these unexpected differences between animal models and humans is currently unclear. Therefore, the aim of this study was to investigate the effect of inhibiting the enzymatic activity of sPLA2-IIA in a novel humanized tg mouse model of atherosclerosis, hsPLA2-IIA/hapoB/hCETP triple transgenic (TTT) mice. Our results demonstrate that inhibition of sPLA2-IIA activity does not alter atherosclerotic lesion formation in this mouse model probably because it induced a proatherogenic plasma lipoprotein profile. In additional experiments we delineated the potential underlying mechanism, namely that sPLA2-IIA decreased CETP activity because of a decrease in the apoE content of sPLA2-IIA-modified very low-density lipoprotein (VLDL) particles. On sPLA2-IIA inhibition, CETP activity increased resulting in elevated plasma levels of proatherogenic apoB-containing lipoproteins. In a clinical perspective, these experimental data suggest that in patients with stable CVD sPLA2-IIA inhibition in combination with a statin or eventually also a CETP inhibitor to decrease VLDL/LDL cholesterol might represent a valid therapeutic approach.

Materials and Methods

Materials and methods are available in the online-only Supplement.

Results

sPLA2-IIA Inhibition Efficiently Reduces Plasma sPLA2-IIA Activity, but Not Atherosclerosis, in TTT Mice

Addition of the sPLA2-IIA inhibitor to the diet resulted in a marked decrease in plasma sPLA2-IIA activity in the TTT mice indicating efficient inhibition of the enzyme (400±50 versus 30±5 U/L; P<0.001). Plasma levels of sPLA2-IIA as determined by ELISA also decreased (581±300 versus 114±35 µg/mL; P<0.001).

Introducing the human sPLA2-IIA transgene into hapoB/hCETP double tg mice resulted in increased atherosclerotic lesion formation determined either as total plaque area (P<0.01; Figure 1A) or as oil-red-O–positive–stained area (P<0.05; Figure 1B). These results are consistent with a proatherosclerotic role of sPLA2-IIA previously described in single tg mice.20 Surprisingly, however, administration of the sPLA2-IIA inhibitor did not change atherosclerotic lesion formation in the TTT mice evaluated in whole aorta, brachiocephalic artery, and aortic root sections after oil-red staining (Figure 1A and 1B). Representative aortic root sections are shown in Figure 1C.

sPLA2-IIA Inhibition Results in a Proatherogenic Plasma Lipoprotein Profile in CETP-Expressing TTT Mice

Dyslipidemia and inflammation are major contributing factors to atherogenesis. To explain the surprising findings of sPLA2-IIA inhibition on atherosclerotic lesion formation, we first evaluated the effect on plasma lipid and lipoprotein profiles. Plasma total cholesterol (Figure 2A) and triglyceride (Figure 2B) levels were not different between hapoB/hCETP double tg mice and TTT mice. However, administration of
the sPLA₂-IIA inhibitor resulted in a significant increase in plasma total cholesterol levels in the TTT mice (P<0.05; Figure 2A) and a trend toward higher levels for plasma triglycerides (P<0.09; Figure 2B). Feeding the sPLA-IIA inhibitor to hApoB/hCETP double tg mice had essentially no effect on plasma total cholesterol or triglycerides (Figure I in the online-only Data Supplement) indicating that the changes in plasma lipids were related to sPLA-IIA expression. Plasma VLDL, LDL, and HDL cholesterol levels were not significantly affected by sPLA-IIA expression in TTT compared with hApoB/hCETP double tg mice on Western diet (Figure 2C). However, sPLA-IIA inhibition in TTT resulted in increased plasma cholesterol levels within the apoB-containing lipoproteins VLDL (P<0.001; Figure 2C) and LDL (P<0.01; Figure 2C) and decreased levels of HDL cholesterol (P<0.001; Figure 2C). The VLDL phospholipid content was increased on varespladib acid treatment in TTT to a similar extent as the VLDL cholesterol levels (0.08±0.03 versus 0.48±0.24; P<0.01).

These data demonstrate that inhibition of sPLA₂-IIA results in a proatherogenic plasma lipoprotein profile specifically in the functional context of CETP expression.

sPLA₂-IIA Expression Decreases Plasma CETP Activity

Because the changes in the plasma lipoprotein profile induced by sPLA₂ inhibition closely resemble alterations because of increased CETP activity, we hypothesized that sPLA₂-IIA expression might inhibit CETP activity. Consistent with this hypothesis, CETP activity decreased in hCETP/hApoB double tg mice when the hsPLA₂-IIA transgene was also expressed (38.1±19.7 versus 13.3±10.1 pmol/h; P<0.01; Figure 3). Interestingly, inhibition of sPLA₂-IIA with varespladib acid resulted in a striking 7-fold increase in CETP activity compared with TTT treated with vehicle (69.4±21.1 pmol/h; P<0.001; Figure 3). The inhibitory effect of sPLA₂-IIA on plasma CETP activity was independent of the atherogenic diet as evidenced by significantly lower plasma CETP activity in chow-fed TTT compared with chow-fed hApoB/hCETP double tg mice (P<0.05; Figure IV in the online-only Data Supplement). However, the inhibitor varespladib acid had no effect on plasma CETP activity in the absence of sPLA₂-IIA in hApoB/hCETP double tg mice (Figure II in the online-only Data Supplement). This indicates that the observed increase in CETP activity, after varespladib acid administration, is because of inhibition of sPLA₂-IIA activity. These data demonstrate that sPLA₂-IIA expression results in decreased CETP activity.

Figure 2. Type IIa secretory phospholipase A2 (sPLA₂-IIA) inhibition results in the development of a proatherogenic plasma lipid and lipoprotein profile in human cholesteryl ester transfer protein (CETP)/human apolipoprotein B (apoB)/human sPLA₂-IIA triple transgenic (TTT) mice. Human CETP/human apoB double transgenic (TT) mice and TTT were fed an atherogenic diet for 17 weeks with or without the sPLA₂ inhibitor varespladib acid (VA). Then (A) total cholesterol, (B) triglycerides, and (C) cholesterol distribution over the different lipoprotein subclasses were determined as indicated and as detailed in Methods in the online-only Data Supplement. Data are given as mean±SD. n=9 to 10 in each group. Significant differences from vehicle-treated TTT are indicated as *P<0.05, **P<0.01, and ***P<0.001. HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and VLDL-C, very low-density lipoprotein.

Figure 3. Type IIa secretory phospholipase A2 (sPLA₂-IIA) inhibition increases cholesteryl ester transfer protein (CETP) activity in vivo. CETP activity was assayed as described under Methods in the online-only Data Supplement in plasma from human CETP/human apolipoprotein B (apoB) double transgenic mice (TT) as well as in plasma from human CETP/human apoB/human sPLA₂-IIA triple transgenic mice (TTT) fed a Western-type diet containing either vehicle or the specific sPLA₂-IIA inhibitor varespladib acid (+VA). Data are given as mean±SD. n=10 in each group. a indicates statistically significant differences from the TT; b, statistically significant differences from the TTT; and c, statistically significant differences from the TTT+VA group (at least P<0.05).
sPLA2-IIA Inhibition Increases the Acceptor Properties of VLDL for CETP-Mediated Lipid Transfer in TTT Mice

Next, we investigated whether the differences in plasma CETP activity observed between TTT mice treated with either varespladib acid or vehicle might be because of different amounts of CETP protein present in plasma. As determined by Western blot, plasma CETP protein levels were identical in both groups (varespladib acid: 4.49±1.47 mg/L versus vehicle: 4.22±1.55 mg/L; ns; Figure 4A), indicating that administration of varespladib acid does not affect plasma CETP protein levels but results in a selective increase in CETP activity.

Because plasma CETP activity is influenced by the properties of apoB-containing lipoproteins and HDL to serve as acceptors and donors of cholesterol, respectively, we next differentially explored these features using lipoprotein subclasses isolated by ultracentrifugation from TTT treated with varespladib acid or vehicle. On incubation with fixed amounts of exogenous purified CETP, CETP activity was significantly higher, when VLDL (P<0.05; Figure 4B) or LDL (P<0.05; Figure 4C) from TTT treated with the sPLA2-IIA inhibitor varespladib acid was added to the assay compared with apoB-containing lipoproteins from vehicle-treated TTT. However, although sPLA2-IIA expression results in major changes in HDL composition,11,22 the donor properties of HDL isolated from TTT with and without varespladib acid treatment were not affected (ns; Figure 4D). Using the infranatant as CETP source and exogenous donor and acceptor liposomes, CETP activities were identical, when TTT receiving the inhibitor were compared with those that were vehicle treated (ns; Figure 4E). These data are consistent with the unchanged plasma CETP protein levels in both groups of mice.

Taken together, these results indicate that sPLA2-IIA expression might specifically decrease the acceptor properties of VLDL for CETP-mediated lipid transfer translating into reduced plasma CETP activity.

sPLA2-IIA Modification of VLDL In Vivo Decreases the Acceptor Properties for CETP-Mediated Lipid Transfer in sPLA2-IIA Single tg Mice

To distinguish whether the observed alterations in the cholesterol content of the different lipoprotein subclasses in response to sPLA2-IIA inhibition represent the underlying cause or the consequence of increased plasma CETP activity, human sPLA2-IIA single tg mice that lack endogenous or transgenically expressed CETP were placed on a...
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Western-type diet and treated with either the sPLA2-IIA inhibitor varespladib acid or vehicle. In contrast to the TTT mice expressing CETP, treatment with varespladib acid did not affect cholesterol content of VLDL. Human sPLA2-IIA single transgenic mice were fed a Western-type diet containing either vehicle (vehicle) or the specific sPLA2-IIA inhibitor varespladib acid (inhibitor). Cholesterol content (A) of the different lipoprotein subclasses isolated from the respective groups. B–D, CETP activity assays performed using VLDL (B; 0.5 µg protein/assay), LDL (C; 2 µg protein/assay), and high-density lipoprotein (HDL; D; 2 µg protein/assay) isolated by sequential ultracentrifugation. E, Western blot for apoE within VLDL isolated by sequential ultracentrifugation from 3 individual mice from each of the respective experimental groups as indicated. HDL from wild-type C57BL/6 mice was used as a positive control. Data are given as mean±SD. n=6 in each group. *Significantly different from control (vehicle treatment) values (at least P<0.05).

Figure 5. Type IIA secretory phospholipase A2 (sPLA2-IIA) activity impairs the acceptor properties of very low-density lipoprotein (VLDL) particles for cholesteryl ester transfer protein (CETP)-mediated lipid transfer in human sPLA2-IIA single transgenic mice by decreasing the apolipoprotein E (apoE) content of VLDL. Human sPLA2-IIA single transgenic mice were fed a Western-type diet containing either vehicle (vehicle) or the specific sPLA2-IIA inhibitor varespladib acid (inhibitor). Cholesterol content (A) of the different lipoprotein subclasses isolated from the respective groups. B–D, CETP activity assays performed using VLDL (B; 0.5 µg protein/assay), LDL (C; 2 µg protein/assay), and high-density lipoprotein (HDL; D; 2 µg protein/assay) isolated by sequential ultracentrifugation. E, Western blot for apoE within VLDL isolated by sequential ultracentrifugation from 3 individual mice from each of the respective experimental groups as indicated. HDL from wild-type C57BL/6 mice was used as a positive control. Data are given as mean±SD. n=6 in each group. *Significantly different from control (vehicle treatment) values (at least P<0.05).

Western-type diet and treated with either the sPLA2-IIA inhibitor varespladib acid or vehicle. In contrast to the TTT mice expressing CETP, treatment with varespladib acid did not affect cholesterol content of VLDL (ns; Figure 5A), whereas LDL (P<0.05; Figure 5A) and HDL (P<0.05; Figure 5A) cholesterol contents were increased compared with vehicle-treated mice. The latter data are consistent with sPLA2-IIA expression resulting in decreased plasma HDL cholesterol levels because of increased catabolism.13,14 The TG:protein ratio remained unchanged within VLDL (0.80±0.198 versus 0.77±0.256, respectively; ns) and LDL (0.32±0.042 versus 0.33±0.165, respectively; ns) from mice receiving varespladib acid compared with vehicle treatment, whereas being decreased within HDL (0.058±0.013 versus 0.079±0.012, respectively; P<0.05). The TG:cholesterol ratio of VLDL (1.27±0.555 versus 1.86±0.704, respectively; ns), LDL (0.178±0.019 versus 0.183±0.087, respectively; ns), and HDL (0.081±0.016 versus 0.090±0.017, respectively; ns) was also not affected by varespladib acid compared with vehicle treatment. In addition, also the phospholipid content of VLDL was identical between the 2 groups (0.03±0.009 versus 0.028±0.010 mmol/L; ns), and the phospholipid:cholesterol ratio remained unaffected by varespladib acid administration (0.745±0.081 versus 0.778±0.065; ns). These data indicate that the altered cholesterol and phospholipid contents within VLDL of varespladib acid–treated TTT are a consequence of increased CETP activity rather than its cause.

Next, CETP activity assays were performed with purified CETP, testing VLDL, LDL, and HDL for their ability to interact with purified CETP. Interestingly, CETP activity was 2.8-fold higher when VLDL from single sPLA2-IIA tg mice treated with varespladib acid was used compared with VLDL from mice receiving vehicle (P<0.01; Figure 5B). In addition, there was no impact of cholic acid in the diet on the acceptor properties of VLDL from sPLA2-IIA single tg mice because VLDL isolated from chow-fed versus 0.5% cholic acid–fed mice gave comparable results in CETP transfer assays (Figure V in the online-only Data Supplement). However, there were no differences on the substrate properties of LDL (ns; Figure 5C) and HDL (ns; Figure 5D) when lipoproteins isolated from varespladib acid–treated mice and controls were compared. These data confirm that even in the absence of altered VLDL lipid composition the cholesterol acceptor function of sPLA2-IIA–modified VLDL for CETP-mediated transfer is severely impaired translating into significantly decreased plasma CETP activity.

Because the lipid composition of sPLA2-IIA–modified VLDL did not represent the underlying basis for decreased CETP activity, we next determined the protein composition of VLDL using SDS-PAGE followed by silver staining. VLDL isolated from sPLA2-IIA tg mice receiving vehicle showed a
significantly decreased band in the molecular weight range of apoE (data not shown) as the only difference, when compared with VLDL from varespladib–treated sPLA₂-IIA tg mice. Subsequently, a striking 3.4-fold increase (P<0.05) in the apoE content of VLDL isolated from sPLA₂-IIA inhibitor–treated single tg mice was confirmed by Western blot using lipoproteins isolated by ultracentrifugation (Figure 5E) and fast protein liquid chromatography (Figure VIA in the online-only Data Supplement). Also in VLDL from TTT mice isolated by fast protein liquid chromatography, a substantial increase in apoE was observed on varespladib acid administration (Figure VIB in the online-only Data Supplement). In addition, by comparing VLDL from wild-type control mice with single sPLA₂-IIA tg mice on chow diet, we further demonstrate that sPLA₂-IIA expression is associated with a substantially lower apoE content of VLDL (Figure VIC in the online-only Data Supplement). The apoE content of VLDL might be an important determinant of CETP activity by facilitating the binding of CETP to VLDL particles.21 Taken together, these data suggest that decreased VLDL-apoE content is the major metabolic change induced by sPLA₂-IIA activity subsequently resulting in decreased plasma CETP activity. By the use of the sPLA₂ inhibitor varespladib acid, this effect is reverse leading to a substantial increase in CETP activity and consequently a proatherogenic plasma lipid and lipoprotein profile in the TTT mouse model.

Hepatic ApoE Overexpression in sPLA₂-IIA Single tg Mice Increases the VLDL-ApoE Content and Subsequently the Acceptor Properties for CETP

Next, apoE was overexpressed in livers of sPLA₂-IIA tg mice by means of a recombinant adenovirus to establish that increasing the apoE content of VLDL in this model translates into increased CETP activity. ApoE overexpression did not affect plasma sPLA₂-IIA activity (537±83 versus 515±54 U/L). VLDL particles isolated from sPLA₂-IIA single tg mice on day 3 after injection with AdhapoE3 were 1.4-fold enriched in apoE compared with VLDL from control adenovirus-administered mice (P<0.05; Figure 6A). Consistent with our hypothesis, CETP activity was significantly increased when VLDL from sPLA₂-IIA tg mice injected with the apoE-expressing adenovirus was used as acceptor particles for CETP-mediated transfer compared with VLDL from controls (P<0.01; Figure 6B). These data demonstrate that increasing the VLDL-apoE content improves the acceptor properties of VLDL from sPLA₂-IIA tg mice for CETP-mediated transfer translating into increased CETP activity.

Discussion

Based on evidence that the acute phase protein sPLA₂-IIA is a cardiovascular risk factor6–12 with intrinsic proatherosclerotic biological activity,24,25 specific inhibition of sPLA₂-IIA activity in vivo seems to be an attractive target for the treatment of atherosclerotic CVD. As opposed to consistent data in preclinical models supporting this concept,21 clinical trials conducted with the sPLA₂ inhibitor varespladib did not deliver the desired antiatherosclerotic results, leading to the discontinuation of the further clinical development of this drug (Anthera Pharmaceuticals, Inc, 9 March 2012, press release). The present study tested the effect of sPLA₂-IIA inhibition in a novel humanized mouse model of lipoprotein metabolism, human apoB/human CETP/human sPLA₂-IIA triple tg mice (TTT) fed a cholesterol-containing diet. However, when TTT were given the sPLA₂-IIA inhibitor varespladib acid there was no impact on atherosclerosis, probably because of the development of a proatherogenic plasma lipoprotein profile with increased apoB-containing lipoproteins and decreased HDL. The latter result was especially unexpected because tg sPLA₂-IIA expression in wild-type mice has a strong decreasing effect on plasma HDL levels by increasing the HDL catabolic rate.13–15

The changes in the plasma lipoprotein profile after administration of varespladib acid were consistent with increased plasma CETP activity. Subsequently, our data confirmed that inhibition of sPLA₂-IIA resulted in a marked increase in plasma CETP activity in the TTT group. Increased CETP activity has been linked to increased risk for atherosclerotic CVD in human populations.26,27 CETP activity in vivo mainly depends on 2 factors: (1) the plasma concentration of the CETP protein and (2) the quantity and the quality of the lipoprotein substrates.28,29 The large increase in CETP activity after sPLA₂-IIA inhibition could not be explained by changes in plasma CETP mass levels, as shown by quantitative Western blot and by the unchanged cholesteryl ester.
transfer activity measured in plasma infranatants devoid of lipoproteins. Instead, our data demonstrate that increased CETP activity on sPLA₂-IIA inhibition was because of an enhanced ability of VLDL to serve as cholesteryl ester acceptors in the presence of purified CETP and liposome donors, whereas LDL and HDL substrate properties were only slightly or not at all modified. The increased ability of VLDL from TTT treated with the sPLA₂-IIA inhibitor to interact with CETP was accompanied by a cholesteryl enrichment of the VLDL fraction. Although some studies demonstrated that VLDL cholesteryl content can have an impact on the magnitude of the CETP-mediated neutral lipid exchange between lipoproteins,30–32 we observed a similar improvement of VLDL acceptor properties after sPLA₂-IIA inhibition, when VLDL from hsPLA₂ single tg mice lacking CETP was used that displayed identical cholesteryl content within VLDL. These results demonstrate that (1) compared with vehicle-treated sPLA₂-IIA single tg mice the dramatic cholesteryl enrichment of VLDL from TTT treated with the sPLA₂-IIA inhibitor is a consequence rather than the cause of increased CETP activity and (2) the increased ability of VLDL to interact with CETP after sPLA₂-IIA inhibition is not because of alterations of the lipid composition of VLDL but possibly because of a modification of the protein composition of the particles.

The finding that altered protein composition of VLDL represents the underlying basis of changes in CETP activity in vivo, we think that it is a novel observation because most studies published today focused on the protein composition and related cholesterol donor properties of HDL. Indeed, the impact of apoA-I, apoA-II, and apoC-I content was studied in HDL,33,34 and the influence of the Lipid Transfer Protein, also known as apoF, was investigated in LDL and HDL.35 As judged from silver-stained SDS-PAGE gels, the only significant difference between VLDL from sPLA₂-IIA inhibitor–treated mice and control animals was a striking increase in the apoE content in mice receiving the inhibitor. When the VLDL-apoE content was increased in sPLA₂-IIA single tg mice by means of adenovirus-mediated gene transfer, the CETP acceptor properties of these particles were significantly improved. These data are in line with a case study on VLDL from a single apoE-deficient subject that reported lower CETP activity using the apoE-deficient VLDL as acceptor compared with VLDL from controls.23

The potential physiological role of an inhibition of CETP by the acute phase protein sPLA₂-IIA has not been explored in the present study. Although largely speculative, we think that 1 possibility is that inflammatory conditions require increased synthesis of steroid hormones and that HDL cholesteryl ester is a known source of cholesterol for steroid hormone synthesis.35 sPLA₂-IIA could make HDL cholesteryl ester more accessible to steroidogenic tissues by increasing SR-BI–mediated selective uptake.13,14 Although inflammation already downregulates CETP expression,36 additional inhibition of CETP by sPLA₂-IIA in this context might further increase the availability of HDL cholesteryl ester for steroid hormone synthesis and might thus represent a protective mechanism during the host response. In this context, it might be important to point out that the cholate added to the diet in our present study induces a certain degree of hepatic inflammation, as shown in Table I in the online-only Data Supplement. However, based on several additional control experiments that we performed, we do not think that this dietary choice affected the validity of our results.

The lack of an HDL-raising effect on sPLA₂-IIA inhibition in patients contrasts the biological effects that would be predicted to occur on sPLA₂-IIA inhibition.13,21,37 Experiments in mice showed that the predominant phenotype of tg expression of human sPLA₂-IIA on the C57BL/6 genetic background is decreased plasma HDL cholesteryl because of an increased catabolic rate.13,14 In support of this mechanism, sPLA₂ inhibition in our present study significantly increased plasma HDL cholesterol in a CETP-deficient model. In mice expressing CETP, however, inhibition of CETP by sPLA₂-IIA expression did not result in a beneficial effect on the plasma lipoprotein profile. Conceivably, a potential HDL-raising effect is counteracted by the direct impact sPLA₂-IIA has independent of CETP by increasing HDL catabolism and thereby decreasing plasma HDL-C levels. In the same direction, a decrease in the apoE content of apoB-containing lipoproteins would be expected to decrease their catabolic rate and thereby counteract a potential decrease in the plasma levels of these lipoproteins caused by the inhibition of CETP.

High-risk patients with atherosclerotic CVD and a significant inflammatory load have increased plasma levels of sPLA₂-IIA6,7,19 and for this patient group sPLA₂-IIA inhibitors have been developed.36 However, in stable patients with CVD, no HDL cholesterol increase was observed.21 Our data suggest that modulation of CETP activity might be the mechanistic basis for this observation. However, our data have been generated in a mouse model with all the potential limitations, so that still validation in a human setting would be required. Although a prospective trial in patients with acute coronary syndrome was discontinued because of lack of efficacy of the sPLA₂-IIA inhibitor varespladib (Anthera Pharmaceuticals, Inc, 9 March 2012, press release), in our view a clinical trial with sufficient duration to investigate atherosclerosis-mediated cardiovascular events in stable patients with CVD combining a sPLA₂-IIA inhibitor with a statin and potentially also with a CETP inhibitor, once a compound with proven clinical benefit becomes available, would still provide scientifically valuable information.

In conclusion, the results from this study performed in a tg humanized mouse model with hypercholesterolemia show proatherogenic effects of sPLA₂-IIA inhibition on the plasma lipoprotein profile that might counteract the local antiatherogenic effects in the vessel wall.

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Disclosures

E. Hurt-Camejo, B. Rosengren, and M. Behrendt are employees of AstraZeneca. D.S. Grass is an employee of Taconic. This work constitutes a scientific collaboration between AstraZeneca and academic institutions without any financial interest involved. The other authors report no conflicts.

References

35. Rigotti A, Edelman ER, Seifert P, Iqbal SN, DeMattos RB, Temel RE, Krieger M, Williams DL. Regulation by adrenocorticotropin hormone of the in vivo expression of scavenger receptor class B type I (SR-BI), a high


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**Significance**

The group IIA secretory phospholipase A2 (sPLA2-IIA) represents a cardiovascular risk factor with intrinsic proatherosclerotic biological activity. Therefore, inhibitors of sPLA2-IIA were developed as treatment for atherosclerotic cardiovascular disease. Although effective in rodent models, human clinical trials did not deliver the expected results. A major difference compared with rodents is the expression of the cholesteryl ester transfer protein in humans. To explore the impact of this difference, the present study tested sPLA2-IIA inhibition with varespladib acid in a novel humanized mouse model of atherosclerosis, human apolipoprotein B/human cholesteryl ester transfer protein/human sPLA2-IIA triple transgenic mice. Our results demonstrate that sPLA2-IIA inhibition in this model does not decrease atherosclerosis. Mechanistically, sPLA2-IIA inhibition increased cholesteryl ester transfer protein activity resulting in a proatherogenic lipoprotein profile. On the basis of these data, more effective strategies using sPLA2-IIA inhibitors to treat atherosclerosis can be developed for clinical use.
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**Supplemental table I:** Plasma parameters of liver inflammation in the different mouse models investigated

<table>
<thead>
<tr>
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<th>TT (chow, vehicle)</th>
<th>TTT (chow, vehicle)</th>
<th>TT (athero, vehicle)</th>
<th>TT (athero, VA)</th>
<th>TTT (athero, vehicle)</th>
<th>TTT (athero, VA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT (U/l)</strong></td>
<td>37 ± 8</td>
<td>41 ± 10</td>
<td>126 ± 41*</td>
<td>137 ± 58*</td>
<td>160 ± 42*</td>
<td>139 ± 49*</td>
</tr>
<tr>
<td><strong>AST (U/l)</strong></td>
<td>33 ± 4</td>
<td>32 ± 7</td>
<td>157 ± 62*</td>
<td>145 ± 75*</td>
<td>196 ± 60*</td>
<td>152 ± 63*</td>
</tr>
<tr>
<td><strong>SAA (µg/ml)</strong></td>
<td>7 ± 3</td>
<td>5 ± 3</td>
<td>60 ± 25*</td>
<td>63 ± 29*</td>
<td>61 ± 40*</td>
<td>52 ± 13*</td>
</tr>
</tbody>
</table>

Data are given as means ± SD, n=5-6 mice/group. athero, atherogenic diet; VA, varespladib acid. * significantly different (p<0.001) from the respective chow group.
Supplemental figure I: sPLA₂-IIA inhibitor treatment does not result in alterations in plasma lipids in TT mice not expressing human sPLA₂-IIA. Human CETP/ human apoB double transgenic (TT) mice were fed an atherogenic diet with or without varespladib acid (VA). Then (A) total cholesterol, (B) triglycerides and (C) phospholipids were determined as detailed in methods. Data are given as means ± SD. n=9 in each group.
Supplemental figure II: sPLA$_2$-IIA inhibitor treatment does not alter plasma CETP activity or protein levels in TT mice not expressing human sPLA$_2$-IIA. Human CETP/ human apoB double transgenic (TT) mice were fed an atherogenic diet with or without varespladib acid (VA). Then (A) CETP activity and (B) CETP mass using Western blot were determined as detailed in methods. Data in (A) are given as means ± SD, n=9 in each group.
Supplemental figure III: sPLA2-IIA inhibitor treatment does not change the donor or acceptor properties of lipoproteins isolated by ultracentrifugation for CETP-mediated transfer in TT mice not expressing human sPLA2-IIA. (A) VLDL, (B) LDL, (C) HDL, and (D) infranatant. Assays were performed as detailed in methods. Data are given as means ± SD. n=5 in each group.
Supplemental figure IV: sPLA$_2$-IIA expression decreases plasma CETP activity in chow-fed mice. CETP activity was significantly lower in TTT mice expressing sPLA$_2$-IIA than in TT mice not expressing human sPLA$_2$-IIA. CETP activity was determined as detailed in methods. Data are given as means ± SD. n=5 per group.
Supplemental figure V: Addition of cholic acid (CA) to the diet does not change the effect of VLDL isolated from sPLA$_2$-IIA single transgenic mice on CETP activity. VLDL from mice fed the respective diets as indicated were isolated by ultracentrifugation and the acceptor properties for CETP-mediated lipid transfer were determined as detailed in methods. Data are given as means ± SD. n=6 per group.
Supplemental figure VI: Western blots to assess the impact of sPLA$_2$-IIA on the apoE content of VLDL using different experimental conditions. (A) VLDL isolated by FPLC from plasma of sPLA$_2$-IIA single tg mice on an atherogenic diet treated with vehicle or VA, (B) VLDL isolated by FPLC from TTT mice on an atherogenic diet treated with vehicle or VA, (C) VLDL isolated by ultracentrifugation from chow-fed sPLA$_2$-IIA single tg mice compared with wild-type controls. Western blots were carried out as detailed in methods.
Materials and Methods

Experimental animals.
The generation of the human sPLA\textsubscript{2}-IIA tg \textsuperscript{1}, the human apoB tg and the human CETP tg mice \textsuperscript{2} on the C57BL/6J genetic background used in this study has been described. The respective models were crossed to obtain hsPLA\textsubscript{2}/hapoB/hCETP triple tg (TTT) mice as well as hCETP/hapoB controls (Taconic, Hudson, NY). The animals were kept in animal rooms with alternating 12-hour periods of light (from 7:00 a.m. to 7:00 p.m.) and dark (from 7:00 p.m. to 7:00 a.m.), with ad libitum access to water and were fed a high fat diet containing 15% cacao fat, 1.25 % cholesterol, 0.5 % cholic acid. This specific diet was chosen to assure atherosclerotic lesion formation in this model that otherwise does not develop atherosclerosis easily. Of note, none of the mice exhibited signs of distress or suffering during the course of the study. The sPLA\textsubscript{2}-IIA inhibitor varespladib acid was administered in the food at a dose of 100 µmol/kg/day. Plasma concentration was 2 µM at the end of the study (in vitro IC\textsubscript{50} = 9nM for human sPLA\textsubscript{2}-IIA). Animals (females) were 12 weeks of age when the treatment started. The total treatment time was 17 weeks. Treatment of TTT as well as of the double transgenic mice not expressing sPLA\textsubscript{2}-IIA with the sPLA\textsubscript{2} inhibitor was not associated with liver toxicity, as judged by measurements of plasma levels of transaminases (Spinreact S.A., Girona, Spain) and the acute phase protein SAA (BioSource, Camarillo, CA, USA; supplemental table I). Key data obtained with the use of varespladib acid in TT mice not expressing the human sPLA\textsubscript{2}-IIA transgene to ascertain specificity of the pharmacological approach are shown as supplemental figures I-III. Procedures for perfusion, fixation and quantitation of atherosclerosis in the whole aorta, brachio-cephalic artery and aortic root sections after oil-red staining were carried out as described \textsuperscript{3}. Briefly, for quantitation, 10 serial sections, each 2-3 µm thick and 100 µm apart were cut and stained with Weigert’s haematoxylin/van Gieson or oil-red-O. Animal experiments were performed in accordance with national laws and all protocols were approved by the responsible local Ethics Review Committees on Animal Experiments in Göteborg, Sweden and at the University of Burgundy.

Generation of recombinant adenoviruses.
Generation of the human apoE3 expressing adenovirus AdapoE3 as well as of the empty control adenovirus AdNull has been described \textsuperscript{4}. Recombinant adenoviruses were amplified and purified as reported previously \textsuperscript{5}. In vivo experiments were carried out using a dose of 1x10\textsuperscript{11} particles of each of these adenoviruses per mouse.

Plasma lipid and lipoprotein analyses.
Mice were bled from the heart after a 4 h-fast using heparinized capillary tubes. Aliquots of plasma were stored at −80°C until analysis. Plasma total cholesterol, triglycerides, and phospholipids were measured enzymatically using commercially available reagents (Wako Pure Chemical Industries, Neuss, Germany). The distribution of cholesterol, triglycerides and phospholipids over the different lipoprotein subfractions was determined after separation of the lipoproteins by fast-protein liquid chromatography (FPLC) using a Superose 6 column (GE Healthcare, Uppsala, Sweden)\textsuperscript{6}.

Plasma sPLA\textsubscript{2}-IIA mass and activity measurements.
Plasma levels of sPLA\textsubscript{2}-IIA protein were measured by ELISA (Cayman Chemicals, Ann Arbor, MI, USA) and ranged between 750 and 1000 ng/ml in the TTT mice on chow diet. Enzymatic activity of sPLA\textsubscript{2}-IIa was measured using radioactively labeled phospholipids as substrate essentially as described before \textsuperscript{7}. Plasma sPLA\textsubscript{2}-IIa activity levels in TTT mice were on average 400 U/liter.

Isolation and characterization of plasma VLDL, LDL and HDL.
VLDL, LDL and HDL were isolated from mouse plasma by sequential ultracentrifugation as the d < 1.019 g/ml, the 1.019 g/ml < d < 1.063 g/ml, and the 1.063 g/ml
Densities were adjusted with KBr solutions. Each centrifugation step consisted of a 3-h run at 100,000 rpm in a TL-100 rotor on a Beckman Optima TLX ultracentrifuge (Fullerton, CA). Cholesterol, triglyceride and phospholipid contents were measured as detailed above, protein levels were determined by the bicinchoninic acid method (BCA Assay, Pierce, Rockford, IL).

Measurement of cholesteryl ester transfer activity.
CETP activity was measured using a fluorescent assay that was performed in microplates using donor liposomes enriched with NBD (nitrobenz-oxadiazol)-labelled cholesteryl esters (ROAR Biomedical, New York, NY) following the general procedure described previously. For determination of plasma CETP activity, incubation media contained 4 µl of donor liposomes and 10 µl of individual total plasma. For the ability of plasma lipoproteins to interact with purified CETP, incubation media contained 4 µl of donor liposomes, 3 µl of purified CETP (kindly provided by Dr. Jean-Paul Pais de Barros, INSERM U498, Dijon, France) and VLDL, LDL or HDL isolated by ultracentrifugation from individual mouse plasma. For CETP activity in plasma infranatant, 4 µl of donor liposomes, 10 µl of the d > 1.21 g/ml fraction ultracentrifugally isolated from individual mouse plasma, and 4 µl of acceptor triglyceride-rich particles (ROAR Biomedical) were used. In all cases, final volumes were adjusted to 200 µl with PBS and incubations were performed in triplicate for 3 h in a FL600 Microplate Fluorescence Reader (Bio-Tek, Winooski, VT). The CETP-mediated transfer of NBD-CE from self-quenched donors to lipoprotein acceptors was monitored by the increase in fluorescence intensity (excitation, 465 nm; emission, 535 nm). The amounts of NBD-cholesteryl esters transferred (pmol) were calculated using a standard curve plotting fluorescence intensity against concentrations of NBD-cholesteryl esters dispersed in isopropanol. Results were expressed as the amount of labeled cholesteryl esters transferred per hour after deduction of blank values.

Determination of the apoE content within VLDL.
The d < 1.019 g/ml VLDL fraction was isolated by ultracentrifugation from mouse sera as described above. Alternatively, VLDL from the different mouse models was isolated by FPLC as detailed above. Isolated VLDL (protein, 0.06 g/l) were diluted (1:4, v/v) in TBS (100 mM, pH 6.8) containing SDS (25 g/l) and dithiothreitol (33 g/l), incubated at 80°C for 5 min and then applied to a SDS polyacrylamide gradient gel (Phastgel 8/25, Amersham Pharmacia Biotech, Pittsburgh, PA), and migration was conducted as recommended by the manufacturer. Apolipoproteins were then either silver-stained as described previously or transferred to nitrocellulose membranes (Trans Blot; Bio-Rad) for apoE immunodetection. The resulting blots were blocked for 2 h in 10 % low-fat dry milk in PBS (100 mM, pH 7.4) containing 0.1 % Tween and then washed with PBS/Tween. Mouse apoE was detected by successive incubations with rabbit anti-mouse apoE antibodies cross-reacting with human apoE (Santa Cruz Biotechnology, SantaCruz, CA, USA) and horseradish peroxidase-coupled goat anti-rabbit secondary antibodies (DakoCytomation, Glostrup, Denmark). Blots were finally developed using the ECL kit (Amersham Biosciences). Apparent molecular weights of individual bands were determined by comparison with protein standards (for silver staining, Low Molecular Weight calibration kit, Pharmacia; for Western blot, Pre-Stained SDS-Page Standards, Low Range, Bio-Rad) that were electrophoresed together with the samples.

Immunoassay of CETP mass levels.
CETP mass levels in mouse plasma were determined by a specific immunoassay with TP1 anti-CETP monoclonal antibodies (Heart Institute, Ottawa, Canada). In brief, equal volumes of plasma samples were diluted (1:9, v/v), electrophoresed, electrobotted and blocked as described above except that 8-14 % discontinuous polyacrylamide gels in a mini-protean device (Bio-Rad) were used. Human CETP was revealed by successive incubations with TP1 anti-CETP antibodies and horseradish peroxidase-coupled secondary antibodies as previously described. Blots were finally developed using the ECL kit (Amersham Biosciences), and the intensity of CETP bands was determined with a Bio-Rad
chemoluminescence analysis system using the Quantity One-4.5.2 software. The CETP mass level in each plasma sample was estimated by comparison with a calibration curve that was obtained with serial dilutions of purified CETP submitted to electrophoresis together with the samples. Measurements were made in duplicate for each individual mouse plasma using a different gel for each repeat.

**Statistical analysis.**

Statistical analyses were performed using the statistical package for social sciences (SPSS, SPSS Inc., Chicago, IL). Data are presented as means ± SD. Statistical differences between two groups were assessed with an independent samples Student’s t-test. To compare more than two groups ANOVA followed by a Bonferroni post-test was used. Statistical significance for all comparisons was assigned at p<0.05.
References

2. Grass DS, Saini U, Felkner RH, Wallace RE, Lago WJ, Young SG, Swanson ME. Transgenic mice expressing both human apolipoprotein b and human cetp have a lipoprotein cholesterol distribution similar to that of normolipidemic humans. J Lipid Res. 1995;36:1082-1091
8. Tietge UJF, Maugeais C, Cain W, Grass D, Glick JM, deBeer FC, Rader DJ. Overexpression of secretory phospholipase a2 causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesteryl ester and apolipoprotein a-i. J Biol Chem. 2000;275:10077-10084