Extrahepatic High-Density Lipoprotein Receptor SR-BI and ApoA-I Protect Against Deep Vein Thrombosis in Mice

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Objective—Deep vein thrombosis (DVT) and pulmonary embolism are frequent causes of morbidity and mortality. We would like to determine whether plasma high-density lipoprotein (HDL), which inversely correlates with the risk of cardiovascular events, affects DVT.

Methods and Results—Using a murine DVT model of inferior vena cava stenosis, we demonstrated that deficiency of the HDL receptor, scavenger receptor class B type I (SR-BI), promotes venous thrombosis. As SR-BI−/− mice have increased plasma cholesterol levels and abnormal HDL particles, we tested SR-BI−/− mice with an SR-BI liver transgene that normalizes both parameters. These mice also exhibited increased susceptibility to DVT, indicating a protective role of extrahepatic SR-BI. Mice lacking the major HDL apolipoprotein apoA-I or endothelial nitric oxide synthase (eNOS) (a downstream target of endothelial SR-BI signaling) also had a prothrombotic phenotype. Intravenous infusion of human apoA-I, an HDL component, and SR-BI ligand prevented DVT in wild-type but not SR-BI−/− or eNOS−/− mice, suggesting that its effect is mediated by SR-BI and eNOS. Intravenous apoA-I infusion abolished histamine-induced platelet–endothelial interactions, which are important for DVT initiation.

Conclusion—An apoA-I (HDL)–SR-BI–eNOS axis is highly protective in DVT and may provide new targets for prophylaxis and treatment of venous thrombosis. (Arterioscler Thromb Vasc Biol. 2012;00:0–0)

Key Words: deep vein thrombosis • scavenger receptor class B type I • apolipoprotein A-I • high-density lipoprotein • endothelial nitric oxide synthase

Venous thromboembolism, which includes deep vein thrombosis (DVT) and its life-threatening complication pulmonary embolism, is a major health problem in the Western world. In the United States alone, venous thromboembolism affects ≈900 000 people/year and leads to hundreds of thousands of hospitalizations and deaths. Various factors predisposing to DVT, such as cancer, obesity, or hereditary blood hypercoagulability, have been described.1 Distortion of blood flow resulting from immobilization, for example, attributable to postsurgery bed-ridden position, long airplane flights, and limb paralysis, constitutes the major provoking factor of idiopathic DVT. In contrast to arterial thrombosis that is initiated by vessel wall injury, for example, when an atherosclerotic plaque ruptures, in venous thrombosis, the endothelial layer remains largely intact. As a consequence, the mechanisms initiating venous thrombosis are different from those underlying arterial thrombosis.

The metabolism of high-density lipoprotein (HDL) has been the focus of much research, in part, because the levels of plasma HDL cholesterol are inversely correlated with the risk of cardiovascular events (ie, diseases based on arterial thrombosis). In contrast, the relationship of plasma HDL levels and venous thromboembolism in humans is unclear, with some studies demonstrating an inverse correlation6,7 and others reporting no such correlation8 or even a direct association of high HDL cholesterol with increased risk of venous thromboembolism in women.9 This discrepancy may reflect, in part, the differences between the studied populations, as HDLs are heterogeneous populations of lipoprotein particles whose characteristics may vary depending on the physiological state of an individual.10

A major function proposed for HDL is reverse cholesterol transport (ie, transport of cholesterol from peripheral tissues to the liver and subsequent excretion into the bile).11 One mechanism by which hepatocytes take up cholesterol from HDL is selective lipid uptake12,13 mediated by the HDL receptor called scavenger receptor, class B, type I (SR-BI).14 During
selective lipid uptake, SR-BI binds to HDL particles via their apolipoproteins, particularly apoA-I, and the receptor mediates the transfer of cholesteryl esters from HDL into the cells. The lipid-depleted HDL particles are then released into the extracellular fluid.

SR-BI, a member of the CD36 family of proteins, is a multiligand receptor expressed on many cells, such as hepatocytes, macrophages, endothelial cells (including endothelium in veins), and platelets.14-16,18 It plays an important role in protection against murine atherosclerosis.14,19 Some studies also implicated SR-BI in HDL-induced signal transduction in endothelial cells.20,21 For example, SR-BI mediates HDL-induced activation of endothelial nitric oxide synthase (eNOS),20 whose product, NO, is antiinflammatory, antithrombogenic, and plays a crucial role in vascular homeostasis.22 HDL binding to SR-BI activates eNOS20 via the following pathway: HDL→SR-BI→src−phosphatidylinositol 3-kinase (Akt or HDL→HDL→HDL→PDZK1−/− mice exhibit impaired postinjury reendothelialization.27 In PDZK1−/− mice, HDL can also stimulate prostaglandin I2 synthesis23 and deliver sphingosine-1-phosphate to control endothelial functions.24

A cytoplasmic adaptor protein, PDZK1, binds to the intracellular C terminus of SR-BI and influences SR-BI abundance and activity in a tissue-specific fashion.20,26 In cultured endothelial cells, PDZK1 is involved in SR-BI-dependent activation of eNOS27 but is not required for normal SR-BI expression levels or binding to HDL.27,28 Similar to SR-BI−/− mice,20 PDZK1−/− mice exhibit impaired postinjury reendothelialization.27 In PDZK1−/− mice, hepatic SR-BI levels fall to <5% of normal, resulting in abnormal plasma HDL (large particles, increased HDL cholesterol) that is reminiscent of, but not as severe as, that seen in SR-BI−/− mice.30 Mice lacking PDZK1 exhibit some changes21 but not all28-30 of the abnormalities seen in SR-BI−/− mice, although with significantly less severity.

SR-BI expressed on platelets and hepatocytes modulates platelet function and arterial thrombosis. SR-BI−/− mice are thrombocytopenic, with ≈38% of the platelet count seen in SR-BI−/− mice attributable to abnormally high-platelet cholesterol levels and the consequent reduced platelet life span in the circulation.32 Platelets lacking SR-BI exhibit moderate changes in aggregation response to some platelet agonists in vitro.32-34 It has recently been reported that deficiency of SR-BI on platelets protects them from hyperactivation mediated by increased platelet cholesterol content and also protects mice from arterial thrombosis induced by endothelial injury.34 Infusion of human apoA-I Milano (a mutant form of apoA-I identified in an Italian family) has been reported to delay FeCl3-induced thrombus formation in rat aorta.35 The question I identified in an Italian family has been reported to delay infusion of human apoA-I Milano (a mutant form of apoA-I)−SR-BI−eNOS axis protects against flow restriction–induced venous thrombosis and that infusion of apoA-I protects mice from DVT.

**Materials and Methods**

An expanded Materials and Methods section is available in the online-only Data Supplement.

**Mice**

SR-BI++-, SR-BI−/−,37 and SR-BI−/[Liver Tg]18 mice were generated and housed as previously described. The SR-BI−/[Liver Tg] mice express a liver-specific murine SR-BI transgene (+0.7-fold of the hepatic expression level in SR-BI++ mice).38 For DVT and blood coagulation experiments not involving apoA-I infusion, these mice were on mixed 75:25 129-S4:C57BL/6 background. Littermates used in these experiments were obtained by crossing transgenic or nontransgenic SR-BI−/+ males to nontransgenic SR-BI−/− or transgenic SR-BI−/+ females, respectively. SR-BI−/+ mice used in experiments involving apoA-I infusion followed by DVT were on mixed 50:50 129-S4:C57BL/6 background. Littermates used in these experiments were obtained by crossing SR-BI−/+ males to SR-BI−/+ females.38 Mice with homozygous null alleles for Nos3 (eNOS−/−) and APOA1 (apoA-I−/−) as well as their control mice were purchased from the Jackson Laboratory and were on a C57BL/6 background. PDZK1−/− mice30 and their PDZK1++ littermate controls were on 129SvEv background. All protocols for animal use were reviewed and approved by the Animal Care and Use Committee of the Immune Disease Institute.

**Flow Restriction Surgery**

Eight- to 9-week-old male mice (weight 23–28 g) were anesthetized with isoflurane–oxygen mixture, placed in a supine position, and the inferior vena cava (IVC) was accessed through medial abdominal incision. For each mouse, the aorta was deliberately separated from the IVC just below the renal veins (in caudal direction) and the IVC was ligated with a polypropylene monofilament 7.0 over a 30G spacer, as previously described.36 The spacer was placed externally in a way excluding any injury to the vessel, then theligature was closed, and the spacer was removed. This procedure decreases the IVC lumen by ≈90%.35 All side branches of the IVC were completely closed as well. After surgery, the peritoneum and skin were sutured separately. In 2, 3, 6, or 8 hours, after initiating IVC stenosis, mice were anesthetized again, opened, and any thrombus present was taken and its weight and length determined. According to our experience, weight and length of thrombi may substantially vary between mice, which makes thrombi prevalence an important readout in this model. Our preliminary experiments showed that about 20% to 25% of wild-type (WT) mice on C57BL/6 background formed a thrombus within 2 to 3 hours in this model, whereas the same percent age of mice on mixed 129-S4:C57BL/6 background and about 40% of WT mice on the mixed or 129SvEv background developed a thrombus within 6 hours.

**Platelet–Endothelial Interactions in Mice**

Intravital microscopy was performed as described.39 In brief, mice were anesthetized with 2.5% tribromoethanol (150 μL per 10 g body weight), and apoA-I (3.5 mg/kg) or vehicle was intravenously infused through the retro-orbital plexus. Three minutes after apoA-I infusion, mesentery was exteriorized, and veins (200–250 μm) were examined using an inverted microscope (Axiovert 135; Carl Zeiss, Inc., objective 10x) connected to a video camera (C2400; Hamamatsu Photonics). Washed syngeneic platelets from C57BL/6 mice (2.5 × 10^9/kg) fluorescently labeled with CFSE (Molecular Probes, Eugene, OR) were infused. Observations from 1 vein per animal were recorded for 2 minutes and then histamine (50 μL, 5 mM/L, Sigma) was superfused and the recording from the same vessel continued for 15 minutes thereafter. Platelets interacting with the endothelium were counted by a blinded observer for 60 seconds starting 1 minute before histamine application and 5, 7, and 10 minutes after histamine. Platelet attachment to the vessel wall lasting more than 0.3 seconds was considered a platelet–endothelial interaction.
Statistics
The percentage of mice with thrombus in different experimental groups was compared using a contingency table and the Fisher exact test. Coagulation onset and coagulation rate were compared using 1-way ANOVA followed by the Tukey multiple comparison test. Plasma D-dimer levels, platelet aggregation amplitude, and number of platelets interacting with vascular wall with and without apoA-I were compared using Student t test. The weight and length of the thrombi were compared with the Mann-Whitney test except for the experiment of apoA-I infusion into C57BL/6J mice (Figure 4), in which the Mann-Whitney test was inapplicable because of uniformity of the results in all mice and therefore the Kruskal-Wallis test was used. Statistics was calculated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant for P<0.05.

Results
Extrahepatic SR-BI Deficiency Enhances Flow Restriction–Induced Thrombosis
To explore the role of SR-BI in murine DVT, we used a flow restriction (stenosis) model in the IVC. We chose a relatively short (2–8 hours as compared with the usual 48 hours)36 stenosis period to be able to detect a prothrombotic phenotype in the experimental mice. Stenosis of the IVC for 6 hours resulted in thrombus development in only 17% of the control SR-BI−/+ mice (2/12, Figure 1). In contrast, 100% of the SR-BI−/− littermates (9/9) developed a thrombus under these conditions. Thus, expression of SR-BI protects mice against DVT.

Hepatic SR-BI expression influences the structure and abundance of plasma HDL and biliary cholesterol secretion.37,40 To determine whether loss of hepatic SR-BI expression was responsible for the prothrombotic phenotype of SR-BI−/− mice, we studied DVT in transgenic SR-BI+/− mice (SR-BI+/−[Liver Tg]), which express in the liver essentially WT levels of a murine SR-BI transgene driven by a primarily liver-specific promoter (Liver Tg, see Methods). SR-BI+/−[Liver Tg] mice exhibit essentially WT plasma cholesterol levels, HDL particle size distributions, and plasma unesterified cholesterol:total plasma cholesterol ratios.38 We reported previously that the total plasma cholesterol for the SR-BI+/−[Liver Tg] mice is lower than that for the control nontransgenic SR-BI+/− mice (128±1 versus 143 ± 4 mg/dL, respectively) or the SR-BI−/− mice (206±7 mg/dL).38 Figure 1 shows that 89% of the SR-BI−/−[Liver Tg] mice (8/9) developed thrombi after 6 hours of IVC flow restriction. No statistically significant differences in thrombus weight (P=0.19) and length (P=0.44) were observed between the nontransgenic and SR-BI+/−[Liver Tg] mice. Because WT levels of hepatic SR-BI did not ameliorate the prothrombotic phenotype in SR-BI−/− mice, we conclude that their common deficiency in extrahepatic SR-BI was the likely cause of their enhanced DVT.

It has been shown in vitro that the adaptor protein PDZK1 is implicated in SR-BI downstream signaling.21,27 We examined flow restriction–induced thrombosis (6- to 8-hour periods of stenosis) in PDZK1−/− and PDZK1+/+ littermates. Surprisingly, thrombosis prevalence did not significantly differ between PDZK1−/− and PDZK1+/+ controls (Figure I in the online-only Data Supplement).

SR-BI and Blood Coagulation
Procoagulant state of blood is frequently associated with venous thrombosis in humans,3 and HDL may influence coagulation as shown in vitro.6 We, therefore, tested ex vivo coagulation of recalcified citrated whole blood drawn from SR-BI−/−, SR-BI−/−, and SR-BI−/−[Liver Tg] mice. SR-BI−/− mice had shorter coagulation onset times (Figure 2A) and higher coagulation rates (Figure 2B) than SR-BI−/− controls (Figure 2A and 2B, shaded bars). The SR-BI−/−[Liver Tg] mice had the longest onset time and the lowest coagulation rate (Figure 2A and 2B, open bars). Clot formation in recalcified blood from these mice was inversely correlated to their rank order of hepatic SR-BI protein expression (SR-BI−/−[Liver...
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Tg]:SR-BI+/−:SR-BI−/−≈0.7:0.5:0) and directly correlated with their rank order of plasma cholesterol levels (1.00:1.12:1.61, respectively), but no correlation with DVT susceptibility was observed. SR-BI+/- and SR-BI−/−[Liver Tg] littermates (n=8) and stabilized by sodium citrate. Calcium chloride was added to the blood within a minute after drawing, and clotting was monitored by a Sonoclot analyzer. A, Coagulation onset (time to first fibrin formation) and (B) coagulation rate. C, D-dimer levels in plasma of SR-BI−/− mice (n=5) and SR-BI+/- controls (n=7). Error bars represent SEM.

Figure 3. ApoA-I and endothelial nitric oxide synthase (eNOS) protect mice from flow restriction–induced thrombosis. Stenosis of inferior vena cava was applied in wild-type (n=11), eNOS+/− (n=11), and apoA-I−/− (n=8) mice, and thrombus formation was examined after 2 hours. A, thrombus weight in mg. B, thrombus length in mm. C, thrombus prevalence. Horizontal bars in dot plots represent median values.

Figure 2. In vivo expression of scavenger receptor class B type I (SR-BI) can influence in vitro clot formation whereas in vivo plasma fibrin D-dimer levels remain unaltered. Whole blood was collected from SR-BI+/− (n=16), SR-BI−/− (n=12), or SR-BI−/−[Liver Tg] littermates (n=8) and stabilized by sodium citrate. Calcium chloride was added to the blood within a minute after drawing, and clotting was monitored by a Sonoclot analyzer. A, Coagulation onset (time to first fibrin formation) and (B) coagulation rate. C, D-dimer levels in plasma of SR-BI−/− mice (n=5) and SR-BI+/- controls. P <0.001. P >0.05.

Deficiencies in ApoA-I or eNOS Predispose Mice to Flow Restriction–Induced Thrombosis

We next examined flow restriction–induced thrombosis in eNOS+/− and apoA-I+/- (reduced HDL) mice. Because we anticipated prothrombotic phenotypes in the mutant mice, we performed the assays using 2-hour rather than 6-hour periods of IVC stenosis. Although only 27% (3/11) of control C57BL/6J mice developed thrombi under these conditions, 88% (7/8) of apoA-I−/− mice (P<0.02 versus control) and 82% (9/11) of eNOS−/− mice (P<0.04 versus control) developed thrombi (Figure 3). These results do not establish directly that the SR-BI antithrombotic effect involves its main ligand apoA-I and eNOS, but they do show that both apoA-I and eNOS play protective roles in DVT.

Infusion of ApoA-I Protects WT but Not SR-BI+/- or eNOS−/− Mice From Venous Thrombosis

Because apoA-I deficiency increased DVT susceptibility, we examined the effects of intravenous administration of exogenous human apoA-I on DVT. Indeed, Figure 4A to 4C shows...
that 1 bolus infusion of apoA-I immediately after application of IVC stenosis for 6 hours completely protected C57BL/6J mice from DVT, decreasing thrombi prevalence from 55% in vehicle-infused mice (n=11) to 0 (n=11). This potent anti-thrombotic effect of apoA-I was SR–BI– and eNOS-dependent: apoA-I administration to SR–BI– or eNOS–/– mice did not significantly influence thrombosis (Figure 4). Thus, apoA-I is unable to exert its protective effect in the absence of either SR-BI or eNOS, raising the possibility that these molecules lay downstream of apoA-I in a signaling pathway that helps protect against venous thrombus development.

Because SR-BI is expressed on platelets, we asked whether in vitro incubation of apoA-I (45 μg/mL) with platelets from C57BL/6J mice affects platelet aggregation induced by a set of agonists. The concentration used is similar to that expected in the blood of mice infused, as described above, with a bolus of 3.5 mg/kg of apoA-I. We observed no statistically significant effect of apoA-I on platelet aggregation induced by high or low concentrations of collagen, calcium ionophore A23187, or a high dose of thrombin, as determined by aggregation amplitude 10 minutes after the addition of agonist (Figure 5A–5D). There was a statistically insignificant trend of decreased aggregation of apoA-I–treated platelets with low-dose thrombin (Figure 5E and 5F). Therefore, infusion of apoA-I protected WT mice against flow restriction–induced thrombosis via a mechanism that apparently either directly or indirectly depends on SR-BI and may not involve direct alteration of platelet aggregation. These results raised the possibility that apoA-I infusion suppressed flow restriction–induced activation of venous endothelium.

**Infusion of ApoA-I Suppresses Endothelial Stimulation-Induced Platelet Rolling**

Platelet interactions with the venous endothelium are crucial for DVT in our stenosis model. Platelet recruitment to the endothelium is largely dependent on von Willebrand factor, a constituent of endothelial Weibel–Palade bodies, whose release is a key step in the initiation of DVT in the murine model used in this study. Therefore, we used platelet–endothelial interactions as an experimental model entirely dependent on the secretion of von Willebrand factor from Weibel–Palade bodies and tested the effects of apoA-I on platelet–endothelial interactions in vivo using intravital microscopy. After intravenous administration of a bolus of apoA-I (3.5 mg/kg) or vehicle, we surgically exposed the mesenteric venules, infused fluorescently labeled platelets into the mice, and then determined the number of platelets interacting with the endothelium before and after activation of the endothelium by histamine. Figure 6 shows that in vehicle-treated WT control mice, platelet–endothelium interactions increased with time from 19±6 at baseline to 84±39 platelets/view field per minute at 10 minutes after histamine superfusion (black squares). In mice pretreated with apoA-I (open circles), baseline platelet–vessel wall interactions were reduced compared with vehicle-treated controls, and there was no significant histamine-mediated increase with time. Thus, apoA-I inhibits platelet recruitment by the venous endothelium, presumably by suppressing Weibel-Palade bodies release.

**Discussion**

In this study, we showed that apoA-I, the major apolipoprotein in HDL, and extrahepatic expression of the HDL receptor SR-BI protected mice from flow restriction–induced DVT. Mice with homozygous null mutations in the APOA1 (apoA-I–/–) or Scarb1 (SR-BI–/–) genes exhibited dramatically increased susceptibility to DVT relative to control WT or SR-BI–/– mice, respectively. SR-BI–/– mice expressing an essentially liver-specific murine SR-BI transgene (SR-BI–/–[Liver Tg]) exhibited susceptibility to DVT that was indistinguishable from that of nontransgenic SR-BI–/– mice. Thus, it appears that SR-BI deficiency in extrahepatic tissues
apoA-I by vehicle

A

A23187, 10 μM

Platelet aggregation, %

Time (min)

B

Collagen, 10 μg/ml

Platelet aggregation, %

Time (min)

C

Collagen, 1 μg/ml

Platelet aggregation, %

Time (min)

D

Thrombin, 1 U/ml

Platelet aggregation, %

Time (min)

E

Thrombin, 0.1 U/ml

Platelet aggregation, %

Time (min)

F

apoA-I Vehicle

Platelet aggregation, %

Time (min)

Figure 5. ApoA-I does not affect platelet aggregation in vitro. Washed platelets from wild-type C57BL/6J mice were incubated with apoA-I (45 μg/mL) or vehicle for 5 minutes before adding (A) calcium ionophore A23187 (10 μmol/L); (B and C) collagen (10 or 1 μg/mL); or (D–F) thrombin (1 U/mL (D) or 0.1 U/mL (E and F)). Light transmission was recorded for 10 minutes. Each curve is a mean of 3 to 7 independent experiments. Arrows indicate time of agonist addition. Horizontal bars represent median values. Aggregation amplitudes of vehicle- and apoA-I-treated platelets at the end of a 10-minute period were compared statistically.

was responsible for the increased susceptibility of SR-BI−/− mice to venous thrombosis.

Some6,7 but not all8–9 studies have shown that plasma HDL inversely correlates with the risk of DVT. Cholesterol enrichment of monocytes/macrophages can induce release of highly procoagulant microparticles from monocytes,37 whereas intact HDL and apoA-I can activate fibrinolysis44 and, therefore, alter thrombus formation and stability. SR-BI−/− mice exhibit a distinctive dyslipidemia (elevated plasma levels of total plasma cholesterol and unesterified cholesterol, increased unesterified cholesterol:total plasma cholesterol ratio, and abnormal HDL particles),37,45 which is associated with increased unesterified cholesterol accumulation in red blood cells46 and platelets,32 and hypercoagulability in an ex vivo assay (this study). Indeed, enhanced blood coagulation is known to predispose humans to venous thrombosis.2 However, hepatic expression of the SR-BI transgene in SR-BI−/−[Liver Tg] mice corrected both the dyslipidemia and abnormal clot formation in recalcified blood but did not influence the increased susceptibility to DVT. PDZK1−/− mice exhibit a dyslipidemia similar to, but milder than, that of SR-BI−/− mice9 yet do not show increased susceptibility to DVT. Also, the lack of SR-BI did not affect plasma D-dimer levels, which indicates that the dyslipidemia itself in SR-BI−/− mice does not cause a marked change in constitutive fibrin formation. Together these findings indicate that dyslipidemia and hypercoagulability were not major causes of the enhanced susceptibility of SR-BI−/− mice to DVT, although SR-BI may regulate blood coagulation potential indirectly through controlling plasma lipids.

Two sites of extrahepatic SR-BI expression appeared to be particularly relevant with regard to the DVT susceptibility of SR-BI−/− mice: platelets and endothelial cells. Platelet SR-BI deficiency has been shown to protect mice from dyslipidemia-mediated arterial thrombosis in vivo.24 Also, SR-BI-deficient platelets have moderately decreased in vitro aggregation in response to some (but not all) agonists, such as adenosine diphosphate, collagen, platelet monocyte aggregates, and convulxin.23,34 Thus, platelet dysfunction attributable to platelet SR-BI deficiency in SR-BI−/− mice might be expected to be antithrombotic, and thus, it is an unlikely mechanism underlying the observed prothrombotic phenotype.

In cultured endothelial cells, SR-BI can serve as a signaling receptor that regulates endothelial physiology by mediating HDL induction of eNOS activity20 via the following pathway: HDL → SR-BI → src → phosphatidylinositol 3-kinase (Akt or mitogen-activated protein kinase) → eNOS.21 The cytoplasmic SR-BI adaptor protein PDZK1 has been shown to influence this eNOS activation pathway in vitro and carotid artery reendothelialization after perivascular injury in vivo.22 We explored the potential role of this pathway in the enhanced susceptibility of SR-BI−/− mice to DVT by assessing flow restriction–induced DVT in mice with homozygous null mutations in the APOA1, Nos3, or Pdzk1 genes. Both apoA-I−/− (which have decreased numbers of HDL particles and decreased total plasma cholesterol)42 and eNOS−/− mice exhibited markedly increased venous thrombosis compared with WT controls, suggesting that this pathway may contribute to an antithrombotic phenotype. In contrast, PDZK1−/− mice were not prone to DVT. It is possible that there is a PDZK1-independent pathway that links SR-BI to eNOS and mediates protection from venous thrombosis in vivo. For example, it has been reported that lysophospholipids present in HDL...
induce eNOS activation through their receptor sphingosine-1 phosphate-receptor 3, a process in which SR-BI participates.47

Because both apoA-I−/− mice and SR-BI−/− mice were prone to venous thrombosis, we examined the effects of intravenous administration of exogenous apoA-I protein on flow restriction–induced DVT. Infusion of apoA-I into mice has been reported to result in increased plasma concentrations of both relatively lipid-free apoA-I and apoA-I associated with HDL particles.48 We found that infusion of apoA-I into WT C57BL/6 animals virtually abolished flow restriction–induced DVT. This dramatic and protective effect of apoA-I was SR-BI–dependent, as there was no protection by apoA-I infusion in SR-BI−/− mice. Moreover, infusion of apoA-I did not protect eNOS-deficient mice from DVT. This result raises the possibility that the antithrombotic effect of apoA-I is eNOS-mediated. Taken together, these data suggest that a signal transduction pathway apoA-I(HDL)→SR-BI→eNOS, previously observed in vitro, may contribute to the protection from venous thrombosis. It is possible that mechanisms not directly involving HDL signaling via SR-BI also contribute to this apoA-I effect in WT mice.49 However, our results support a pathway in which apoA-I–enriched HDL interacts directly with SR-BI on extrahepatic cells. Potential target cells include platelets and the venous endothelium. Indeed, using intravital microscopy, we showed that infusion of apoA-I completely inhibited histamine-induced platelet–endothelial interactions in vivo. Treatment of isolated platelets with concentrations of apoA-I comparable with those expected in vivo after the apoA-I infusion did not affect platelet aggregation induced by a high dose of thrombin, A23187, or collagen. Thus, the antithrombotic effect of apoA-I infusion is unlikely to be a consequence of a direct apoA-I effect on platelets. However, we cannot completely rule out apoA-I effect on platelets in vivo.

HDL can activate eNOS in endothelial cells in vitro via an SR-BI–dependent signaling pathway.50,51 Activation of eNOS and release of NO, in turn, can inhibit the release of Weibel–Palade bodies50 and, thus, interfere with the activation of the endothelium required for platelet–endothelium interactions and thrombosis.52 Thus, it appears that an apoA-I/HDL pathway may operate in vivo to protect mice from venous thrombosis, at least in part, via suppression of endothelial activation and subsequent platelet and leukocyte recruitment.53

In summary, our studies support the hypothesis that HDL has beneficial effects on endothelium54 and expand range of benefits of HDL from arterial to venous circulation. The HDL receptor SR-BI appears to mediate, possibly via signaling through eNOS, some of these effects. Our observation that eNOS−/− mice exhibit increased DVT is consistent with this mechanism and suggests that eNOS can suppress endothelial activation, and thus the release of von Willebrand factor and platelet adhesion, key features in the initiation of DVT.53 We found that apoA-I infusion prevents flow restriction–induced DVT in WT C57BL/6J mice. Thus, it may be possible that apoA-I mimetics, or other agonists of SR-BI, might provide a novel medical approach to prevent or treat DVT in humans.

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Disclosures

None.

References


Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O”Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell. 2003;115:139–150.


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Supplement Material

Materials and Methods

Sonoclot coagulation analysis
Whole blood was collected from the retro-orbital plexus and coagulation was immediately assessed using the Sonoclot analyzer and non-activated clotting test kit (Sienco). The analysis cuvettes were preheated at 37°C. Citrated whole blood (0.28 mL) was recalcified with 20 µL of calcium chloride (150 mmol/L) and analyzed. We measured 1) onset of clot formation (time in seconds to first fibrin formation) and 2) the rate of fibrin polymerization (clot rate), which is an index of fibrinogen conversion into fibrin gel.

ApoA-I infusion for DVT experiments
Native human apoA-I (Genway; 3.5 mg/kg body weight) in vehicle buffer (10 mmol/L NH₄HCO₃, pH 7.4) was infused into WT C57BL/6J, SR-BI⁻/⁻ or eNOS⁻/⁻ mice as a bolus intravenously via retro-orbital plexus immediately after IVC stenosis induction. Control mice received the same volume of the vehicle alone. Mice were sacrificed 6 h later and thrombi weight and length were measured.

Platelet isolation and aggregation
Whole blood was drawn from the retro-orbital venous plexus and stabilized with heparin (7.5 U/mL). Blood was centrifuged (80 x g, 10 min), platelet-rich plasma was transferred to a fresh tube, incubated with PGI₂ (1 µg/mL, 5 min, 37°C) and centrifuged at 600 x g
for 3.5 min. The platelet pellet was resuspended in Tyrode’s-HEPES buffer (137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO₃, 0.3 mmol/L NaH₂PO₄, 5.5 mmol/L glucose, 5 mmol/L HEPES, 0.35% bovine serum albumin), and stored at 37°C for no longer than 1 h before use in experiments.

Platelet aggregation was evaluated using a Chrono-Log 4-channel optical aggregation system (Chrono-Log). Washed platelets (2.5 x 10⁵/μL) were incubated with apoA-I (45 μg/mL) for 5 min at 37°C, after which thrombin (Sigma, 0.1 or 1 U/mL), collagen (Nycomed, 1 or 10 μg/mL) or calcium ionophore (A23187, Sigma, 10 μmol/L), and light transmission was recorded over 10 min. The apoA-I concentration corresponds to the expected concentration of apoA-I reached in the blood of a mouse of 25 g with a blood volume of 2 mL after infusion of 3.5 mg/kg of apoA-I, assuming no losses due to distribution into tissues or degradation/excretion.

**ELISA for D-dimers**

Blood was drawn into 1/9 of its volume of 3.8% sodium citrate from the mouse retroorbital plexus. Plasma was obtained by centrifugation at 2300 x g and stored at -80°C until the D-dimer level was determined using an ELISA kit (Diagnostica Stago, Parsippany, NJ) according to the manufacturer’s instructions.
Figure I

A

![Graph showing thrombus weight, mg](image)

Thrombus weight, mg

PDZK1\(^{+/+}\)  PDZK1\(^{-/-}\)

\[ p = 0.49 \]

B

![Graph showing thrombus length, mm](image)

Thrombus length, mm

PDZK1\(^{+/+}\)  PDZK1\(^{-/-}\)

\[ p = 0.54 \]

C

![Graph showing thrombus in % of mice](image)

Thrombus in % of mice

PDZK1\(^{+/+}\)  PDZK1\(^{-/-}\)

\[ p = 0.38 \]
Figure I. PDZK1 deficiency does not promote DVT. IVC stenosis surgery was performed in WT (n = 9) and PDZK1⁻/⁻ (n = 13) mice. For both genotypes, mice were sacrificed after either 6 or 8 h (two independent experiments), thrombi excised and their weights and lengths measured. As no differences were observed between results for 6 and 8 h DVT, the results of the two experiments were combined. (A) thrombus weight in mg; (B) thrombus length in mm; (C) percent of mice that developed a thrombus (thrombi prevalence). Horizontal bars in dot plots represent median values. These results indicate that PDZK⁻/⁻ mice do not have a prothrombotic phenotype.